Alteracions vasculars pulmonars induïdes per l'exposició a fum de tabac en un model animal de MPOC

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ALTERACIONS VASCULARS PULMONARS INDUÏDES PER L'EXPOSICIÓ A FUM DE TABAC EN UN MODEL ANIMAL DE MPOC

Memòria presentada per

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per optar al títol de Doctora en Biologia

Treball realitzat sota la direcció del **Dr. Víctor I. Peinado** i el **Dr. Joan Albert Barberà**

al laboratori de Malalties Respiratòries de l'IDIBPAS

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Tesi inscrita en el programa de doctorat de Medicina

Departament de Medicina, Facultat de Medicina

Als meus pares

A les meves germanes

Al Carles

La present tesi Doctoral ha estat realitzada dins el programa de Doctorat de Medicina de la Universitat de Barcelona. La Comissió de Doctorat de la Facultat de Medicina ha avaluat i acceptat aquesta memòria per a la seva publicació i presentació com a tesi Doctoral. Seguint la normativa vigent aquesta memòria es presenta com a compendi d'articles originals publicats en revistes indexades situades dins dels dos primers quartils de l'àrea de coneixement.

L'estructura d'aquesta tesi consta d'una introducció amb els antecedents més rellevants relacionats amb l'àrea temàtica de la memòria. També s'inclou un apartat de fonaments de la tesi adreçat a ressaltar els motius pels quals es va desenvolupar el treball. A continuació es presenten les hipòtesis, objectius i resultats dels dos articles principals que fonamenten aquest treball i dels quals la doctoranda n'és la primera autora. El primer d'aquests articles es va publicar l'any 2009 i es presenta en el format electrònic de la revista. El segon article ha estat acceptat el mes de gener de 2011. Dins la secció de resultats s'ha afegit un annex en el qual es presenten dos articles publicats en revistes del primer quartil de l'àrea temàtica d'aquesta tesi i dels quals la doctoranda n'és co-autora. Aquestes publicacions complementen els dos articles principals ja que utilitzen el model animal desenvolupat en aquesta tesi Doctoral i donen un valor afegit al conjunt del treball. Finalment es discuteixen els dos treballs principals i es presenten les respectives conclusions.

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INTRODUCCIÓ

1.- MALALTIA PULMONAR OBSTRUCTIVA CRÒNICA

1.1.- Definició i aspectes generals

La Malaltia pulmonar obstructiva crònica (MPOC) és una malaltia prevenible i tractable que es caracteritza per obstrucció al flux aeri, dispnea, excessiva producció de moc i tos crònica. Aquestes alteracions resulten com a conseqüència d'un procés inflamatori anormal en resposta a l'exposició de partícules inhalades[1]. A més, la malaltia presenta efectes sistèmics importants, els quals poden respondre a tractament.

Les darreres estimacions (2007) de la Organització Mundial de la Salut (OMS), indiquen que uns 210 milions de persones pateixen MPOC i que 3 milions de persones van morir per aquesta causa al 2005. A Espanya, aquesta patologia afecta a un total del 10,2% de la població entre 40 i 80 anys, és a dir, més de 2,1 milions persones. Tot i així, un 75% dels casos o més segueixen sense ser diagnosticats. L'OMS preveu que la mortalitat deguda a la MPOC incrementarà un 30% en els propers 10 anys sent la quarta causa de mort a tot el món[2].

1.2.- Factors de risc

Consum de tabac

L'exposició activa a fum de tabac és la principal causa de la MPOC. Factors com l'edat d'inici, el nombre de paquets/any i l'hàbit actual són importants en el desenvolupament de la malaltia. S'ha demostrat una associació dosi-resposta entre la reducció del volum d'aire en el primer segon de l'expiració forçada (FEV_1) i la dosi de tabac[3].

Tot i la relació directe del tabac amb la MPOC, el tabac no és causa suficient de MPOC ja que només el 10-20% dels fumadors desenvolupa la malaltia[4], i tampoc és causa necessària per que alguns malalts no han estat mai fumadors. Hi ha altres factors de risc que intervenen en l'aparició de la malaltia, potser potenciant els efectes del tabac en la reducció de la funció pulmonar.

Factors genètics

La única causa genètica clara que s'ha relacionat fermament amb la MPOC és el dèficit de l'enzim inhibidor de la proteasa α1-antitripsina, present en un percentatge molt petit de la població, de manera que només se li atribueix un 1% dels casos de MPOC[5].

Pol·lució i contaminació de l'aire en locals tancats

La pol·lució dels ambients urbans proporciona una base per malalties respiratòries, com l'asma, al·lèrgies i MPOC. En països subdesenvolupats, l'exposició a biomassa sòlida de combustibles tals com femtes d'animals, restes de collites i fusta utilitzada per cuinar o com a calefacció són la major font de contaminació en ambients tancats. Aquest tipus de contaminació afecta preferentment la salut de les dones[6].

Altres

Pacients amb hiperreactivitat bronquial, atòpia i asma solen presentar major obstrucció de les vies aèries i major nombre de símptomes. Per contra, les infeccions respiratòries durant la infància poden modular el sistema immunitari protegint de l'aparició d'atòpia. Altres factors com la nutrició, l'ambient en la vida intrauterina o les condicions ambientals del domicili poden modular el grau d'afectació de la malaltia.

1.3.- Alteracions de la via aèria

La MPOC inclou clàssicament dues presentacions patològiques, la bronquitis crònica i l'emfisema. La bronquitis crònica es defineix com a producció de moc, donant lloc a tos amb expectoració, mentre que l'emfisema es descriu com un procés patològic en el que es produeix destrucció alveolar sense fibrosis aparent[7]. Tant l'emfisema com la bronquitis crònica comparteixen la reducció del FEV_1, paràmetre diagnòstic que caracteritza la limitació del flux aeri.

1.3.1.- Inflamació

Durant l'exposició a fum de tabac, els neutròfils són les primeres cèl·lules inflamatòries en respondre. Tot i que no es coneix el mecanisme pel qual el tabac recluta aquestes cèl·lules

se sap que la nicotina és quimiotàctica per als neutròfils[8]. El més probable és que el fum de tabac inicií una irritació de l'epiteli que doni lloc a una resposta inflamatòria. Els neutròfils són cèl·lules capaces d'alliberar radicals d'oxigen, elastasa i citocines, elements essencials en la patogènesi de la MPOC ja que tenen efectes en les cèl·lules caliciformes i les glàndules de la submucosa i indueixen emfisema i inflamació. Els neutròfils es localitzen especialment a les glàndules i l'epiteli bronquial[9], en zones properes a la capa muscular del bronqui[10] i al lumen de la via aèria, recoberts en esput. L'extracte de fum de tabac empitjora la capacitat fagocítica dels neutròfils mitjançant la supressió de l'activitat de la caspasa 3 d'aquestes cèl·lules[11]. Aquesta situació té lloc en els primers estadis però es pot perpetuar durant la vida del fumador i podria ser una de les causes de risc de infeccions respiratòries que pateixen els fumadors i els pacients MPOC.

El paper dels eosinòfils en la patogènesi de la MPOC és poc clar. Es creu que els pacients amb elevat nombre d'eosinòfils en l'esput i el rentat broncoalveolar podrien ser un subgrup diferent de MPOC ja que s'ha vist que aquests pacients són bons responedors al tractament amb corticoesteroides[12-14].

Altres tipus cel·lulars implicats en la resposta inflamatòria a la MPOC són les cèl·lules T CD8+ i CD4+. El nombre de cèl·lules T CD8+ es troba augmentat en el parènquima[15] pulmonar i les vies respiratòries en la MPOC. Tot i la seva elevada presència en aquesta patologia no es coneix exactament quin és el paper d'aquest tipus cel·lular. Estudis realitzats en ratolins deficients en cèl·lules T CD8+ exposats a fum de tabac indiquen un possible paper en la resposta inflamatòria i la inducció d'emfisema a través de la producció de quimiocines induïdes pel interferó γ. També s'ha trobat una relació entre el nombre de cèl·lules apoptòtiques i el nombre de cèl·lules T CD8+ a la paret alveolar[16] indicant una possible inducció de l'apoptosi de les cèl·lules epitelials i endotelials per part de les cèl·lules CD8+. Per altre banda, les cèl·lules CD4+ també es presenten en nombres elevats a la via aèria petita de fumadors i pacients amb MPOC[17], les quals expressen la quimiocina CXCL10 que controla l'alliberació de metal·loproteïnases elastòliques de la matriu[18].

Els macròfags són cèl·lules efectores molt potents degut a la seva capacitat per produir i alliberar ROS, proteïnes de la matriu extracel·lular i mediadors com leucotriens, prostaglandines, citocines, quimiocines i metal·loproteïnases de la matriu (MMPs)[19]. Els macròfags són especialment proliferants en fumadors[20] i es troben formant agregats al voltant de la via aèria petita que s'associa a fibrosis peribronquiolar. En pacients amb MPOC, els macròfags tenen una activitat fagocítica deficient que actua contra les cèl·lules epitelials apoptòtiques[21;22].

La inflamació causada pel fum de tabac és un factor crític que promociona l'excessiva producció de moc de forma crònica. El increment en el nombre de cèl·lules productores de moc és degut a una gran proliferació (hiperplàsia) i una major producció i secreció de moc (hipertròfia). Es creu que el lloc dominant en l'obstrucció al flux aeri es troba als bronquis inferiors a 2mm de diàmetre. Per contra, la localització crítica de secreció de moc clínicament més rellevant és la via aèria superior. Existeix una relació directe entre el FEV_1 i l'àrea luminal del bronqui i una relació inversa entre el FEV_1 i el percentatge de l'àrea de la paret del bronqui, especialment en els bronquis de menor diàmetre[23].

1.3.2.- Emfisema

L'emfisema es defineix com un augment dels espais aeris distals als bronquíols terminals que s'associa a la destrucció de la paret alveolar. La destrucció de l'elastina pulmonar s'ha establert com el mecanisme principal de la destrucció alveolar possiblement causat per l'alliberament de metal·loproteïnases i d'elastasa de les cèl·lules inflamatòries, com els macròfags o neutròfils. Aquesta producció excessiva de proteïnases per part de les cèl·lules inflamatòries estaria sobrepassant les defenses d'antiproteases induint així la destrucció alveolar. A aquest desequilibri entre producció i destrucció s'afegeix l'efecte inhibidor del fum de tabac sobre la síntesi de col·làgena i elastina[24].

Al 1963 Laurel i Eriksson van descriure per primer cop la relació entre la deficiència de la proteïna α-1antitripsina circulant en plasma amb l'emfisema pulmonar[25]. Posteriorment s'ha relacionat aquesta alteració genètica amb altres patologies com l'asma[26] o les

bronquièctasis[27]. La principal funció d'aquesta proteïna és la inhibició de l'elastasa neutrofílica[28], d'aquí la seva relació amb la destrucció del parènquima alveolar. A la taula 1 es descriuen breuement els mecanismes implicats en l'emfisema:

Mecanismes d'emfisema en la MPOC
Desequilibri proteasa-antiproteasa (activació de MMP-9, MMP-12, serina proteases, elastasa de neutròfil, inactivació de l'α1-antitripsina)
Activació de cèl·lules T CD8+ i alliberació de perforines
Apoptosi de cèl·lules alveolars (disminució VEGF, el qual estimula la supervivència i creixement de la cèl·lula alveolar i endotelial
Envelliment i senescència que dóna lloc a una mala reparació i manteniment pulmonar (el fum de tabac inhibeix la reparació alveolar)
Neteja inefectiva de les cèl·lules apoptòtiques per part dels macròfags que dóna lloc a disminució de l'efectivitat dels mecanismes anti-inflamatoris
Disfunció mitocondrial amb increment d'estrès oxidatiu i increment d'apoptosi cel·lular

Taula 1. Mecanismes implicats en l'aparició d'emfisema a la MPOC. Abreviacions: VEGF: Factor de creixement de l'endoteli vascular. (Adaptació K.F. Chung i I.M. Adcock ERJ, 2008).

1.4.- Alteracions de la circulació pulmonar

Durant molts anys la clínica ha centrat l'atenció en les de la via aèria i el parènquima. Amb el temps les alteracions vasculars han guanyat importància ja que s'ha demostrat el paper que juguen en el intercanvi de gasos, la hipertensió pulmonar (HP) o les afectacions cardíaques secundàries.

1.4.1.- Estructura vascular

Les artèries són conductes membranosos, elàstics, amb ramificacions encarregades de distribuir la sang per l'organisme. Cada vas arterial muscular consta de 3 capes:

1.- *Capa interna o íntima*: està en contacte directe amb la sang del lumen. Constituïda per l'endoteli (cèl·lules endotelials i pericits), una làmina basal i una capa conjuntiva subendotelial.

2.- *Capa mitja*: formada per fibres musculars llises disposades de forma concèntrica, fibres elàstiques i fibres de col·làgena, en proporció variable segons el tipus de vas. Les fibres elàstiques fan la contracció i relaxació mentre que l'elastina permet que els vasos s'estirin i es contraguin controlant el diàmetre del vas.

3.- *Capa externa o adventícia*: formada per teixit conjuntiu laxa, que consta fonamentalment de fibroblasts i col·làgena amb la funció d'ancorar el vas a les estructures adjacents.

Els límits entre les capes estan generalment ben definits. Les artèries musculars presenten sempre una làmina elàstica interna separant la capa íntima de la capa mitja i una làmina elàstica externa que separa la capa mitja de l'adventícia.

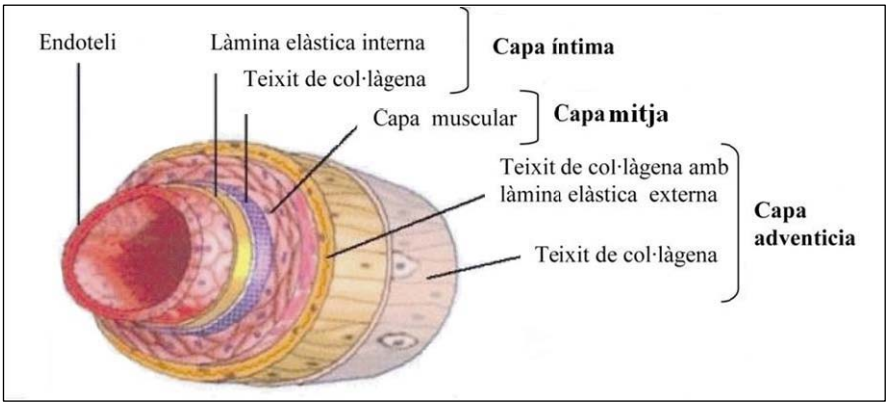

Figura 1. Estructura de les artèries musculars. Tres capes concèntriques constitueixen l'estructura del vas sanguini: capa interna (o íntima), capa mitja i capa externa (o adventícia).

1.4.2.- Paper de l'endoteli: funció endotelial

L'endoteli vascular és, tot i la seva simplicitat estructural, un òrgan funcionalment complex. Tal com es menciona a l'apartat anterior, l'endoteli consisteix en una capa unicel·lular que cobreix la superfície interna dels vasos sanguinis i conforma la paret dels capil·lars. Lluny de ser només una barrera mecànica entre la sang i els teixits, és un òrgan activament compromès amb una gran varietat de processos fisiològics i patològics. Degut a la seva estratègica localització detecta els canvis en les forces hemodinàmiques que actuen sobre la paret vascular, així com senyals químics transportats per la sang, i respon a aquests

alliberant substàncies vasoactives, algunes de les quals tenen funcions antagòniques com vasodilatadors i vasoconstrictors.

El resultat net d'aquest balanç de mediadors vasoactius es tradueix en diferents efectes o funcions de l'endoteli com la disminució del to vascular degut a la relaxació del múscul llis de la paret vascular, la inhibició de la proliferació cel·lular, l'adhesió i l'agregació plaquetària, la depleció de l'activació del sistema de coagulació, l'estimulació de la fibrinòlisi, la disminució de la permeabilitat i inhibició de l'adhesió i la migració de neutròfils i macròfags generadors de inflamació.

Una de les funcions principals que compleix l'endoteli és el control del to vascular mitjançant la regulació de l'expressió balancejada dels diferents principis vasoactius que produeix. Clàssicament es destaquen tres vies principals implicades a la cèl·lula endotelial:

a) Via de l'endotelina

L'endotelina (ET) és un pèptid de 21 aminoàcids que prové de la transformació de la pro-ET mitjançant l'acció de l'enzim convertidor d'endotelina (ECE). L'ET-1 exerceix els seus efectes vasculars a traves de dos receptors: receptor tipus A (ET_A) localitzat a les cèl·lules musculars llises i receptor tipus B (ET_B) localitzat a les cèl·lula endotelial vascular i a la cèl·lula muscular llisa[29]. L'activació dels dos receptors ET_A i ET_B a les cèl·lules musculars indueix vasoconstricció, proliferació cel·lular i hipertròfia. Per contra, l'estimulació del receptor ET_B sobre la cèl·lula endotelial resulta en la producció de vasodilatadors. A més ET_B està implicat en l'aclariment de ET-1 del sistema circulatori[30].

l'ET és un vasoconstrictor amb acció mitogènica[31] sobre les cèl·lules musculars que juga un paper important en la HP. En aquesta patologia, els nivells elevats d'ET-1 estan relacionats amb la pressió sanguina pulmonar i amb el cabdal cardíac[32].

b) Via de l'òxid nítric

L' òxid nítric (NO) es produeix a partir de l'aminoàcid bàsic L-arginina mitjançant l'acció de tres isoformes diferents de la NO-sintasa (NOS I o neuronal (nNOS), NOS II o induïble (iNOS)

i NOS III o endotelial (eNOS)) expressades pràcticament a tots els tipus cel·lulars vasculars. Mentre que eNOS i nNOS són proteïnes constitutivament expressades, l'expressió de iNOS requereix la interacció de la cèl·lula amb estímuls que generin senyals nuclears apropiats. Les tres isoformes de NOS comparteixen un cicle catalític similar, que consisteix en l'oxidació de L-arginina per formar NO i citrulina.

La presència de NO generat a la cèl·lula endotelial indueix l'activació de la guanilat-ciclasa (GC) que converteix el trifosfat de guanosina (GTP) en monofosfat de guanosina cíclic (GMPc), el qual és degradat per la fosfodiesterasa (PDE). Així doncs, els efectes del NO estan mediats per un segon missatger, el GMPc, que manté la relaxació de les cèl·lules musculars llises de l'artèria pulmonar i inhibeix la seva proliferació.

El NO alliberat per l'endoteli és la molècula clau per al manteniment de l'homeòstasi, el to vascular, la pressió i el flux sanguíni[33;34]. A més de tenir una funció vasoreguladora, disminueix l'agregació plaquetària i l'adhesió de monòcits, evita la proliferació de les cèl·lules musculars llises i preserva de l'oxidació a les lipoproteïnes de baixa densitat (LDL). Aquesta molècula també té un paper antiinflamatori, augmentant la producció del factor nuclear kappa β (NF-κ β), un factor de transcripció implicat en la regulació de múltiples gens proinflamatoris que expressen citosines.

c) Via de la prostaciclina

La prostaciclina (prostaglandina I_2 (PGI_2)) és un metabòlit derivat de l'àcid araquidònic (AA) que és produït tant a la cèl·lula endotelial com a la cèl·lula muscular llisa. Està catalitzada per la prostaciclina sintasa (PS) a les cèl·lules endotelials. És un vasodilatador molt potent que estimula l'adenilat ciclasa (AC) donant lloc al increment de la producció monofosfat d'adenosina cíclic (AMPc) a partir de trifosfat d'adenosina (ATP), un segon missatger que manté les cèl·lules musculars llises de l'artèria pulmonar relaxades i inhibeix la seva proliferació. La prostaciclina també inhibeix fortament l'agregació plaquetària. Aquest mediador clau es troba disminuït en pacients amb hipertensió arterial pulmonar comparat

amb subjectes control[35] segurament degut a una reducció en la producció de PS en artèries de petit i mitjà calibre d'aquests pacients[36].

Les tres vies interaccionen entre elles modulant els efectes les unes de les altres.

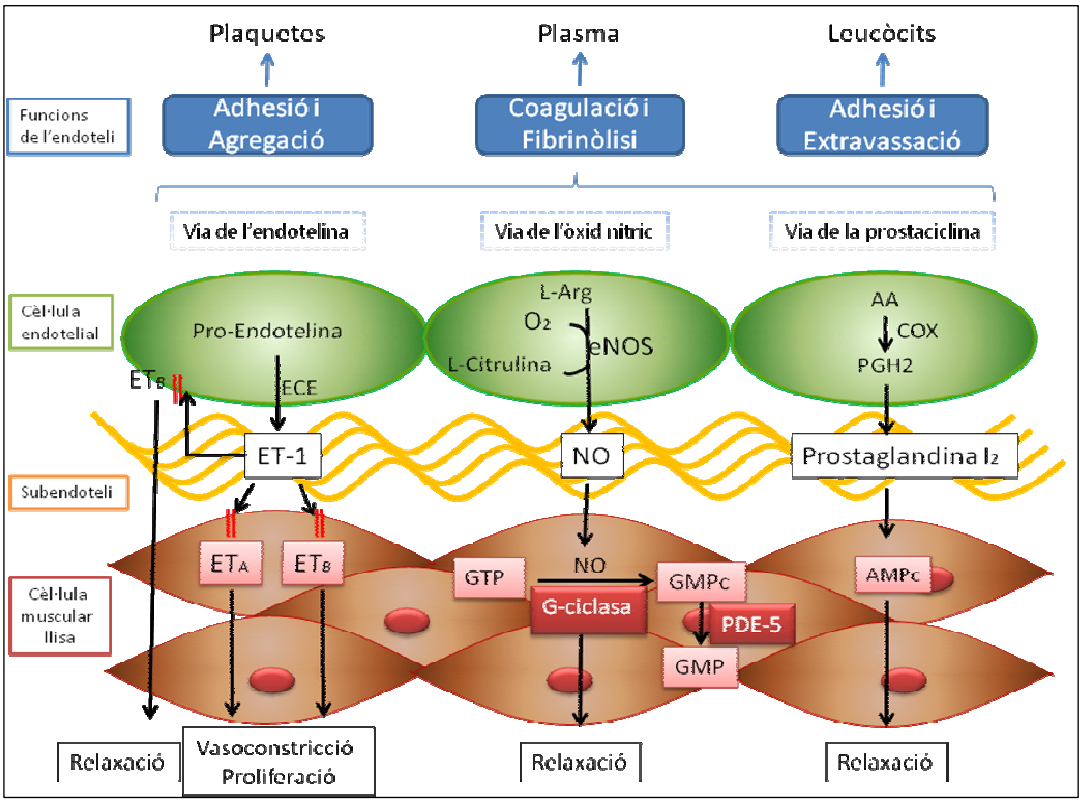

Figura 2. L'endoteli pulmonar és un regulador del to vascular pulmonar mitjançant l'alliberament de substàncies vasoactives com l'endotelina-1 (ET-1) i de factors relaxadors derivats de l'endoteli com l'òxid nítric (NO) i la prostaciclina (PGI_2). Aquests mediadors també són capaços de regular el flux sanguini pulmonar i la tendència de la sang a coagular així com induir i/o inhibir la migració i proliferació de les cèl·lules musculars llises vasculars[34;37].

El terme de disfunció endotelial indica que l'endoteli no compleix apropiadament les seves funcions. Una menor biodisponibilitat de NO, una alteració de la producció de prostanoides (incloent prostaciclina, tromboxà-A2 i/o isoprostanoides), un deteriorament de la hiperpolarització depenent de l'endoteli, així com una major alliberació d'ET-1, poden individualment o associats contribuir a la disfunció endotelial. No obstant, la menor biodisponibilitat de NO causada per una disminució de la seva síntesi o un augment de la

velocitat de degradació constitueix el fenomen més primerenc i important de la disfunció endotelial.

En un gran nombre de malalties cardiopulmonars com la HP primària i secundària[31], la MPOC i la insuficiència cardíaca congestiva, el balanç de la producció endotelial pulmonar de mediadors vasoconstrictors i vasodilatadors es troba alterada donant lloc a disfunció endotelial. Així ho va demostrar Dinh-Xuan [38] en un estudi realitzat en pulmons explantats de pacients amb MPOC a qui se'ls va practicar transplantament pulmonar. S'observà que les artèries pulmonars no només desenvolupen una major tensió en resposta a un vasoconstrictor (fenilefrina) sinó que només són capaces de relaxar un 41% en front a un 81% dels controls a vasodilatodors dependents de la síntesi de NO (acetilcolina i ADP (difosfat d'adenosina)). Però la disfunció endotelial no és una característica exclusiva de persones amb malaltia sinó que també està present en les artèries de fumadors sans. Peinado et al[39] varen demostrar que la resposta vasodilatadora de les artèries pulmonars de fumadors amb funció pulmonar normal es localitzava en un estadi entremig entre els pacients amb MPOC moderada i subjectes no fumadors (Figura 3).

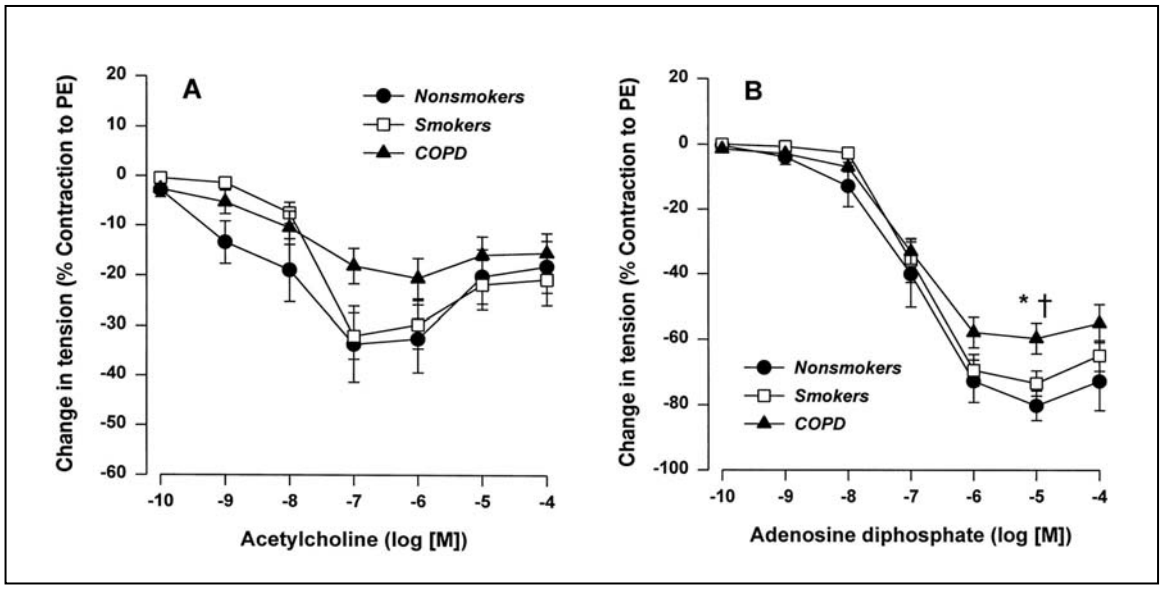

Figura 3. Corba dosi-resposta expressada en percentatge de canvi respecte a la contracció a fenilefrina (PE). Canvi de tensió d'artèries pulmonars en resposta a dosis acumulatives d'acetilcolina (A) i ADP (B) en no fumadors, fumadors i pacients MPOC. * *P*<0.05 comparat amb fumadors; † *P*<0.05 comparat amb no fumadors. (Adaptació de Peinado et al, 1998. Am.J.Physiol).

Aquestes dades suggereixen per primer cop que el fum del tabac podria ser la causa original de la lesió endotelial a la MPOC i que estaria actuant temps abans de que es desenvolupi o detecti la malaltia.

1.4.3.- Remodelat Vascular

El remodelat vascular és un procés patològic que causa engruiximent de la paret arterial fent que aquesta envaeixi el lumen i en redueixi el seu diàmetre i, conseqüentment, incrementi la resistència al flux sanguini. Aquesta alteració pot afectar a qualsevol tipus de vas i òrgan. Concretament, en el llit vascular pulmonar el remodelat afecta preferentment a vasos petits (per sota de 500 µm de diàmetre) i a artèries precapil·lars, característica patològica principal de la HP[39-41].

A la MPOC l'engruiximent de la íntima és el tret més destacat del remodelat vascular i es caracteritza per la proliferació cel·lular i l'acumulació de proteïnes de la matriu extracel·lular com la col·làgena i l'elastina[42]. Els canvis en la capa mitja són menys evidents i la majoria d'estudis no han demostrat diferències en el gruix de la muscular en aquests pacients. No obstant això, el remodelat de les artèries pulmonars no està restringit als pacients amb MPOC. De fet, l'engruiximent de la intima també està present en fumadors amb funció pulmonar normal i no difereix en magnitud de la que es troba en pacients amb MPOC[42]. Santos et al[42] varen caracteritzar el component cel·lular dels vasos de pacients MPOC i de fumadors i varen observar dos fenotips cel·lulars diferents responsables del remodelat de la íntima: cèl·lules α-actina$^+$/desmina$^+$ i cèl·lules α-actina$^+$/desmina$^-$ responent a una capacitat contràctil o sintètica respectivament. Aquests resultats assenyalen al tabac com al factor iniciador del remodelat en pacients MPOC ja que té lloc no només en estadis molt inicials de la malaltia sinó abans de que aquesta es desenvolupi.

Histològicament, els canvis arterials en HP inclouen totes les capes de la paret vascular i es caracteritzen per hiperplàsia de la íntima, hipertròfia de la mitja, proliferació a l'adventícia i trombosis *in situ*. Els canvis estructurals de la paret vascular varien en funció del factor estimulador, així doncs, humans que viuen a grans alçades (ambients hipòxics)

mostren hipertròfia de la capa mitja mentre que els pacients amb MPOC presenten canvis en la capa íntima. Aquestes diferències també estan presents en models animals, on s'ha observat que la ultraestructura de les cèl·lules musculars d'arterioles pulmonars de rates tractades amb monocrotalina divergeix de la de les rates sotmeses a hipoxia[43]. Així, en el cas de la monocrotalina, es troben miofilaments gruixuts perifèrics envoltats per làmines elàstiques, mentre que en el cas de la hipòxia, les cèl·lules musculars són madures amb miofilaments fins envoltats de làmines gruixudes. Aquestes diferències en les característiques patològiques segons el tipus d'estímul podria explicar la irreversibilitat de la HP associada a la MPOC. Mentre que la HP associada a grans alçades és reversible al tornar a condicions normòxiques, la HP i el remodelat vascular de pacients MPOC és només parcialment reversible amb oxigenteràpia.

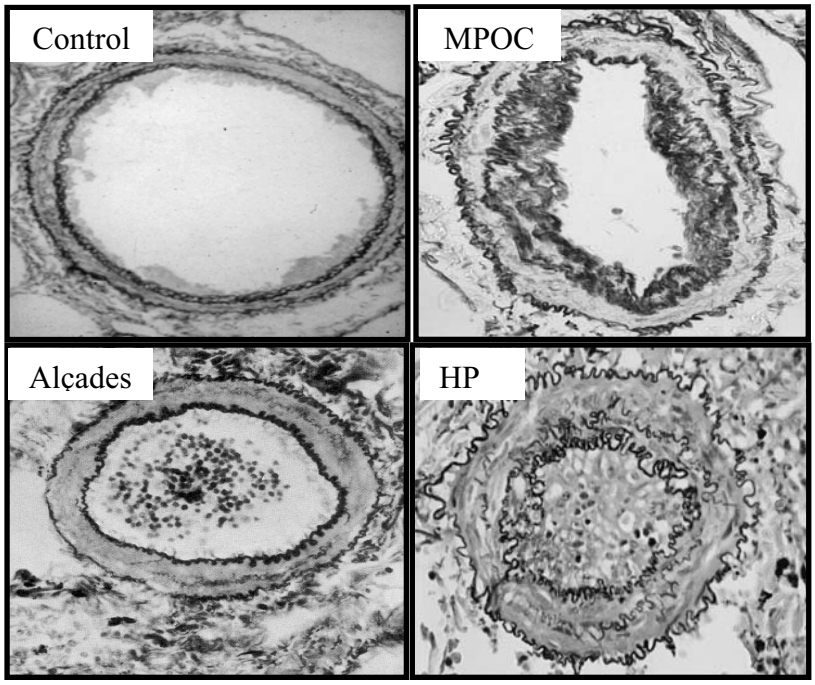

Figura 4. Tinció d'orceïna en seccions transversals d'artèries intrapulmonars de pacients amb MPOC, habitants en alçada i malalts amb HP. El remodelat vascular en pacients MPOC destaca per la hipertròfia a la capa íntima (més propera al lumen del vas) mentre que en HP i habitants d'alçades s'observa un engruiximent en la capa muscular delimitat per la làmina elàstica.

1.5.- Efectes sistèmics

Inicialment es considerava la MPOC com una malaltia exclusivament pulmonar, però la realitat és que també es tracta d'una malaltia sistèmica on estan implicats el sistema cardiovascular, musculatura esquelètica, moll d'os i metabolisme entre d'altres[44].

Un efecte sistèmic comú en aquests pacients és la limitació a l'exercici[1;2;45] que s'explica pel gran esforç requerit al respirar. Aquesta limitació a l'exercici no és deguda exclusivament a la dispnea o a l'edat avançada d'aquest tipus de pacient sinó a una fatiga a les cames[46;47]. La disfunció de la musculatura esquelètica és comú en pacients MPOC i contribueix significativament a la limitació de la capacitat d'exercici i la qualitat de vida[48].

La disfunció del múscul esquelètic a la MPOC afecta tant a músculs respiratoris, que pateixen una pèrdua de força i resistència, com a músculs perifèrics, especialment a les extremitats inferiors. Existeixen dos mecanismes diferents però a l'hora relacionats que expliquen aquests fenòmens: 1) pèrdua de la massa muscular i 2) disfunció de la musculatura restant. La disfunció muscular podria ser secundària a les alteracions intrínseques del múscul (anomalies de les mitocòndries, pèrdua de proteïnes contràctils) o a alteracions en les condicions a les que treballa el múscul (hipòxia, hipercàpnia, acidosis) que resulten del intercanvi pulmonar de gasos característic de la MPOC[49].

Un altre efecte sistèmic relacionat amb la MPOC és la pèrdua de pes. Aquest és un factor de mal pronòstic[50;51] independent d'altres indicadors que té l'avantatge de ser reversible si es tracta amb una teràpia apropiada fent que el pronòstic del pacient millori[51]. La desnutrició no només reflexa la pèrdua de pes sinó la pèrdua de massa corporal amb predilecció de la massa magra, sobretot els músculs. Les causes són múltiples però la més acceptada és la presència de inflamació sistèmica, tal com suggereixen altes concentracions sèriques del factor de necrosi tumoral alfa (TNF-α) en pacients amb valors baixos de bioimpedància[52]

La MPOC s'associa no només a una resposta inflamatòria anormal en el parènquima pulmonar[2] sinó també a una inflamació sistèmica que inclou estrès oxidatiu sistèmic, activació de les cèl·lules inflamatòries circulants i nivells elevats de citocines proinflamatòries[53]. La taula 2 resumeix el efectes sistèmics relacionats amb la MPOC.

Efectes sistèmics de la MPOC
Inflamació Sistèmica
Estrès oxidatiu Activació de cèl·lules inflamatòries (neutròfils/limfòcits) Increment dels nivells plasmàtics de citocines i proteïnes en fase aguda
Pèrdua de pes i anormalitats nutricionals
Increment de la despesa energètica in repòs Composició anormal del cos Metabolisme aminoàcids anormal
Disfunció de la musculatura esquelètica
Pèrdua de massa corporal Estructura i funció anormal Limitació de l'exercici
Altres efectes sistèmics potencials
Efectes cardiovasculars Efectes en el sistema nerviós Efectes osteoesquelètics [54]

Taula 2. Esquema dels efectes sistèmics associats a la MPOC. (Adaptació A. Agustí. ERJ. 2003).

2.- HIPERTENSIÓ PULMONAR ASSOCIADA A LA MPOC

La HP és una malaltia hemodinàmica definida per l'augment anòmal de la pressió arterial pulmonar mitjana (PAPm) ≥25mmHg en repòs i una pressió enclavada ≤15mmHg avaluada per cateterisme cardíac dret[55]. La HP pot presentar-se en múltiples situacions clíniques (taula 3).

Dins del grup de HP associada a malalties pulmonars, la MPOC és la malaltia més freqüent. És difícil estimar la incidència exacte de la HP a la MPOC ja que no s'ha analitzat utilitzant mètodes diagnòstics fiables. S'estima un valor elevat en la prevalença d'HP induïda per esforç (58% segons Kessles *et al)* mentre que en repòs és menys freqüent (5-30%)[56]. La progressió de la HP en pacients MPOC és lenta, i la PAP roman estable durant períodes de 3-10 anys[57].

Els canvis patològics de la HP associada a la MPOC inclouen la hipertrofia de la mitja i la proliferació obstructiva de la íntima de les artèries pulmonars distals. També es pot produir un grau variable de destrucció del llit vascular en zones emfisematoses o fibròtiques. Els

mecanismes biopatològics i fisiopatològics implicats en aquest procés són múltiples i inclouen la vasoconstricció hipòxica, la tensió mecànica dels pulmons hiperinsuflats, la pèrdua de capil·lars, la inflamació i els efectes tòxics del fum del tabac.

1. Hipertensió arterial pulmonar (HAP)
1.1. PAH idiopàtica
1.2.Heretable
1.2.1. BMPR2
1.2.2. ALK1, endoglina (amb o sense telangièctasis hemorràgica heretable)
1.2.3. Desconegut
1.3. Induïda per toxines o drogues
1.4. Associat a
1.4.1. Malalties del teixit connectiu
1.4.2. Infecció per VIH
1.4.3. Hipertensió portal
1.4.4. Malalties cardíaques congènites
1.4.5. Schistosomiasis
1.4.6. Anèmia hemolítica crònica
1.5. Hipertensió pulmonar persistent del nounat
1'. Malaltia pulmonar veno-oclusiva (PVOD) i/o hemangiomatosi capil·lar pulmonar (PCH)
2. Hipertensió pulmonar pertanyent al malaltia cardíaca esquerre
2.1 Disfunció sistòlica
2.2 Disfunció diastòlica
2.3 Malaltia vascular
3. Hipertensió pulmonar pertanyent a malalties pulmonars i/o hipòxia
3.1 Malaltia pulmonar obstructiva crònica
3.2. Malaltia pulmonar intersticial
3.3 Altres malalties pulmonars amb patró obstructiu i restrictiu
3.4 Desordres respiratoris de la son
3.5 Desordres de hipoventilació alveolar
3.6 Exposició crònica a grans alçades
3.7. Anormalitats del desenvolupament
4. Hipertensió pulmonar tromboembòlica crònica
5. Hipertensió pulmonar amb mecanismes multifactorials poc clars
5.1. Desordres hematològics: desordres mieloproliferatius, esplenectomia
5.2. desordres sistèmics: sarcoïdosis, histiocitosi pulmonar de les cèl·lules de Langerhans: limfangioleiomatosis, neurofibromatosis, vasculitis.
5.3. Desordres metabòlics: malaltia de l'emmagatzematge de glicogen, Malaltia de Gaucher, desordres de tiroides
5.4. Altres: obstrucció tumoral, mediastinitis fibrosant, fallada renal crònica en diàlisi
AKT1: activin receptor-like kinase type 1; BMPR2: bone morphometric protein recetor type 2: HIV human immunodeficiency virus

Taula 3: Classificació clínica actualitzada de la HP del 4rt Simposium Mundial de la HP de Dana Point, CA, USA[58].

Nombrosos estudis han demostrat que la presència de HP, tot i ser moderada, té una rellevància en el pronòstic de pacients amb MPOC. Al 1972, un estudi longitudinal de 7 anys amb 50 pacients amb MPOC va demostrar que la supervivència estava inversament relacionada amb la resistència vascular pulmonar[59]. Set anys més tard, el mateix autor va publicar un estudi de seguiment a 200 pacients durant 15 anys on demostrava que la presència o absència de HP era un dels factors predictius més potents de mortalitat[60]. Al 1981 Weitzenblum determina també el valor de PAP com a factor de predicció de supervivència[61] dividint la seva població d'estudi segons PAP per sobre o per sota de 20mmHg (Figura 5). En un estudi més recent, Oswald-Mammosser demostra que la PAPm és el millor factor predictiu de mortalitat[62] i estableix el límit de PAP en 25mmHg per sobre del qual la taxa de supervivència en la seva cohort de pacients en 5 anys és del 36% i per sota del qual la taxa de supervivència és del 62%. En aquest estudi ni el FEV_1, ni el grau de hipoxèmia o hipercàpnia varen tenir cap valor pronòstic.

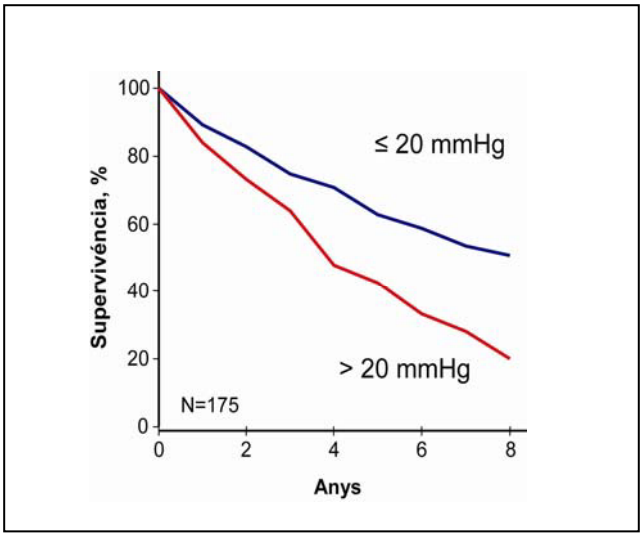

Figura 5. Percentatge de supervivència en un grup de 175 pacients amb MPOC segons els valors inicials de PAP (< 20 mmHg línea en blau, o > 20 mmHg línea vermella). Les diferències entre grups són significatives després de 4 i 7 anys. (Adaptació E. Weitzenblum, Thorax 1981).

2.1.- Factors implicats en la hipertensió pulmonar associada a la MPOC

La HP és una alteració hemodinàmica freqüent a la MPOC que pot estar causada pels mateixos factors relacionats amb el dany vascular. Principalment en diferenciem dos, el fum de tabac i la hipòxia.

Fum de tabac

El fum de tabac és un aerosol que conté 10^{10} partícules/mL, entre les quals hi ha metalls pesants, hidrocarburs aromàtics policíclics, aza-arenes, N-nitrosamines i altres substàncies orgàniques. Els components bàsics del fum de tabac identificats són: quitrà que es composa d'una gran varietat de productes químics orgànics i inorgànics (nitrogen, oxigen, hidrogen, diòxid de carboni i monòxid de carboni), nicotina, òxids de nitrogen, àcid cianhídric, metalls i compostos radioactius.

En la combustió d'una cigarreta el fum inhalat (o mainstream) representa aproximadament el 45% del total de la biomassa de la cigarreta, mentre que el 55% restant correspon al fum que es desprèn de la part incandescent de la cigarreta a l'ambient i que no s'inhala (sidestream).

El fum "mainstream" es divideix en una fase sòlida particulada (quitrà) i una fase gasosa (gasos tòxics, components orgànics volàtils, radicals lliures, etc). El quitrà de la cigarreta conté elevades concentracions de radicals lliures estables amb temps de vida molt llargs. El fum "sidestream" es divideix també en una fase gasosa i una fase sòlida, contenint concentracions més elevades de components tòxics i carcinògens i altres compostos volàtils i semivolàtils[63]. Els radicals lliures i els oxidants de la fase gasosa romanen en un estat de creació continua en el qual s'estan creant i destruint constantment i on la seva concentració augmenta a mesura que el fum es va formant[64;65]. La fase gasosa suposa al voltant de 0.4-0.5g/cigarreta i conté substàncies volàtils orgàniques i inorgàniques. La part particulada (quitrà) consisteix en partícules fines (0.1-1.0 μm) que penetren en profunditat dins l'alvèol. Alguns dels components solubles de la part aquosa del quitrà poden produir anions superòxids ($O_2^{\cdot-}$) i subseqüentment H_2O_2 i el radical hidroxil reactiu ($HO^{\cdot}$), que poden causar dany oxidatiu als lípids de membrana cel·lular, enzims i DNA[66]. El fum "sidestream" consisteix en compostos químics similars en la fase aquosa i gasosa i a més és ric en

radicals lliures de vida curta i altament reactius. S'ha demostrat que l'exposició ambiental a fum de tabac (fumadors passius) és també un risc per la salut i una càrrega que afavoreix la majoria de les malalties respiratòries. L'exposició a tabac involuntària o ambiental es defineix com una exposició combinada del fum "mainstream" exhalat i del fum ambiental de la combustió de la part final de la cigarreta (sidestream)[67;68].

Hipòxia alveolar

Diversos processos fisiològics estan implicats en la HP associada a la MPOC. Entre ells es troba la hipòxia, un potent estimulador de la vasoconstricció pulmonar. En estat d'hipòxia aguda, les artèries pulmonars adjacents a l'alvèol hipòxic es contrauen. Com a resultat, la sang es redistribueix als segments pulmonars millor ventilats per tal de mantenir la relació ventilació-perfusió (V_A/Q) [69;70]. Aquest procés de vasoconstricció pulmonar hipòxica (VPH) afecta principalment a les artèries musculars de resistència i a venes de petit calibre (<200μm[71] i <900 μm[72] respectivament). La VPH s'inicia segons després de la hipòxia alveolar i reverteix quan es restaura la normoxia. La resposta vasoconstrictora a hipòxia és específica del pulmó ja que, tant en humans com en animals, la hipòxia dilata les artèries sistèmiques[73].

Amb exposicions prolongades a hipòxia les artèries pulmonars musculars i les arterioles pateixen un procés de remodelat vascular que es caracteritza per la hipertròfia de la capa muscular. Aquest fenomen dóna lloc a un increment en la resistència vascular pulmonar persistent i un increment en la càrrega del ventricle dret que finalitza amb una hipertrofia ventricular. És interessant el fet de que, a diferència d'altres hipertensions pulmonars (ex. Idiopàtica), no hi ha hipertròfia de la capa íntima, suggerint que el mecanisme responsable de la HP hipòxica es localitza a la cèl·lula muscular llisa de les artèries pulmonars de menor calibre.

Diversos estudis han intentat esbrinar els mecanismes de la resposta VPH. Els estudis més recents s'han centrat en els mecanismes de sensibilitat de l'oxigen en cèl·lules musculars llises de les artèries pulmonars ja que sembla que es contrauen quan les artèries de

resistència pulmonars estan exposades a hipòxia en absència d'altres tipus cel·lulars[73;74]. Per contra, l'alliberació de NO des de l'endoteli pulmonar sovint suprimeix la VPH de tal manera que pot ser alterada per l'estat fisiològic de la circulació pulmonar i la progressió dels processos vasculars de la malaltia[75;76].

Exposicions prolongades de la circulació pulmonar a hipòxia donen lloc a remodelat vascular, especialment a la capa muscular, probablement originant canvis en l'equilibri entre el NO i els mecanismes implicats en detectar hipòxia. Per contra als seus efectes en la circulació pulmonar, la hipòxia normalment produeix vasodilatació en la circulació perifèrica associada a una millora de l'aportació d'oxigen als teixits. Les teories actuals de com es detecten els nivells d'oxigen per part de la capa muscular llisa de les artèries pulmonars es mou al voltant de la modulació de la biosíntesi de les espècies reactives d'oxigen (ROS) tals com el peròxid d'hidrogen (H_2O_2) i/o canvis en l'oxidació/reducció o l'estat redox dels sistemes metabòlics cel·lulars com el NADPH/NADP, les quals estan estretament relacionades amb la generació i metabolisme de les ROS[77-79].

Altres factors relacionats

Policitèmia

La policitèmia secundaria pot ser una resposta adaptativa a la hipoxèmia. La policitèmia pot donar lloc a alteracions en el to vascular i a increments en la PAP[80] degut a un increment en la viscositat de la sang. En estudis realitzats en gossos[81] Mc Grath demostra que la policitèmia per si sola incrementa la resistència vascular pulmonar un 112% i la hipòxia sola un 141%. Quan es combinen hipòxia i policitèmia la resistència vascular pulmonar incrementa un 308%.

Inflamació

La inflamació induïda pel fum de tabac és evident a totes les estructures del pulmó inclosos els vasos pulmonars. En pacients amb MPOC fumadors amb PAP normal es troben nombres elevats de leucòcits infiltrant l'adventícia, especialment limfòcits CD8+. És interessant recalcar que el increment del nombre de limfòcits es correlaciona amb la

disminució de la relaxació vascular dependent d'endoteli en anells d'artèries pulmonars així com un engruiximent del gruix de la íntima de les artèries pulmonars[39].

Citosines inflamatòries com la Interleukina-6 (IL-6), la proteïna C reactiva (PCR), el TNF-α s'associen a PAP elevada en la MPOC[82;83]. A més, existeix una associació entre el polimorfisme del gen de la IL-6 amb nivells elevats de IL-6 en sèrum i amb una PAP més elevada en la MPOC[82].

Estrès de Fricció

Líquids viscosos movent-se per una estructura cilíndrica creen un estrès friccional sobre la paret del vas. Si el flux es manté constant, a mesura que el diàmetre luminal disminueix amb la vasoconstricció, l'estrès de fricció augmenta dins el vas. Aquest estrès constant dins la paret del vas causa un increment de la degranulació de les plaquetes, increment de l'alliberament de citosines inflamatòries, increment de l'expressió de molècules d'adhesió, disminució de la producció de NO derivat d'endoteli i major producció d'ET[84;85].

Disfunció endotelial.

Els pulmons dels pacients MPOC mostren una pitjor vasodilatació mediada per l'endoteli. A més, estudis in vitro d'anells d'artèries intrapulmonars d'aquests pacients revelen una resposta a ADP disminuïda[39].

3.- MODELS ANIMALS EN LA MALALTIA PULMONAR

La primera pregunta a respondre és què constituiria un model animal perfecte de MPOC. Aquesta no és una pregunta fàcil ja que la MPOC en humans es produeixen 4 canvis anatòmics com a mínim: emfisema, remodelat bronquial (inclou metaplàsia de cèl·lules caliciformes), bronquitis crònica i HP.

Un bon model animal hauria de tenir una anatomia pulmonar semblant a la humana. Per contra el disseny d'un model animal de MPOC acostuma a trobar inconvenients tals com 1) diferències considerables entre espècies en el desenvolupament i maduració del teixit

pulmonar, 2) diferències entre espècies en l'anatomia pulmonar 3) susceptibilitat diferent als agents causants de la malaltia entre espècies i soques d'una mateixa espècie.

S'han desenvolupat diferents models animals d'alteracions pulmonars atenent als canvis anatòmics més importants relacionats amb la MPOC. En molts casos s'ha utilitzat un agent específic per induir específicament algunes de les alteracions més significatives de la MPOC. Aquests models són:

3.1.- Models d'emfisema

Elastasa

Actualment no es coneix del cert la causa de la formació de l'emfisema però la hipòtesi més acceptada és la proteasa-antiproteasa. Aquesta hipòtesi sorgeix de l'observació de dos fets claus: 1) el descobriment de que pacients amb un desenvolupament primerenc de la malaltia i historial familiar amb emfisema eren deficients en α-1-antitripsina[86] i 2) la descripció en hàmsters de la inducció d'emfisema per instil·lació de la proteasa de la papaïna[87]. Aquestes observacions van donar lloc a la idea de que l'emfisema associat a la MPOC es desenvolupa com a resultat del flux inflamatori mediat pel fum de tabac cap al tracte respiratori inferior que dóna lloc a l'alliberament de proteases que destrueixen la matriu del parènquima.

Aquesta hipòtesi es va formular en gran part a partir dels experiments en que es provoca emfisema per l'administració d'enzims elastolítics ja sigui per instil·lació traqueal o per inhalació de l'aerosol. S'ha utilitzat un gran nombre d'enzims amb el propòsit d'induir emfisema[88-91] i a partir d'aquests estudis s'ha trobat que els únics enzims capaços de degradar elastina intacta eren els que provocaven emfisema. Les col·lagenases per si mateixes són ineficaces[91;92].

Mentre que la papaïna s'utilitzava en els primers estudis, gairebé tots els estudis actuals utilitzen elastasa pancreàtica porcina (PPE) o elastasa de neutròfil humà (HNE). És important destacar que l'acció de PPE i HNE no tenen el mateix espectre proteolític; tenen

diferents inhibidors endògens primaris (α_1-antitripsina per HNE i α_2-macroglobulina per PPE, tot i que α_1-antitripsina també és efectiva) i el que és més important, un inhibidor exogen particular pot no inhibir tots dos enzims.

La inducció d'emfisema sever amb la instil·lació d'elastasa és relativament fàcil, contràriament al que passa amb altres models en que només s'indueix un lleu emfisema. No obstant, els mecanismes pels quals l'elastasa indueix emfisema no estan del tot clars. Els estudis realitzats demostren que immediatament després de la instil·lació de l'enzim hi ha una pèrdua ràpida d'elastina acompanyada d'hemorràgia i exsudat inflamatori que inclou macròfags i neutròfils en el tracte respiratori baix. També hi ha sobrexpressió d'una gran varietat de mediadors proinflamatoris com el TNFα, IL-1β, IL-6 i IL-8[93], però la lesió progressa més enllà un cop ja no es detecten nivells d'elastasa.

A més de l'emfisema, la instil·lació d'elastasa a pulmons dóna lloc a alveolitis aguda (infiltrat neutrofílic i de cèl·lules limfomononuclears), metaplàsia de cèl·lules caliciformes, edema pulmonar, hemorràgia i trencament de l'epiteli respiratori[94-97]. Aquests canvis donen lloc a alteracions en la funció pulmonar consistents amb els canvis característics de la MPOC[94;95]. Una de les desavantatges més grans d'aquest model és el fet de que la inflamació és temporal i es resol després d'una setmana de l'administració de l'elastasa. Un aspecte interessant d'aquest model és que els canvis emfisematosos són progressius fins a unes 8 setmanes, molt després de que la inflamació ja hagi desaparegut, suggerint la contribució d'altres factors, no coneguts, a la destrucció progressiva del pulmó.

L'avantatge més gran que aporta el model de l'elastasa per sobre d'altres models MPOC *in vivo* és la rapidesa del inici de l'aparició de l'emfisema. La destrucció de les parets alveolars comença a les 2 setmanes després de l'administració d'elastasa, empitjora al llarg del temps i és irreversible[94;95]. Per aquest motiu el model de l'elastasa és ideal per testar teràpies que intentin revertir i reparar el dany emfisematós.

Fam

En humans, la desnutrició crònica s'associa a alteracions semblants a les de l'emfisema del parènquima pulmonar[98]. Estudis realitzats en models de fam en rata difereixen en la quantitat de reducció del menjar, en el grau d'alteració de l'estructura pulmonar i fins i tot en els canvis fisiològics[99-103]. En tot cas, les alteracions en la mecànica pulmonar d'aquests animals es caracteritza per una disminució en el volum pulmonar en relació a les pressions transpulmonars. Aquest no és un model irreversible ja que en retornar a la ingesta normal d'aliment els canvis mecànics reverteixen[100]. Cicles de fam i realimentació resulten en els mateixos efectes, però a més s'observa una reducció en l'àrea de la superfície alveolar interpretat com una disminució del nombre d'alvèols[104].

Sorprenentment, la depleció de calories acompanyada de depleció de proteïnes resulta en un emfisema més lleu que el d'aquells animals amb una depleció similar de calories però amb una aportació normal de proteïnes. Els grups d'animals privats d'ingesta calòrica mostren volums pulmonars menors i menor nombre d'alvèols que els controls indicant que els efectes de la fam són deguts a una manca de creixement.

Un estudi comparatiu entre el model de fam (45% de pèrdua de pes) i el model d'emfisema induït per elastasa mostra diferències considerables entre els dos models[105]. El model d'elastasa reprodueix l'emfisema humà amb un increment del volum pulmonar, un desplaçament a la dreta de la corba ventilació-perfusió i una reducció del flux expiratori. No obstant, en el model de fam els volums pulmonars no incrementen i no hi ha disminució del flux aeri tot i que sí s'observa un desplaçament a la dreta de la corba ventilació-perfusió. Tots dos grups mostren espais aeris incrementats suggerint que els dos models poden representar destrucció pulmonar, emfisema en el cas de l'elastasa, i alteracions en el creixement pulmonar en el cas del model d'emfisema induït per fam. Estudis recents en ratolí[106;107] han qüestionat aquesta explicació ja que els alvèols desapareixen ràpidament en dietes pobres en calories i tornen a aparèixer amb la realimentació. La fam estimula l'expressió del gen de la caspasa i la depressió de la replicació cel·lular, mentre que la

realimentació incrementa la síntesis de DNA cel·lular i un increment en l'expressió de RNA d'un gran nombre de gens implicats en la replicació. Per tant, el model de fam tot i ser un model relativament ràpid que incrementa els espais entre les parets alveolars, no produeix en realitat canvis fisiològics d'emfisema i s'assembla més a un model de creixement pulmonar anormal o de reparació anormal. En aquest model no s'observen alteracions a nivell vascular i de via aèria.

Apoptosi

La idea de que l'emfisema es dóna degut a una fallada en el manteniment i la reparació del pulmó sorgeix dels estudis on s'ha induït apoptosis mitjançant la interferència del VEGF[108], del seu receptor VEGFR[109] o del complex dels dos factors[110].

L'apoptosi generalment implica l'activació de la cascada de caspasa i es pot induir per instil·lació intratraqueal de la caspasa 3 activada[111] o per l'activació de la ceramida, un regulador localitzat per sobre en la cascada[112]. Aquests models indueixen engrandiment dels espais alveolars en un període molt curt de temps però no afecten a les vies respiratòries. A més, la inhibició del receptor de VEGF produeix una reducció del llit vascular i HP[108;113;114] amb muscularització de les artèries pulmonars petites.

Tot i això, cap d'aquests models s'ha demostrat que estigui associat amb el trencament de la matriu, un fet que es crucial en la patogènesis de l'emfisema.

3.2.- Models de inflamació

Lipopolosacàrids

La inflamació és una alteració característica constant en la MPOC i en altres patologies respiratòries a la qual se li atribueixen diversos efectes patològics com la disfunció endotelial, el remodelat vascular o l'emfisema. Per això és de gran rellevància desenvolupar un model animal que impliqui inflamació.

El lipopolisacàrid (LPS) és un glicolípid component de la paret cel·lular dels bacteris gramnegatius que s'utilitza pels seus efectes proinflamatoris. Aquesta endotoxina d'origen bacterià està present de forma concomitant en el fum de tabac, pol·lució i pols orgànics.

La instil·lació de LPS a la via aèria indueix inflamació neutrofílica pronunciada donant un pic entre les 6-24h després de l'administració[115]. L'administració crònica de LPS també dóna lloc a remodelat de la via aèria, emfisema i alteració de la funció pulmonar[116-118]. Tot i ser robust, la rellevància clínica d'aquest model és qüestionable en tant que la inflamació que indueix és inhibida per glucocorticoides[94;95]. A més, administracions repetides de LPS atenuen la inflamació neutrofílica al induir tolerància immune[118],i per tant no reflecteix la inflamació progressiva i insensible a esteroides que caracteritza la MPOC.

En el model agut, LPS indueix un tipus de reacció inflamatòria mixta amb un increment en el nombre de neutròfils[119], increment en TNFα i IL-1β en el rentat broncoalveolar (BAL) i increment de les metal·loproteïnases MMP-9, MMP-12[120;121].

La instil·lació crònica de LPS (durant varies setmanes) produeix engrandiment dels espais alveolars i remodelat de les vies aèries, amb parets més gruixudes i increment en el nombre de cèl·lules caliciformes en la via aèria superior.

3.3.- Models de hipertensió pulmonar

Hipòxia crònica

La hipòxia normobàrica i hipobàrica s'utilitzen freqüentment per induir HP en una gran varietat d'animals. Aquest és un model útil per que és predictible i reproduïble dins una mateixa soca d'animal. No obstant, hi ha certa variabilitat en la resposta a hipòxia entre espècies. A més, les respostes varien significativament amb l'edat ja que els individus joves amb els pulmons en ràpida maduració són més sensibles a aquest factor[122]. Els animals més utilitzats són la rata i el ratolí. La muscularització dels petits vasos no-muscularitzats de les parets alveolars comença ràpidament degut a l'aparició de cèl·lules que expressen α-actina de cèl·lula muscular llisa. En certes soques de rata s'observa

engruiximent i fibrosis de les grans artèries pulmonars proximals que demostren un increment de la seva rigidesa[123]. Després de 2 setmanes d'hipòxia, les rates desenvolupen HP podent duplicar els valors de la pressió mitja de l'artèria pulmonar. Aquest augment correlaciona amb els canvis estructurals. Hi ha presència d'hipertròfia del ventricle dret però no hi ha evidències de fallada del ventricle.

Els ratolins exposats a hipòxia, tot i causar un increment de la pressió de l'artèria pulmonar, només presenten petits canvis en el remodelat vascular, sent marcadament inferior que en el cas de les rates[124-127]. Les respostes a hipòxia en ratolins són dependents de la soca i la comparació intraespècies pot variar significativament dependent de les soques que es comparin.

Monocrotalina

La monocrotalina és un alcaloide de pirrolicidina tòxic present a la planta *Crotalaria spectabilis*. La ingestió de monocrotalina resulta en un desenvolupament progressiu de HP en varies espècies animals. Es va descriure per primera vegada fa més de 40 anys en un model de rata amb repetides ingestions orals [128]. Se sap que les oxidases del fetge activen *in vivo* el pirrol de monocrotalina donant lloc a la seva forma reactiva que desencadena el dany vascular. El models de monocrotalina, particularment en rates, es poden aconseguir amb una única dosi subcutània o intraperitoneal fent que aquest sigui un model molt simple i accessible per un gran ventall de investigadors. Desafortunadament la resposta a monocrotalina és diferent segons les espècies, soques i fins i tot entre individus degut a les diferències en el metabolisme hepàtic de la citocrom P-450. L'espècie més utilitzada pel model de monocrotalina és la rata. En animals més grans s'ha aconseguit desenvolupar HP per injecció directa del metabòlit tòxic dihidromonocrotalina [129].

Tot i no conèixer amb exactitud el mecanisme pel qual la monocrotalina produeix HP, s'especula que directament causa dany endotelial i que després activa el desenvolupament i la progressió de la hipertensió severa i letal[130]. Això es basa en les observacions que mostren que el increment de la pressió arterial pulmonar i el remodelat

vascular està endarrerit fins a 1-2 setmanes després de la dosi de inici[131]. Altres investigadors han suggerit que els increments de la pressió arterial pulmonar i el remodelat vascular són deguts a l'acumulació massiva de cèl·lules inflamatòries mononuclears en la capa adventícia dels petits vasos intraacinars[132]. Aquests canvis tenen lloc a artèries i venes pulmonars i sorgeixen abans de la hipertrofia muscular de la capa mitja. Alguns investigadors han evidenciat canvis en les venes, qüestionant-ne la validesa com a model d'hipertensió arterial pulmonar[132-134].

El model animal de monocrotalina desenvolupa hipertrofia i disfunció del ventricle dret, factor important en els models de HP. Dosis altes de monocrotalina durant 5 setmanes provoquen una pressió sistòlica del ventricle dret de 80 mmHg associada a una baixa taxa de supervivència (35%)[130;131;135]. Malauradament hi ha evidències de que la monocrotalina causa miocarditis que afecta tant al ventricle dret com a l'esquerre, el que complica l'estudi de la hipertròfia del ventricle dret associat a HP[136].

Combinació de Models

S'han dedicat esforços a crear models que indueixin dany pulmonar vascular progressiu amb canvis en la neoíntima encara que aquests models de vegades són més propers a models de HPA que MPOC:

1) model de monocrotalina i pneumonectomia. Segueix la teoria de que en situacions on les condicions hemodinàmiques pulmonars estan alterades, els efectes de la monocrotalina sobre la circulació pulmonar es veurien incrementats[137].

2) hipòxia combinada amb Sugen 5416 (inhibidor del receptor de VEGF). Es basa en el concepte de que VEGF és un factor important pel manteniment i diferenciació de les cèl·lules endotelials vasculars i que al inhibir el seu receptor combinat amb hipòxia s'observa una HP irreversible associada a proliferació endotelial en precapil·lars [113].

3) model de BMPR2, ja que BPMPR2 juga un paper crític en la patogènesis de la hipertensió arterial pulmonar idiopàtica i en la familiar[138;139].

4) sobrexpressió de IL-6 en ratolins[124].

3.4.- Model animal de MPOC

Models genètics

Un pas endavant en el desenvolupament de models animals és l'ús de les eines genètiques. Els ratolins ofereixen els avantatges de baix cost, disponibilitat d'una extensa oferta de gens/proteïnes i seqüències/anticossos, i el que és més important, una gran quantitat de soques mutants naturals (Tight skin, beige, pallid), línies de ratolins knockouts (receptor de l'àcid retinoic $g^{-/-}$, sulfactant $D^{-/-}$) i línies de ratolins transgènics que sobrexpressen certs mediadors (IL-13, IL-18, IFN-γ (interferó gamma)) que desenvolupen engrandiment de l'espai alveolar. Aquestes soques tenen diferents respostes a l'exposició a fum de tabac i donen la possibilitat de conèixer els mecanismes que estan directament implicats en la inflamació i els processos específics que succeeixen dins la MPOC.

Els models animals genèticament alterats permeten la investigació dels efectes específics d'un gen o proteïna en les diferents lesions de la MPOC i són útils en el disseny d'agents terapèutics. Però la MPOC no és una malaltia deguda a l'alteració d'un sol gen o proteïna, per això el model ideal seria una combinació de diverses variants al·lèliques capaç de reproduir un malaltia amb diferents síndromes com la MPOC.

Exposició a fum de tabac com a model de MPOC

Degut a que la causa principal de la MPOC és el tabac, l'ús de l'exposició a tabac com a model animal de MPOC seria el model més lògic a utilitzar. Però dins d'aquest model hi ha diferents variables:

Mètodes d'exposició a fum de tabac: actualment s'han desenvolupat dos tipus d'exposició: exposició directe (nose-only) i exposició ambiental (whole body). En aquest darrer mètode s'afegeix l'inconvenient de que els animals al ser exposats ambientalment poden ingerir nicotina o components del quitrà al netejar-se la pell. No obstant, tot i ser un factor

important, s'ha demostrat que els animals exposats de forma ambiental presenten menys carboxihemoglobina en sang i menys pèrdua de pes que els animals exposats amb el mètode directe (nose-only)[140].

Al establir un mètode d'exposició a fum de tabac és útil mesurar els nivells de cotinina (metabòlit de la nicotina) o la carboxihemoglobina (COHb) en sang per tal de determinar la quantitat relativa de tabac fumat. Wright et al. troben en model agut nivells de COHb 5% i en model crònic nivells del 15-20%. Nivells de cotinina al voltant de 200-250 ng/ml equivalen a un fumador moderat.

Elecció i limitació de les espècies: Actualment s'utilitzen tot tipus d'animals en el model d'exposició a fum de tabac: ratolí, rata, conill porquí. Els conills porquins ofereixen nombrosos avantatges entre els quals destaquen emfisema fàcilment reconeixible, remodelat de la via aèria més marcat que en ratolins i desenvolupament de metaplàsia de cèl·lules caliciformes a la via aèria petita. Per contra, el gran desavantatge que presenta el conill porquí és el seu alt cost, la falta d'eines per estudis moleculars i el nombre limitat d'anticossos. Per altra banda, les rates desenvolupen canvis lleugers i si són exposades massa severament presenten efectes per sobrecàrrega de partícules no específiques[141] que poden donar lloc a magnificar alguns efectes.

L'avantatge més gran que té aquest model respecte els altres models és la capacitat de utilitzar l'agent principal causant de la malaltia per reproduir diferents característiques de la MPOC en animals. Tot i que fa dècades que s'utilitza el fum de tabac per replicar malalties pulmonars en animals petits (com l'estudi de Mertens al 1930 on investiga la relació del fum de tabac i el càncer[142]), no va ser fins al 1990 que es començà a treballar en aquest model com a sistema per mimetitzar la patologia de la MPOC. Diversos grups han demostrat que l'exposició a fum de tabac no només produeix emfisema sinó metaplàsia i hipertròfia epitelial a la via aèria principal així com remodelat fibròtic a la via aèria petita, ambdós consistents amb els canvis observats en pacients MPOC[141;143;144]. Tot i

això, les patologies i els canvis en la funció pulmonar associats a aquest model animal equivaldrien a un grau lleu de severitat en humans.

Contràriament al que passa en altres models animals, l'exposició a fum de tabac produeix inflamació que no reverteix amb el tractament amb glucocorticoides[145] per tant sembla que aquest model sí que reprodueix de maneres efectiva el progrés lent i insensible a esteroides associat a la MPOC en humans.

Model	Tractament	Pros	Contres
Emfisema	Elastasa	Fàcil de mesurar els canvis funcionals Tractament únic, econòmic i fàcil Ràpid Pot produir severitat de forma dosi depenent També produeix inflamació (transitòria)	Rellevància de teràpia poc clara És crucial una bona elecció dels enzims, espècie i edat de l'animal, dosi i protocol Diferent acció de l'elastasa de neutròfil o l'elastasa pancreàtica porcina L'emfisema d'elastasa és diferent al de tabac
	Fam	Model de curta durada Resultats relativament consistents	No encaixa amb els canvis fisiològics humans Pot representar un model de manteniment/reparació anormal del creixement pulmonar Consideracions ètiques de la inducció de pèrdua d'un 45% del pes per fam
	Apoptosi	Produeix engrandiment dels espais aeris i remodelat vascular Model de curt termini	L'apoptosi no està present en el model d'emfisema induït per fum de tabac No hi ha remodelat vascular Emfisema no és permanent
Inflamació	LPS	Model de curta durada amb canvis a la via aèria i parènquima i regulació a l'alça de citocines inflamatòries Útil en recerques adreçades a l'exacerbació	Infiltrat inflamatori no és anàleg al que es troba amb fum de tabac No està clar si els mecanismes implicats recapitulen les lesions induïdes per tabac
Hipertensió Pulmonar	Hipòxia	Reproduïble dins una mateixa soca Ràpid (en 2 setmanes desenvolupa HP)	Variabilitat intra i interespècies Reversible al tornar a condicions de normoxia
	Monocrotalina	Una única dosi Accessible i baix cost	Diferent resposta a monocrotalina segons l'espècie No es coneix el mecanisme d'actuació Afecta a ventricle dret i esquerre
MPOC	Models genètics	Baix cost Gran oferta de gens, proteïnes, anticossos, seqüències i soques	Només seqüenciat el genoma de ratolí
	Exposició a fum de tabac	Induït pels mateixos estímuls que en humans Produeix emfisema, remodelat de la via aèria, remodelat vascular i HP en determinades espècies Alteracions fisiològiques semblants a humans	No produeix la severitat observada en humans Requereix diversos mesos d'exposició Les lesions no progressen un cop es deixa l'exposició a tabac

Taula 4. Taula resum d'avantatges i desavantatges dels models animals utilitzats en recerca per l'estudi d'alteracions pulmonars relacionades amb la MPOC i la HP.

FONAMENT DE LA TESI DOCTORAL

Tal com s'ha exposat a la introducció, el grup de recerca ha proposat la hipòtesi de que les alteracions vasculars pulmonars que tenen lloc a la MPOC poden estar originades pels efectes del fum de tabac. Per tal de confirmar i aprofundir en aquesta hipòtesi és de gran interès disposar d'un model experimental que pugui ser objectiu d'estudis detallats i d'interaccions. El model de cobai exposat a fum de tabac és el que més s'acosta a la malaltia en humans i en el que s'observen canvis consistents a les artèries pulmonars. Per tant, el primer objectiu d'aquesta tesi ha estat desenvolupar aquest model al nostre medi i validar-lo. El segon objectiu ha estat avaluar l'efecte del tabac en la funció endotelial i descriure les característiques cel·lulars dels canvis que ocorren a la paret vascular, ja que no hi ha informació al respecte. El tercer objectiu ha estat comparar els efectes vasculars del tabac i la hipòxia així com els seus efectes, per separat o en combinació, ja que poden explicar el que ocorre en humans.

A més dels estudis assenyalats, que constitueixen el nucli central de la tesi, el model experimental desenvolupat al nostre laboratori ha estat utilitzat per altres equips de recerca i la doctoranda ha contribuït, en el que fa referència al treball experimental amb aquest model, en dos treballs addicionals que es presenten en forma d'annex en aquesta tesi doctoral.

HIPÒTESIS

Les hipòtesis en que es fonamenten els treballs d¡aquesta tesi Doctoral són (Figura 6):

1ª) El consum de tabac és el principal agent etiopatogènic de les alteracions de la circulació pulmonar i dels canvis sistèmics que es produeixen a la MPOC. Per aquest motiu considerem que l'exposició crònica a fum de tabac en cobais produirà canvis estructurals i funcionals a les artèries pulmonars similars als que s'observen en pacients amb MPOC. Específicament esperem trobar canvis en el territori vascular pulmonar, en els que es produirà disfunció endotelial, proliferació cel·lular als vasos intrapulmonars i canvis en l'expressió de substàncies de síntesi endotelial amb propietats vasoactives.

2ª) Els components del fum de tabac produeixen disfunció endotelial i promouen el remodelat vascular pulmonar, alteracions que tenen lloc en els primers estadis de la MPOC. En estadis més avançats de la malaltia disminueix la PO_2 alveolar i apareix hipoxèmia. La hipòtesi que es planteja és que els animals exposats a fum de tabac són més susceptibles a l'acció de la hipòxia, ja que aquesta s'afegeix a canvis preexistents a l'endoteli i a l'estructura de les artèries pulmonars. Per tant, la combinació de fum de tabac i hipòxia donarà lloc a canvis més marcats en les artèries pulmonars que els produïts per cadascun dels estímuls per separat donant explicació a les possibles causes de progressió de la malaltia.

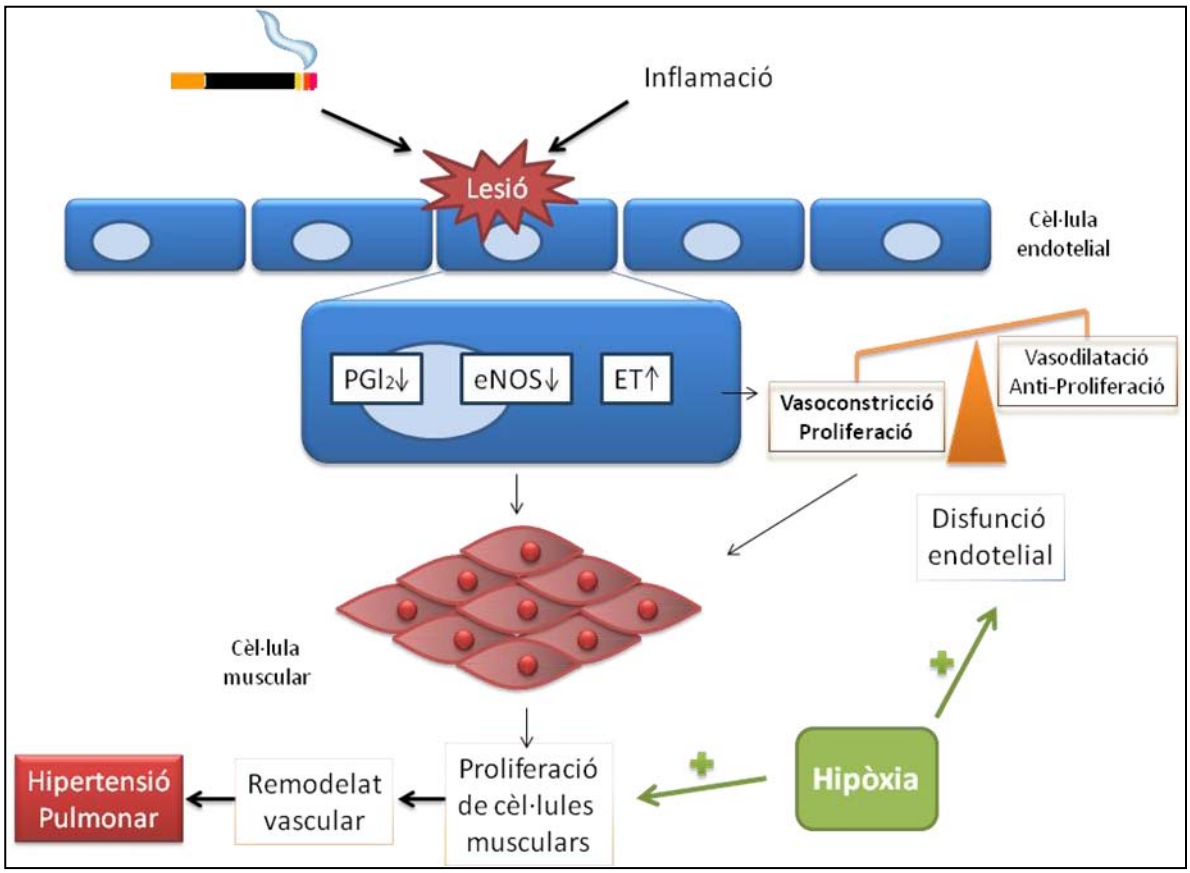

Figura 6. Esquema resum de les hipòtesis dels estudis. L'origen de la lesió endotelial es troba en l'efecte nociu del fum de tabac juntament amb l'acció de mediadors pro-inflamatoris. Aquest atac sobre la cèl·lula endotelial afecta directament l'activitat i síntesi de mediadors vasoactius produint un desequilibri a favor de la síntesi d'agents vasoconstrictors que promouen la proliferació cel·lular com l'ET, i en contra de la síntesi d'agents vasodilatadors i anti-proliferatius com la PGI_2 i el NO. Aquesta alteració té dues conseqüències transcendentals: la disfunció de l'endoteli per regular el to vascular i la proliferació descontrolada de les cèl·lules musculars de la capa mitja del vas. En aquest punt, la hipòxia actua afavorint i potenciant tots dos processos patològics. Si les exposicions a fum de tabac i hipòxia són sostingudes i perduren en el temps, la proliferació cel·lular donarà lloc al remodelat reduint així el diàmetre de la llum del vas i incrementant la resistència vascular. Tota aquesta cadena de fets desembocarà en un increment de la pressió de l'artèria pulmonar.

OBJECTIUS

D'acord amb les hipòtesis descrites, els objectius plantejats en els dos estudis principals que conformen la tesi Doctoral són:

1.- Primer estudi. ***Effects of cigarette smoke on endothelial function of pulmonary arteries in the guinea pig.***

Objectiu general

Desenvolupar un model experimental en cobai de malaltia pulmonar induïda per la exposició a fum de tabac, dirigit a avaluar els mecanismes patogènics de les alteracions que tenen lloc a la circulació pulmonar i establir-ne l'ordre de la seqüència de canvis que es produeixen.

Objectius específics

En aquest model experimental ens proposem determinar el moment de inici i l'evolució de:

1.- la disfunció endotelial

2.- els canvis en l'estructura de l'artèria pulmonar

3.- la composició cel·lular dels vasos intrapulmonars

4.- l'expressió de molècules amb acció sobre la funció i el remodelat vascular (molècules de síntesi endotelial, factors de creixement)

5.- l'emfisema pulmonar

2.- Segon estudi. ***Effects of cigarette smoke and hypoxia on the pulmonary circulation in the guinea pig***

Objectiu general

Avaluar les similituds i diferències en els canvis vasculars pulmonars induïts per l'exposició crònica al fum de tabac i els produïts per la hipòxia sostinguda, així com els efectes sobre la circulació pulmonar al combinar ambdós estímuls.

Objectius específics

Investigar els canvis induïts per l'exposició crònica a fum de tabac i la hipòxiade forma separada o en combinació sobre:

1.- l'hemodinàmica pulmonar

2.- la funció endotelial de les artèries pulmonars i sistèmiques

3.- la morfologia dels vasos pulmonars i del ventricle dret

4.- l'expressió de mediadors vasoactius sintetitzats per l'endoteli

5.- la composició cel·lular dels vasos intrapulmonars

RESULTATS

Els resultats dels dos estudis principals han estat publicats en dos articles que es presenten a continuació i dels quals la doctoranda n'és la primera autora:

Primer article:

Effects of cigarette smoke on endothelial function of pulmonary arteries in the guinea pig.

Elisabet Ferrer, Víctor Ivo Peinado, Marta Díez, Josep Lluís Carrasco, Melina Mara Musri, Anna Martínez, Robert Rodríguez-Roisin, Joan Albert Barberà.

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Segon article:

Effects of cigarette smoke and hypoxia on the pulmonary circulation in the guinea pig.

Elisabet Ferrer, Víctor Ivo Peinado, Javier Castañeda, Jesús Prieto-Lloret, Elena Olea, Mª Carmen González-Martín, Mª Victoria Vega-Agapito, Marta Díez, David Domínguez-Fandos, Ana Obeso, Constancio González, Joan Albert Barberà.

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Effects of cigarette smoke on endothelial function of pulmonary arteries in the guinea pig

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Abstract

Background: Cigarette smoking may contribute to pulmonary hypertension in chronic obstructive pulmonary disease by altering the structure and function of pulmonary vessels at early disease stages. The objectives of this study were to evaluate the effects of long-term exposure to cigarette smoke on endothelial function and smooth muscle-cell proliferation in pulmonary arteries of guinea pigs.

Methods: 19 male Hartley guinea pigs were exposed to the smoke of 7 cigarettes/day, 5 days/week, for 3 and 6 months. 17 control guinea pigs were sham-exposed for the same periods. Endothelial function was evaluated in rings of pulmonary artery and aorta as the relaxation induced by ADP. The proliferation of smooth muscle cells and their phenotype in small pulmonary vessels were evaluated by immunohistochemical expression of α-actin and desmin. Vessel wall thickness, arteriolar muscularization and emphysema were assessed morphometrically. The expression of endothelial nitric oxide synthase (eNOS) was evaluated by Real Time-PCR.

Results: Exposure to cigarette smoke reduced endothelium-dependent vasodilatation in pulmonary arteries (ANOVA $p < 0.05$) but not in the aorta. Endothelial dysfunction was apparent at 3 months of exposure and did not increase further after 6 months of exposure. Smoke-exposed animals showed proliferation of poorly differentiated smooth muscle cells in small vessels ($p < 0.05$) after 3 months of exposure. Prolonged exposure resulted in full muscularization of small pulmonary vessels ($p < 0.05$), wall thickening ($p < 0.01$) and increased contractility of the main pulmonary artery ($p < 0.05$), and enlargement of the alveolar spaces. Lung expression of eNOS was decreased in animals exposed to cigarette smoke.

Conclusion: In the guinea pig, exposure to cigarette smoke induces selective endothelial dysfunction in pulmonary arteries, smooth muscle cell proliferation in small pulmonary vessels and reduced lung expression of eNOS. These changes appear after 3 months of exposure and precede the development of pulmonary emphysema.

Introduction

Patients with chronic obstructive pulmonary disease (COPD) show intimal hyperplasia in pulmonary muscular arteries, which results from the proliferation of smooth muscle cells (SMCs), and an increased proportion of muscularized arterioles. In addition, pulmonary arteries of COPD patients show abnormal endothelium-dependent vascular reactivity [1,2]. Studies conducted in healthy smokers have also revealed intimal hyperplasia in pulmonary muscular arteries, which does not differ from that in patients with mild COPD [3]. Furthermore, endothelial function of pulmonary arteries in healthy smokers lies between that in non-smokers and COPD patients, thereby indicating that endothelial dysfunction is present at the origin of the disease [2]. The impairment of endothelial function results from changes in the expression and release of vasoactive mediators that also regulate cell growth [4]. Overall, these initial alterations may lead to persistent changes in the vascular structure and function that underlie the development of pulmonary hypertension in COPD [5].

Studies performed in animal models have attempted to reproduce some of the pulmonary alterations that occur in COPD [6,7]. Among these, the model of airflow obstruction resulting from exposure to cigarette smoke (CS) is probably the most satisfactory approach. Chronic exposure of the guinea pig to CS is a widely recognized model of COPD [6,8]. In this model, Wright et al. [9-12] have shown muscularization of small pulmonary vessels, which precedes the development of emphysema, as well as changes in the expression of vascular mediators. In a recent study performed in guinea pig precision-cut lung slices, short-term exposure to CS induced a delayed response to vasoactive agents in intracinar arteries [13]. Whether long-term exposure to CS in this animal model produces endothelial dysfunction in pulmonary arteries has not been addressed using the organ-bath methodology, which is the conventional method to assess endothelial function of pulmonary arteries in humans [2,14] and in animal models [15-18]. Furthermore, the extent to which changes in endothelial function are related to vessel remodeling and/or expression of endothelium-derived mediators remains to be established.

We hypothesized that in the guinea pig, long-term exposure to CS alters endothelial function, induces the proliferation of poorly differentiated SMCs in pulmonary vessels, and reduces the expression of endothelium-derived vasodilators, in a similar way as in humans [2-4]. Accordingly, the present study was addressed to investigate in the guinea pig the effects of chronic exposure to CS on the endothelial function of pulmonary arteries and the lung expression of endothelial nitric oxide synthase (eNOS), and to determine whether CS induces muscularization in small pulmonary vessels. We also studied the chronological sequence of the functional and morphological changes induced by CS on pulmonary vessels.

Methods

Animals and cigarette smoke exposure

Thirty-six male Hartley guinea pigs (Harlam Ibérica, Spain), each weighing 300 g, were given a diet of standard chow and water supplemented with vitamin C (1 g/L; Roche Pharma, Madrid, Spain) *ad libitum*. A group of 19 animals was exposed to the smoke of 7 research cigarettes (1R3F; Kentucky University Research; Lexington, KY, USA) per day, 5 days a week, using a nose-only system [6] (Protowerx Design Inc; Langley, British Columbia, Canada) for a period of 3 and 6 months (n = 6 and n = 13, respectively). Controls (n = 17) were sham-exposed during the same periods of time (n = 9 for 3 months, n = 8 for 6 months). Animals that died during the study were excluded from the sample size. All procedures involving animals and their care were approved by the Ethics Committee of the University of Barcelona and were conducted following institutional guidelines that comply with national (Generalitat de Catalunya decree 214/1997, DOGC 2450) and international (Guide for the Care and Use of Laboratory Animals, National Institutes of Health, 85-23, 1985) laws and policies.

Endothelial function

After 3 or 6 months of CS exposure and 24 h after the last exposure, the animals were anesthetized with ketamine (50 mg/ml; 50 mg/kg. Pfizer Pharmaceuticals, Dun Laoghaire, Ireland) and xylazine (2%; 7 mg/kg. Bayer AG, Leverkusen, Germany) and the cardiopulmonary block was quickly removed to isolate a segment of the aorta and the main pulmonary artery. Arteries were cleaned of fat and connective tissue and cut into rings 3 mm in length. Two rings of the thoracic aorta and the left and right branches of the main pulmonary artery were placed in organ bath chambers (Panlab, Barcelona, Spain) filled with Krebs-Henseleit's buffer (containing (in mM) 118 NaCl, 24 $NaHCO_3$, 11.1 glucose, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$), bubbled with a gas mixture of 21% O_2 and 5% CO_2 (pH 7.35–7.45) and kept at 37°C by an outer-water bath warmed by a recirculating heater. Ring preparations were attached to an isometric transducer (Panlab, Barcelona, Spain) and equilibrated for 1 h under a resting tension of 1.75 g for pulmonary artery and 2.3 g for aortic rings, as established in preliminary studies. After a period of stabilization, arteries were contracted with KCl (60 mM) to determine their viability and contractile capacity. On the basis of previous experience, arteries with contractions <1 g were considered not viable. All rings were pre-incubated with indomethacin (1×10^{-5}

M, Sigma Aldrich. St Louis, USA) 30 min before the experiments in order to inhibit the synthesis of cyclo-oxygenase products. Indomethacin was kept in the solution throughout the experiment. The rings were then contracted with norepinephrine (NE; 1×10^{-7} to 0.2×10^{-6} M, Sigma Aldrich.) to obtain a stable plateau of tension. Endothelial function was evaluated by adding adenosine-5'-diphosphate (ADP, Boehringer GmbH, Mannheim, Germany), an endothelial nitric oxide (NO)-dependent vasodilator, to the organ bath. One of the rings of the aorta and the left branch of the pulmonary artery were tested to cumulative concentrations of ADP (10^{-9} to 10^{-5}M). Response to cumulative concentrations of the exogenous NO donor, sodium nitroprusside (SNP; 10^{-10} to 10^{-5} M, Sigma Aldrich.), was also tested in the other two rings. To corroborate the endothelial function assay performed with ADP, all the procedures were repeated in the presence of N^G-monomethyl-L-arginine (L-NAME; 10^{-1} M, Sigma Aldrich.), an inhibitor of eNOS. Endothelium-dependent vasodilator responses were assessed by the maximal relaxation induced by ADP, the dose that caused 50% relaxation (EC_{50}), and the area under the curve (AUC) [19] (Sigmaplot 10.0, Systat Software Inc, San José, CA, USA). Whereas EC_{50} is a single-point estimated value, the AUC is a summary measure obtained from all experimental points in the dose-response curve, providing a complete profile of vessel responsiveness. Each curve was evaluated by an observer without knowledge of the smoke exposure status.

Histological Assessment

Explanted lungs were inflated with 4% formaldehyde at a constant pressure of 25 cm H_2O during 24 h, and then embedded in paraffin. Histological examination was performed in 4-μm sagital sections stained with hematoxylin-eosin. The presence of emphysema was evaluated by measuring the mean linear intercept of alveolar septa in 20 randomly selected fields per slice using an image analysis system (Leica Qwin, Leica Microsystems Image Solutions Ltd, Cambridge, UK). Pulmonary vessels were analyzed in lung tissue sections stained with orcein. To assess the number of muscularized arterioles, all vessels with an external diameter <50 μm and with double elastic laminas were counted.

After the organ bath studies, all artery rings were fixed in 4% formaldehyde and cryo-embedded in optimal cutting temperature (O.C.T). Morphometric studies were performed in 4-μm slices of aorta and main pulmonary artery sections stained with elastin-Van Gienson. The external and internal elastic laminas were outlined and both total and lumen areas were computed using an image analysis system [2] (Leica Qwin). The area of the arterial wall was estimated as the difference between the total and luminal areas. Wall thickness was calculated by dividing the arterial wall area by the external perimeter of the artery [20].

Immunohistochemical studies

The expression of desmin and α-actin in pulmonary vessels (< 50 μm) was assessed by immunohistochemistry using anti-α-actin and anti-desmin antibodies (Dako, Glostrup, Denmark). An avidin-biotin reaction was performed to amplify the signal. The immunoreactions to α-actin and desmin were quantified as the number of positive vessels per mm^2. The intensity and extension of immunoreaction to desmin were also semi-quantitatively evaluated in a scale from 1 to 3 (for intensity, 1: low, 2: medium, 3: high; and for extension, 1: 0–25% of the vessel wall, 2: 26–75%, 3: > 75%).

Real Time-PCR

Total RNA was extracted from lung tissue using TRIzol (Invitrogen, Paisley, Scotland, UK). For reverse transcription, 2.0 mg of total RNA was retrotranscribed using a high-capacity cDNA Archive kit (Applied Biosystems). Quantification of eNOS was done with real-time PCR using SYBR Green I chemistry (SensiMix (dT) DNA Kit, Quantance Ltd, Ballards Lane, London). Normalization of gene expression levels was performed by using β-actin as endogenous housekeeping gene. To generate a standard curve, 7-fold serial dilutions of each purified PCR product were used for templates. Primers were designed based on guinea pig (eNOS) sequence from GeneBank using specific software (Primer Express, Applied Biosystems, Foster City, CA.). Amplification was performed on Chromo4 thermocycler (MJ Research, BioRad, Hercules, CA), and each sample was run in duplicate. The identities of the amplified products were examined using 12% poly-acrylamide gel electrophoresis and melt curve analysis. The primer sequences for eNOS were 3'-AGCCAACGCGGTGAAGATC-5' and 5'-TTAGCCATCACCGTGCCC-3' and for β-actin 3'-ATATCGCTGCGCTCGTTGTC-5' and 5'-AACGATGCCGTGCTCAATG-3'.

Statistics

To evaluate the effect of CS exposure on endothelial function, a general linear model [21] was fitted using time, group and time-by-group interaction as independent factors. The estimates of the factors were adjusted by the effect of contraction to NE by including it in the model. The significance of the independent factors was assessed by the common ANOVA F-test using the type-3 sum of squares. The adequacy of the model was checked by examination of the Pearson residuals. All other variables are expressed as mean ± standard deviation (SD) or as median and inter-quartile range depending on whether or not the variables followed a normal distribution. Comparisons between groups were performed by the Student t-test or

Mann Whitney test. A p-value lower than 0.05 was considered significant.

Results

Five of the 24 guinea pigs exposed to CS died during the study while no deaths occurred in the control group. The animals that died during the experiment were excluded from the analysis.

At the end of the study, animals showed normal behavior and activity. CS-exposed guinea pigs were smaller and had lower body weights than the controls (data not shown). The anesthesia was deep in all cases and anesthetic dosages were identical between controls and CS-exposed animals. The anatomical examination revealed no signs of respiratory infection or other major abnormalities in lung tissue.

Vascular contractility

In pulmonary arteries, maximal contraction to KCl was greater in animals exposed to CS at 3 and 6 months than in controls (Table 1). Maximal contraction to NE was similar in all groups at all times of exposure.

In the aorta, there were no differences in the maximal contraction to KCl between CS-exposed animals and controls at any time of exposure. The ANOVA revealed a time effect in the contractile response to NE (Table 2).

Endothelial function

Guinea pigs exposed to CS showed a shift to the right in the dose-response curve of pulmonary arteries to ADP, as shown by a greater AUC and higher (less diluted) EC_{50}, compared with control guinea pigs (Table 1, Figure 1). The ANOVA failed to show any interaction between CS exposure and time (Table 1), thereby indicating that reduced reactivity of pulmonary arteries in CS-exposed animals was independent of the length of exposure.

The ANOVA also revealed a marked effect of time on endothelium-dependent relaxation. Irrespective of whether the animals were exposed to CS, the endothelium-dependent vasodilatation of pulmonary arteries was lower at 6 months than at 3 months.

The maximal relaxation induced by ADP was close to 100% in all groups. When pulmonary artery rings were exposed to the competitive eNOS inhibitor, L-NAME, the relaxation induced by ADP was almost completely abolished (Table 1), indicating that ADP operated through the L-arginine-NO pathway. In all cases, pulmonary arteries reached maximal relaxation when they were assessed with SNP, an exogenous NO donor.

In rings of the aorta, no effect of CS exposure was found in any of the relaxation responses to the pharmacological agents that were tested (Table 2). Yet, the ANOVA revealed

Table 1: Vascular response of pulmonary artery

	3 months		6 months		ANOVA		
	Control (n = 9)	**CS-Exposed** (n = 6)	**Control** (n = 8)	**CS-Exposed** (n = 12)	**Time**	**CS Exposure**	**Interaction**
Contraction							
KCl (60 mM), mg	1825 ± 164	2318 ± 201	1673 ± 174	2694 ± 142	0.554	<0.001	0.134
NE (10^{-6}M), mg	1043 ± 118	1058 ± 145	990 ± 125	895 ± 107	0.396	0.703	0.660
Relaxation to ADP							
EC_{50}, (-log [M] ADP)	7.48 ± 0.07	7.28 ± 0.09	7.21 ± 0.07	7.15 ± 0.06	0.009	0.110	0.318
AUC†	7.71 ± 0.03	7.78 ± 0.04	7.83 ± 0.03	7.91 ± 0.03	<0.001	0.037	0.881
Relaxation to ADP+L-NAME							
EC_{50}, (-log [M] ADP)	ND	ND	ND	ND	ND	ND	ND
AUC†	8.34 ± 0.05	8.30 ± 0.05	8.28 ± 0.05	8.27 ± 0.04	0.358	0.619	0.711
Relaxation to SNP							
EC_{50}, (-log [M] SNP)	8.66 ± 0.12	8.66 ± 0.14	8.57 ± 0.14	8.36 ± 0.11	0.137	0.370	0.414
AUC†	7.52 ± 0.06	7.42 ± 0.07	7.59 ± 0.07	7.65 ± 0.05	0.022	0.816	0.201

Definition of abbreviation: NE: Norepinephrine; ADP: Adenosine diphosphate; SNP: Sodium nitroprusside; EC_{50}: dose that causes 50% of relaxation; AUC: area under the curve; L-NAME: N^G-monomethyl-L-arginine
Values are mean ± SEM
†log-transformed
ND: not determined

Table 2: Vascular response of aorta

	3 months		6 months		ANOVA		
	Control (n = 9)	**CS-Exposed** (n = 6)	**Control** (n = 8)	**CS-Exposed** (n = 12)	**Time**	**CS Exposure**	**Interaction**
Contraction							
KCl (60 mM), mg	3346 ± 290	3224 ± 336	3596 ± 290	3837 ± 237	0.144	0.755	0.537
NE (10^{-6}M), mg	1347 ± 192	1621 ± 222	922 ± 192	1037 ± 157	0.012	0.341	0.682
Relaxation to ADP							
EC_{50}, (-log [M] ADP)	7.10 ± 0.10	7.29 ± 0.12	6.97 ± 0.10	6.93 ± 0.08	0.038	0.588	0.260
AUC†	8.21 ± 0.04	8.15 ± 0.05	8.25 ± 0.04	8.23 ± 0.03	0.208	0.440	0.651
Relaxation to ADP+L-NAME							
EC_{50}, (-log [M] ADP)	ND	ND	ND	ND	ND	ND	ND
AUC†	8.80 ± 0.06	8.85 ± 0.08	8.76 ± 0.07	8.67 ± 0.05	0.116	0.661	0.268
Relaxation to SNP							
EC_{50}, (-log [M] SNP)	7.53 ± 0.15	7.26 ± 0.26	8.04 ± 0.13	8.15 ± 0.10	<0.001	0.837	0.286
AUC†	7.91 ± 0.07	7.99 ± 0.08	7.93 ± 0.07	7.88 ± 0.05	0.544	0.991	0.343

Definition of abbreviation: NE: Norepinephrine; ADP: Adenosine diphosphate; SNP: Sodium nitroprusside; EC_{50}: dose that causes 50% of relaxation; AUC: area under the curve; L-NAME: N^G-monomethyl-L-arginine
Values are mean ± SEM
†log-transformed
ND: not determined

a time effect in both the endothelium-dependent and –independent relaxation responses.

Morphological evaluation

The thickness of the walls of the right and left main pulmonary arteries was enlarged in CS-exposed animals (Figure 2A and 2B). Wall thickening of pulmonary arteries was due to both smooth muscle cell proliferation and elastic fiber deposition (data not shown). In contrast, the thickness of the aorta was not affected at either 3 or 6 months (Figure 2C and 2D, Table 3).

The percentage of intra-pulmonary vessels with double elastic laminas increased 2-fold after 6 months of CS exposure compared to controls (12.3 ± 4.8 vs. 6.3 ± 5.7 respectively). This effect was not observed at 3 months (6.1 ± 3.9 vs. 7.4 ± 8.1 respectively) (Figure 3). There were no differences in the percentage of muscularized arterioles at 3 months of exposure.

Immunohistochemical evaluation

Immunohistochemical evaluation was performed in vessels with a diameter <50 μm. The number of vessels that were positive to smooth muscle α-actin was significantly greater in CS-exposed animals, at both 3 and 6 months of exposure (Figures 4A, B and 4C). In contrast, no changes were observed in the number of desmin-positive vessels at 3 or 6 months (Figure 4D), nor in the intensity or extension of protein expression (data not shown).

Alveolar space size

An increase in the mean distance between alveolar septa was observed in animals exposed to CS for 6 months (control vs. exposed: 52 ± 8 vs. 59 ± 7 μm, $p < 0.05$). This finding is consistent with the presence of pulmonary emphysema. No differences between control animals and those exposed to CS for 3 months were found.

Gene expression of eNOS

Gene expression of eNOS was evaluated by Real-Time PCR in whole lung homogenates and normalized by the expression of β-actin. Compared with control animals, eNOS expression was decreased at 3 and 6 months of exposure to CS (Figure 5).

Correlation

The wall thickness of pulmonary arteries correlated significantly with the contraction to KCl ($r = 0.63$, $p = 0.003$) (Figure 2E). This correlation was not observed in aortas. There was no correlation between the endothelial function of the main pulmonary artery and the number of α-actin-positive intrapulmonary vessels or muscularized arterioles.

Discussion

Our results show that guinea pigs chronically exposed to CS developed endothelial dysfunction in the pulmonary artery, which was already apparent after 3 months of exposure. In this period of time, animals exposed to CS

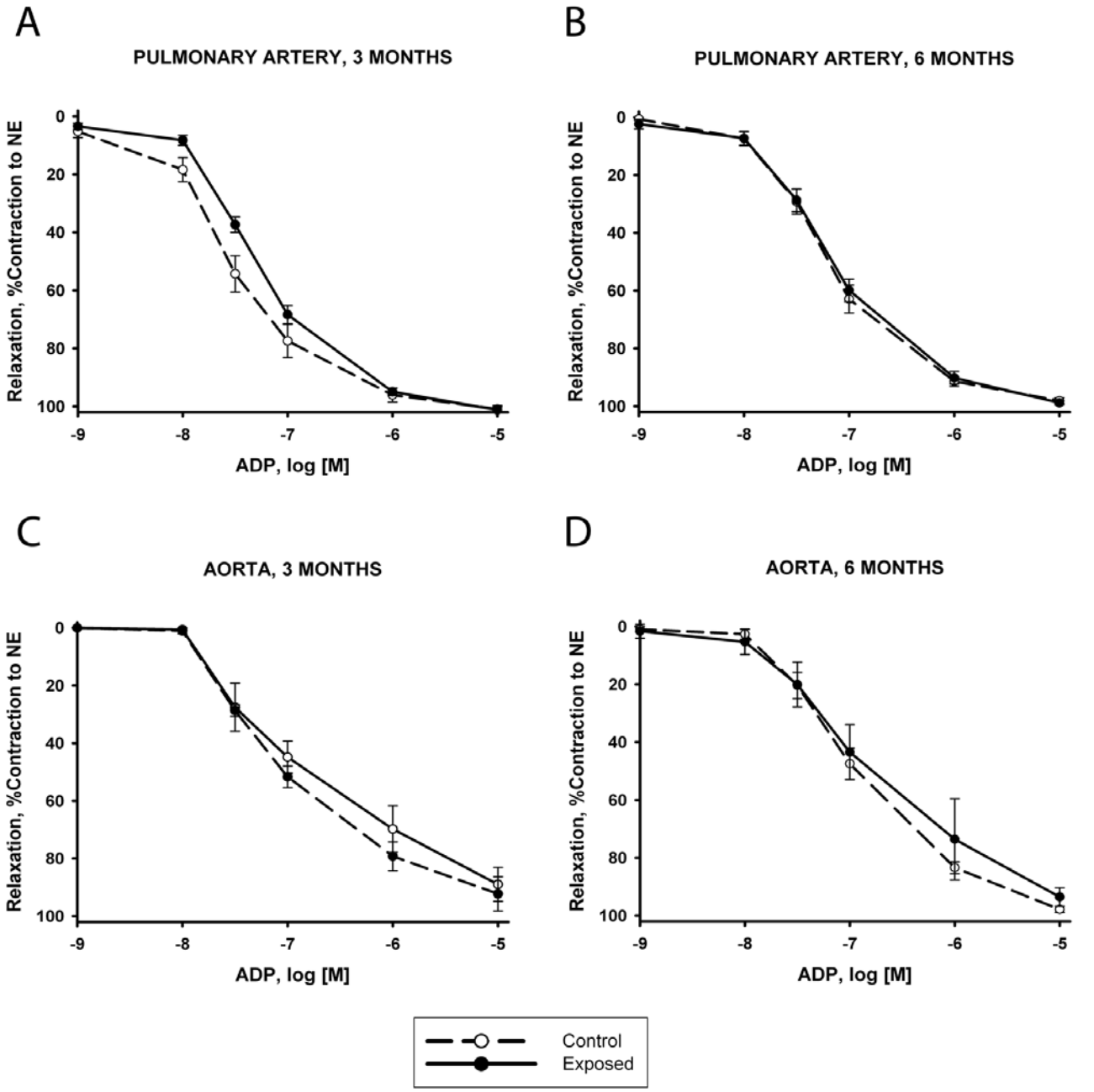

Figure 1

Endothelial function of pulmonary and aorta arteries. Relaxation of main pulmonary artery and aorta to cumulative doses of adenosine-5'-diphosphate (ADP) expressed as % of contraction to norepinephrine (NE). Upper panels show dose-response curves of pulmonary arteries in smoke exposed (continuous line) and control (dashed line) animals at 3 (A) and 6 months (B) of exposure. Lower panels show dose-response curves of aorta at 3 (C) and 6 months (D) of exposure. Values are mean ± SEM.

showed reduced expression of eNOS in lung tissue and developed SMC proliferation in small intrapulmonary arteries. Longer exposure resulted in complete muscularization of small pulmonary vessels, as well as emphysematous changes in the alveolar spaces.

Endothelial dysfunction in pulmonary arteries was shown by a diminished response to the endothelium-dependent vasodilator ADP, which was abolished by the eNOS inhibitor L-NAME. Contrasting with this observation, no differences between groups were found in the endothe-

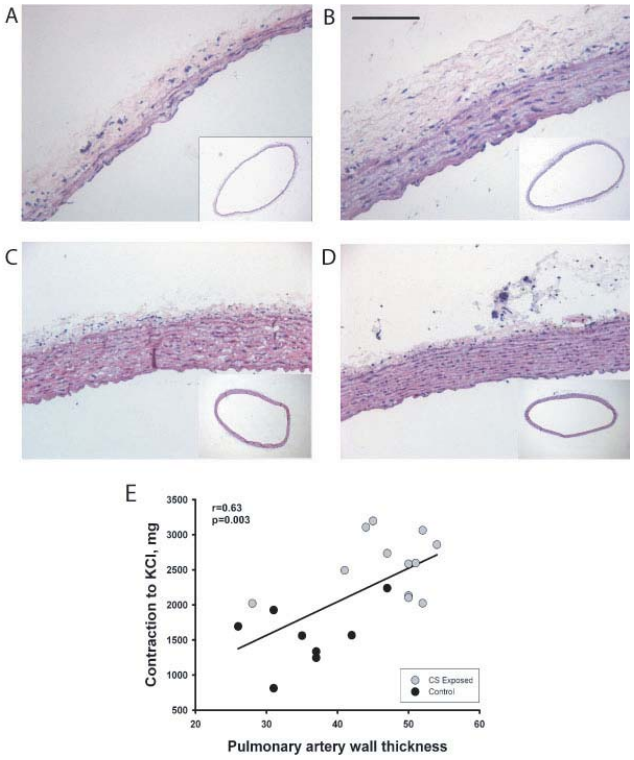

Figure 2
Morphometry of pulmonary artery. Hematoxylin-eosin stained sections of main pulmonary artery (upper panels) and aorta (lower panels) from control (A and C) and cigarette smoke (CS)-exposed (B and D) guinea pigs. Whereas, pulmonary artery of the exposed animal shows prominent thickening of the arterial wall, no difference in wall thickness is noticed in the aorta. Scale bar, 100 μm. (E) Correlation between the contraction to KCl and the wall thickness of the main pulmonary artery in control (black symbols) and CS-exposed (grey symbols) animals after 6 months of exposure.

lium-dependent relaxation of the aorta, thereby suggesting that CS exposure exerted a direct effect on pulmonary circulation. These results are in agreement with previous studies showing diminished endothelium-dependent relaxation in pulmonary arteries of smokers [2,4] and in guinea pigs after a short period of CS exposure [13]. Moreover, in the present study endothelial dysfunction preceded the complete muscularization of small intrapulmonary vessels (vessels with double elastic lamina), thereby supporting the hypothesis that in COPD endothelial dysfunction is an early event that antecedes pulmonary vascular remodeling [22]. The mechanisms by which CS impairs pulmonary endothelium remain to be established. Our results show a decrease in eNOS gene expression in the lungs of animals exposed to CS. This finding is in agreement with those of Su and co-workers [23], who demonstrated that eNOS is down-regulated in endothelial cell cultures exposed to cumulative doses of CS extract. The expression of eNOS is also reduced in pulmonary arteries of smokers and in patients with different degrees of COPD severity [4,24]. Accordingly, it is conceivable that CS might alter endothelium-dependent relaxation by down-regulating eNOS expression in pulmonary arteries of guinea pigs.

We also observed a marked effect of time on the endothelial function of pulmonary arteries. The vascular relaxation induced by ADP was lower at 6 months than at 3 months, irrespective of whether the animals were exposed to CS or not. The effect of time on vascular reactivity was also apparent in the aorta. Since the guinea pigs used in our study were in their growing period (mean weight increased by 231% at 3 months, and by 404% at 6 months), we consider that growth (or maturation) might affect vascular reactivity, in particular the endothelial function of pulmonary arteries, by mechanisms that have

Table 3: Morphometric measurements in main pulmonary artery and aorta

		3 months		6 months	
		Control (n = 8)	**CS-Exposed** (n = 5)	**Control** (n = 8)	**CS-Exposed** (n = 13)
Pulmonary artery	**Diameter (mm)**	2.26 (2.10–2.49)	2.37 (2.11–2.45)	2.26 (2.13–2.39)	2.19 (2.15–2.40)
	Wall area (mm²)	0.26 (0.24–0.33)	0.38 (0.30–0.42)	0.27 (0.24–0.31)	0.35* (0.32–0.41)
	Wall thickness (μm)	38 (36–43)	50 (43–57)	35 (33–40)	51‡ (45–53)
Aorta	**Diameter (mm)**	2.21 (2.14–2.30)	2.34 (2.17–3.80)	2.22 (2.07–2.32)	2.28 (2.24–2.36)
	Wall area (mm²)	0.58 (0.51–0.65)	0.77 (0.59–0.92)	0.50 (0.46–0.65)	0.63 (0.57–0.74)
	Wall thickness (μm)	82 (74–92)	96 (86–117)	77 (70–87)	88 (80–97)

Wall thickness was calculated as total area/external perimeter
Values are median and interquartile range
* $p < 0.05$
‡ $p \leq 0.01$

not been fully elucidated. Indeed, it has been observed that hormonal changes associated with sexual maturity may affect posttranscriptional and/or translational regulation of eNOS protein and result in lower plasma NO levels in adult male pigs, thereby exerting an effect on vascular function [25]. On the other hand, maturation also induces increased production of reactive oxygen species (ROS) in vessels, which, in turn, may impair vessel function as a result of decreased NO bioavailability [26-28].

We characterized the phenotype of the SMC responsible for vascular remodeling in guinea pigs exposed to CS by evaluating the expression of the intermediate filaments smooth muscle α-actin and desmin in small pulmonary vessels. Animals exposed to CS for 3 months showed an increase in the number of α-actin-positive vessels, which persisted after 6 months of exposure. In contrast, there were no differences in the number of vessels positive for the contractile filament desmin, either at 3 or at 6 months. Accordingly, CS-exposed guinea pigs showed proliferation of α-actin^{+}/desmin^{-} SMC, which represent a subpopulation of less differentiated SMCs with synthetic capacity [5]. These results are consistent with those obtained in COPD showing that vascular remodeling is produced by the intimal proliferation of poorly differentiated SMCs [3]. The structural alterations in the pulmonary circulation of CS-exposed guinea pigs might be a consequence of changes in the synthesis and release of vasoactive and cell proliferative mediators, since endothelin and VEGF expression are increased in the arterial wall of remodeled vessels in animals exposed to CS [12]. After 6 months of exposure, we found an increased percentage of vessels with double elastic laminas. In the same experimental model, Wright et al. [9] also reported that the greater number of vessels with double elastic laminas was accompanied by an increase in pulmonary artery pressure (PAP). This observation suggests that muscularization of small vessels induced by CS exposure is associated with pulmonary hypertension. In keeping with this, we observed a wall enlargement in main pulmonary arteries after 6 months of CS exposure, which correlated with the contractility to KCl.

Smooth muscle cell proliferation in small vessels was already present at 3 months of exposure and preceded the development of emphysema. These findings confirm previous observations made by Yamato et al. [29] and are in agreement with studies performed in healthy smokers, who showed pulmonary vascular remodeling and endothelial dysfunction [2]. Although hypoxemia, which is associated with emphysema, is one of the strongest agents producing vasoconstriction and vessel remodeling,

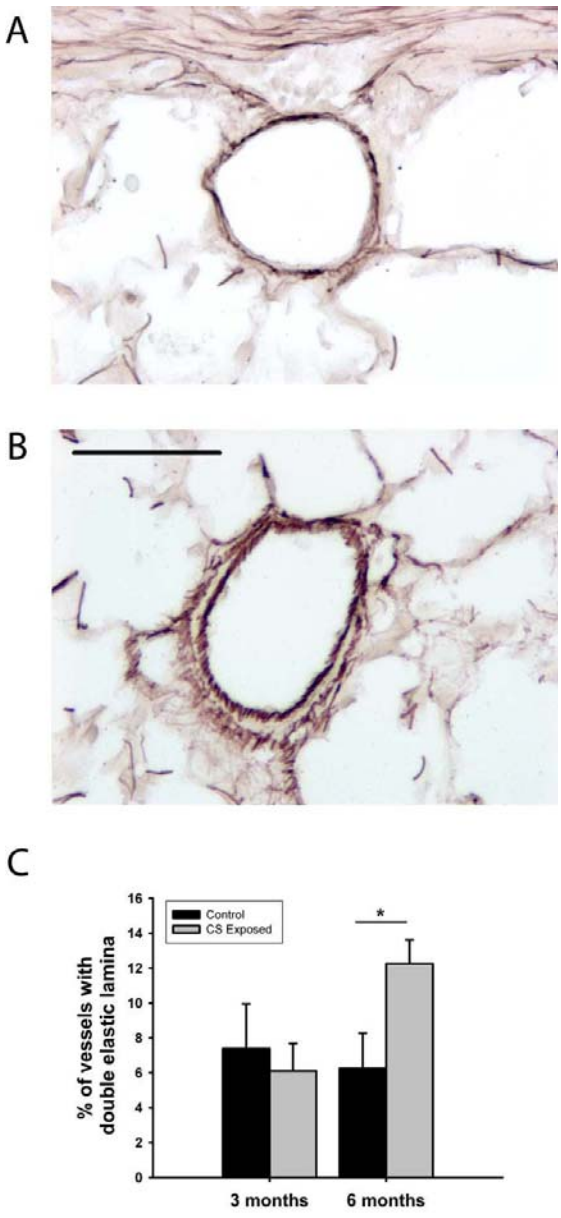

Figure 3
Double elastic lamina presence in small intrapulmonary arteries. Orcein stain of small pulmonary vessels in a control guinea pig (A) and an animal exposed to cigarette smoke (CS) (B). In the exposed animal a double elastic lamina is present, indicating full muscularization of the vessel. Scale bar, 50 μm. (C) Bar graph shows the number of vessels with double elastic laminas expressed as a percentage of the total number of vessels. The number of vessels with double elastic laminas was higher in guinea pigs exposed to CS for 6 months. * $p < 0.05$ compared with control group. Values are mean ± SEM.

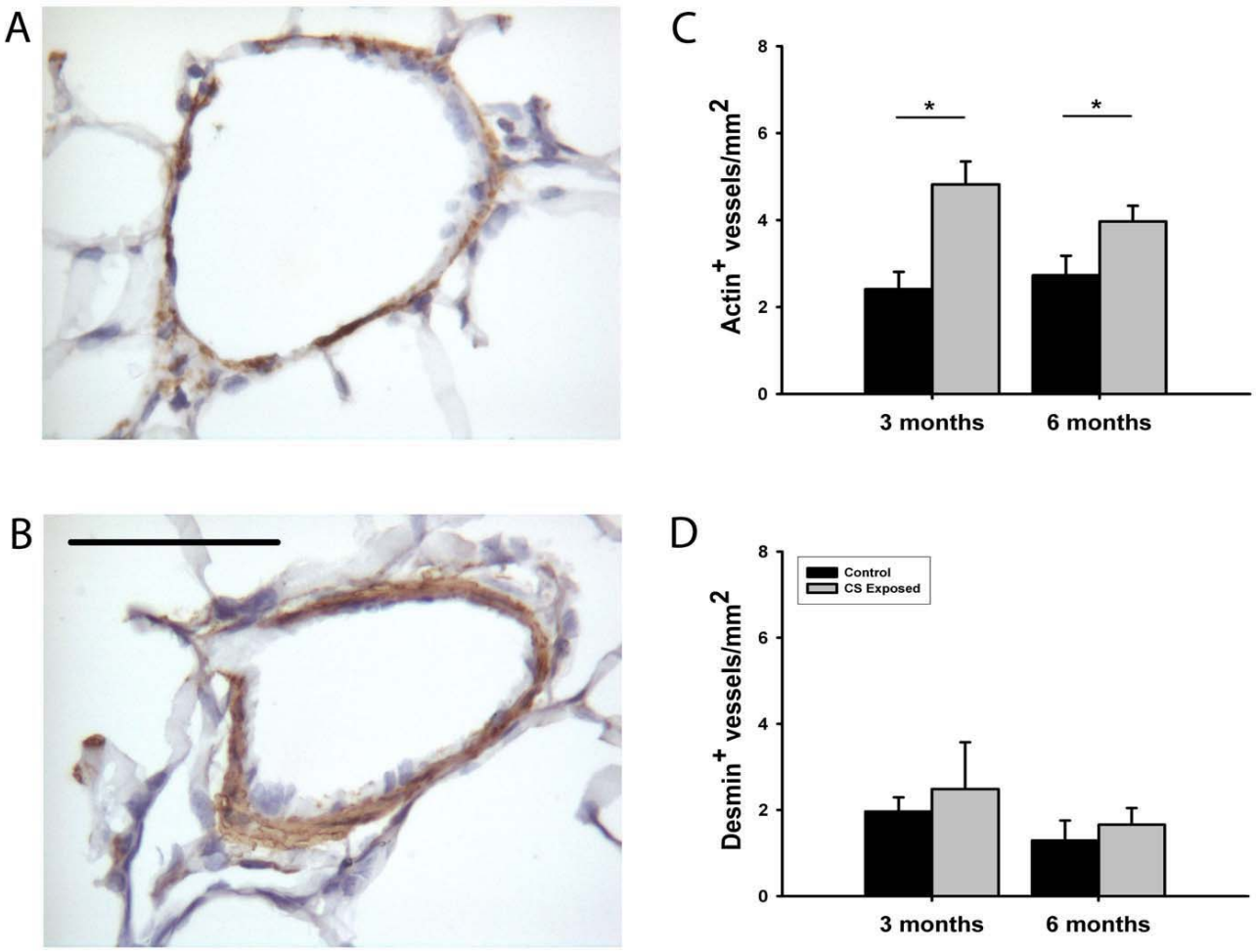

Figure 4
Smooth muscle cell proliferation in small intrapulmonary arteries. Immunohistochemistry for α-actin in small vessels of a control guinea pig (A) and an animal exposed to cigarette smoke (CS) (B). The vessel of the exposed animal shows a thicker wall with a strong immunoreactivity to α-actin. Scale bar, 50 μm. Bar graphs show the number of vessels/mm^2 with positive immunoreactivity to α-actin (C) and desmin (D) in control and CS-exposed guinea pigs, for 3 and 6 months of exposure. * $p < 0.05$ compared with control group. Values are mean ± SEM.

our results corroborate that CS exposure may have a similar effect on pulmonary vessels [30]. Nevertheless, the presence of hypoxemia in patients with COPD might exert a synergistic effect on the pathogenesis of pulmonary hypertension. Further studies are required to elucidate the potential synergism between cigarette smoke and hypoxia in this experimental model.

It is interesting to note that endothelial dysfunction and vessel remodeling associated with CS exposure affected selectively pulmonary arteries while the aorta remained unaltered. We consider this could be due to the fact that pulmonary vessels are exposed to greater concentrations of CS products, whereas the effects on the aorta might be eventually caused by products diffusing to the blood. We do not disregard that longer exposure to CS might exert an effect on the aorta or systemic vessels. Although, it is conceivable that longer exposure would also result in greater structural and functional damage of pulmonary vessels. Yet, it is noteworthy that in this experimental model pulmonary vessels develop changes after a short period of CS exposure that antecede changes in lung structure or systemic vascular involvement.

In conclusion, the guinea pig chronically exposed to CS develops endothelial dysfunction selectively in pulmonary arteries, without presenting changes in systemic arteries. This endothelial dysfunction is accompanied by reduced lung expression of eNOS and proliferation of poorly differentiated SMCs in small pulmonary vessels.

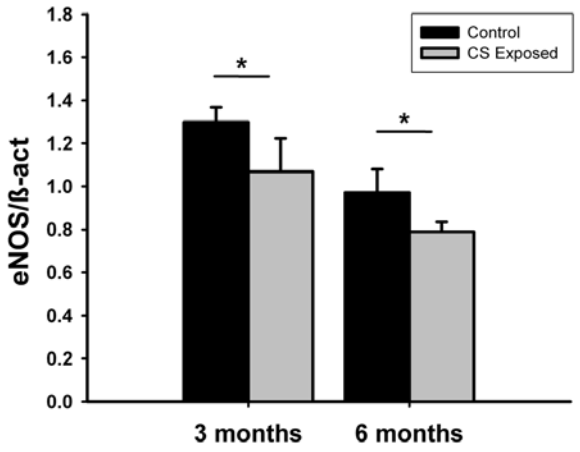

Figure 5
Gene expression of eNOS in whole lungs evaluated by real time-PCR. Bars show the expression of the eNOS gene, normalized by the expression of β-actin (β-act) gene. Compared with control guinea pigs, the expression of eNOS was reduced in animals exposed to cigarette smoke, both at 3 and 6 months of exposure. * $p < 0.05$, compared with control group. Values are mean ± SEM.

These changes are already apparent after a relatively short period of CS exposure and precede the full muscularization of small pulmonary vessels and the development of emphysema. Results in this experimental model confirm that CS has direct and deleterious effects on the structure and function of pulmonary vessels that might contribute to the development of pulmonary hypertension in COPD.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
EF carried out the experimental work, participated in the analysis of the data and in the preparation of the manuscript. VIP was involved in the conception of the study, participated in its design and coordination, the analysis of the data and in the elaboration of the manuscript. MD provided support in the experimental work and data collection. JLC performed the statistical analysis and contributed to the analysis of the data. MMM carried out the RT-PCR experiments. AM contributed in the implementation of the study and in the immunohistochemical studies. RRR provided funding support and contributed in the analysis of the data. JAB conceived the study, raised funding support, participated in the design and implementation of the study, and in the revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Resum del primer article:

Effects of cigarette smoke on endothelial function of pulmonary arteries in the guinea pig.

L'objectiu d'aquest estudi va ser avaluar l'efecte del fum de tabac sobre la funció de la cèl·lula endotelial així com la seqüència dels canvis pulmonars i vasculars en cobais exposats a fum de tabac. Per això, 35 cobais Hartley mascles van ser exposats a fum de 7 cigarretes/dia, 5 dies a la setmana durant 3 (n=15) i 6 (n=20) mesos. 17 cobais controls van ser falsament exposats a fum de tabac durant els mateixos períodes de temps. La funció endotelial de les arteries pulmonars es va mesurar com la relaxació induïda per ADP, un vasodilatador dependent de la síntesi de NO endotelial. També es va avaluar la relaxació no dependent de l'endoteli així com la inhibició de l'eNOS amb L-NAME. Mitjançant anàlisi de imatges es va mesurar el gruix de la paret arterial i la presència d'emfisema.

Els resultats d'aquest primer estudi són:

1.- Funció endotelial

1.1.- Els estudis realitzats al bany d'òrgans demostren una menor resposta vasodilatadora de les artèries pulmonars de cobais exposats a fum de tabac a l'ADP respecte els animals controls. Aquesta alteració es manifesta com a un desplaçament a la dreta de la corba dosi-resposta a ADP. La disfunció endotelial es veu corroborada per l'absència de resposta vasodilatadora de l'artèria en presència de L-NAME, un inhibidor de la NO sintasa. Aquesta disfunció es manifesta als 3 mesos i perdura als 6 mesos d'exposició a fum de tabac.

1.2.- No s'observen canvis en la funció endotelial a l'aorta.

2.- Estructura de l'artèria pulmonar

2.1.- L'exposició a fum de tabac s'associa a l'engruiximent de la paret de l'artèria pulmonar principal, que es fa evident als 3 mesos d'exposició i es manté als 6 mesos.

2.2.- La vasoconstricció de l'artèria pulmonar en resposta a KCl és més pronunciada en els animals exposats a fum de tabac i està directament relacionada amb el gruix de la paret de l'artèria pulmonar

2.3.- No s'observen canvis a l'artèria aorta.

3.- Canvis cel·lulars dels vasos intrapulmonars

3.1.- Per tal d'analitzar els canvis induïts pel fum de tabac en els vasos intrapulmonars es van realitzar estudis immunohistoquímics en seccions transversals de pulmó utilitzant anticossos contra α-actina específica de múscul llis, un marcador de cèl·lula muscular llisa, i contra desmina, un filament contràctil específic de cèl·lules musculars madures. Es van avaluar els vasos amb diàmetre <50µm. Els resultats demostren que els cobais exposats a fum de tabac presenten un major nombre de vasos positius per α-actina mentre que no s'observen canvis en l'expressió de desmina. Aquest fet indica que les cèl·lules responsables del remodelat vascular induït pel tabac són cèl·lules de fenotip poc diferenciat. Aquests canvis són evidents als 3 mesos d'exposició a fum de tabac i es mantenen als 6 mesos.

3.2.- La presència de vasos <50µm amb doble làmina elàstica es va realitzar mitjançant la tinció d'orceïna en seccions transversals de pulmó. Els animals exposats a fum de tabac van presentar una major proporció de vasos intrapulmonars amb doble elàstica als 6 mesos d'exposició. No hi havia diferències als 3 mesos.

4.- Expressió de NO

Per tal de comprovar l'alteració de la via del NO i justificar així la presència de disfunció endotelial, es va analitzar l'expressió de mRNA d'eNOS mitjançant Real Time-PCR (RT-PCR) en homogenats de pulmó. Els resultats demostren que hi ha una disminució de l'expressió de l'eNOS en els cobais exposats a fum de tabac. Aquest dèficit de producció de NO per

part de l'eNOS està present als 3 mesos d'exposició a fum de tabac i empitjora amb el temps d'exposició.

5.-Emfisema pulmonar

La distància interseptal mitjana (Lm), un indicador de la destrucció alveolar, va resultar ser significativament superior en els cobais exposats a fum de tabac respecte els controls, demostrant així la presència d'emfisema als 6 mesos d'exposició.

Segon article:

Effects of cigarette smoke and hypoxia on the pulmonary circulation in the guinea pig

Elisabet Ferrer, Víctor Ivo Peinado, Javier Castañeda, Jesús Prieto-Lloret, Elena Olea, Mª Carmen González-Martín, Mª Victoria Vega-Agapito, Marta Díez, David Domínguez-Fandos, Ana Obeso, Constancio González, Joan Albert Barberà.

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Effects of Cigarette Smoke and Hypoxia on the Pulmonary Circulation in the Guinea Pig

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Running head: Cigarette smoke and hypoxia on pulmonary circulation

ABSTRACT

Rationale: Cigarette smoke (CS) and chronic hypoxia (CH) can produce pulmonary hypertension. Similarities and differences between both exposures and their interaction have not been explored.

Objective: Investigate the effects of CS and CH, as single factors or in combination, on the pulmonary circulation in the guinea pig.

Methods: Fifty-one guinea pigs were exposed 12 weeks to CS, and 32 sham-exposed. 50% of the animals in each group were additionally exposed to hypoxia the last 2 weeks. We measured pulmonary artery pressure (PAP) and the weight ratio between right ventricle (RV) and left ventricle+septum. Pulmonary artery contractility in response to norepinephrine (NE), endothelium-dependent vasodilatation and distensibility were evaluated in organ bath chambers. The number of small intrapulmonary vessels showing immunoreactivity to smooth muscle (SM)-α-actin and double elastic laminas was assessed microscopically.

Results: CS and CH induced similar increases of PAP and RV hypertrophy ($p<0.05$, each), effects that were further enhanced when both factors were combined. CH increased the contractility to NE ($p<0.01$) and reduced the distensibility ($p<0.05$) of pulmonary arteries. Animals exposed to CS showed an increased number of small vessels with positive immunoreactivity to SM-α-actin ($p<0.01$) and those exposed to CH a greater proportion of vessels with double elastic laminas ($p<0.05$).

C**onclusions**: We conclude that chronic hypoxia amplifies the detrimental effects of CS on the pulmonary circulation by altering the mechanical properties of pulmonary arteries and enhancing the remodeling of pulmonary arterioles.

Word count: 232 words

Key words: cigarette smoke, COPD, hypoxia, pulmonary hypertension, vascular remodeling.

INTRODUCTION

Pulmonary hypertension is a common and serious complication of chronic obstructive pulmonary disease (COPD). It is considered to result from the effects of chronic hypoxemia on pulmonary vessels. Indeed, acute and chronic exposure to hypoxia results in smooth muscle cell (SMC) and adventitial fibroblast proliferation[1]. Nevertheless, structural changes in the pulmonary arteries of COPD patients differ from those observed in subjects exposed to a hypoxic environment. Whereas highlanders show medial hypertrophy, COPD patients show prominent changes in the intima[2]. Intimal hypertrophy is present in non-hypoxemic COPD patients and in smokers with normal lung function, suggesting that vascular changes may be triggered by cigarette smoke (CS) before hypoxemia develops.

Cigarette smoking is associated with endothelial dysfunction[3], increased expression of growth factors[4] and inflammatory cell infiltrate in pulmonary arteries[5]. These factors may induce SMC proliferation and increase pulmonary vascular resistance. The molecular mechanisms by which CS induces vascular changes remain unknown, but they might be related to oxidative damage[6].

The effects of hypoxia on human lungs are difficult to characterize because it is usually related to the presence of primary lung diseases that may be associated to vascular remodeling by additional mechanisms (i.e. inflammation). Nevertheless, there is evidence that in humans chronic hypoxia (CH) *per se* may induce endothelial dysfunction[7], increased expression of growth factors[8] and inflammation[9].

Some of the observations in pulmonary vessels of COPD patients have been replicated in a guinea pig model chronically exposed either to CS or hypoxia. Exposure to CS induces muscularization of precapillary vessels and increases pulmonary artery pressure (PAP)[10;11], which runs in parallel to endothelial dysfunction[12;13], and develops before pulmonary emphysema is apparent. CH on the other hand, induces pulmonary hypertension, right ventricle (RV) hypertrophy, vascular remodeling[14] and

increased plasma levels of endothelin-1 (ET-1) in the guinea pig[15].
We have hypothesized that in COPD changes in pulmonary vessels are initiated at early disease stages as a result of a direct effect of CS products on the pulmonary endothelium. Subsequently, with the progression of the disease hypoxemia may add on, producing further vascular damage potentially resulting in pulmonary hypertension. The combined effects of CS and CH on the pulmonary circulation have not yet been assessed in experimental models.
Accordingly, the present study aimed to evaluate the effects of CS exposure and CH, alone and as combined stimuli, on pulmonary hemodynamics, RV structure, pulmonary vascular reactivity and vessel remodeling in the guinea pig.

METHODS

Animals and management

Eighty-three male Hartley guinea pigs weighing 350g were divided into four groups. One group was exposed to CS for 12 weeks (n=33); a second group was kept in a normal atmosphere for 10 weeks and subsequently exposed to FiO_2 0.12 for 2 weeks in a hypoxic chamber (CH) (n=16); a third group was exposed to CS for 12 weeks and to CH for the last two weeks (CSCH) (n=18); and a Control group was sham-exposed to CS and kept in a normal atmosphere (n=16).

Cigarette smoke exposure

Animals were exposed to the smoke of four research cigarettes (2R4F; Kentucky University Research; Lexington, KY, USA) per day, 5 days a week for 12 weeks using a nose-only system[13;16].

Exposure to chronic hypoxia

Animals were placed in hypoxic glass chambers continuously fluxed with gas mixture (12% O_2 in N_2; $PO_2 \approx 85$ mmHg; equivalent to ≈4300m).

Pulmonary hemodynamics

In half of the animals in each group a catheter was placed in the pulmonary artery through the right ventricle. The right carotid artery was canulated to simultaneously measure systemic arterial pressure. Measurements were performed in normoxic and hypoxic conditions (4 min at a 0.10 fraction of inspired oxygen (FiO_2)). The recovery profile was evaluated by pumping again air with a FiO_2 of 0.21.

Vessel distensibility and endothelial function

Half of the animals in each group underwent assessment of vessel distensibility and reactivity. Vessel distensibility (Dis) was evaluatedin rings of pulmonary artery and aorta (3mm length) according to the equation $Dis=\Delta D/\Delta P \cdot D$ (where ΔD is the difference of diameter before and after 1mm of stretching, ΔP is the difference in pressure at the same points and D is the final diameter of the ring) [17;18]. Endothelial function was assessed as the change in wall tension in response to cumulative doses of adenosine-5'-diphosphate (ADP), as previously described[13].

Macroscopic and microscopic morphologic studies

Right ventricular hypertrophy was measured as the ratio between the RV weight and the weight of the left ventricle plus septum.
Wall thickness of the aorta and main pulmonary artery were measured [13]. To further assess the characteristics of extracellular matrix in the vessel wall, the area occupied by mucopolysaccharides, collagen and elastin was evaluated in adjacent sections of main pulmonary artery stained with Alcian blue, Masson trichrome and Orcein, respectively, and expressed as a percent of total vessel area.
The number of intrapulmonary vessels with a diameter <50μm showing positive immunoreactivity to smooth muscle (SM)-α-actin was counted and expressed as percent of total number of small vessels. These vessels were further classified into non-muscularized, partially muscularized and fully muscularized, according to the proportion of vessel wall positive for SM-α-actin [19]. Additionally, the number of intrapulmonary vessels <50μm with double elastic laminas, assessed in Orcein stained sections, were counted and expressed as percent of total number of small intrapulmonary vessels.

Assessment of cell proliferation

Lung tissue sections were immunostained with a monoclonal antibody against proliferating cell nuclear antigen (PCNA) to assess cell proliferation. The number of cells with nuclei showing immunoreactivity to PCNA was counted and expressed as the ratio between positive and negative cells in each vessel wall.

Real-Time PCR

Total RNA was extracted from lung tissue using an RNeasy Microkit. Real-time PCR for endothelial nitric oxide synthase (eNOS) and ß-actin was performed as previously described[13]. Results were normalized to ß-actin expression levels and relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method[20].

Western blot analysis

Endothelial NOS activity was determined in lung homogenates by the ratio between the phosphorylated form (PeNOS) and the total eNOS protein expression, analyzed by western blot.

Plasma chemistry of vasoactive agents

A method based on the Griess reaction[21] was used to measure nitrites and nitrates in the plasma. Endothelin-1 (ET-1) levels were determined by ELISA assay. Plasma levels of norepinephrine (NE), epinephrine and serotonin were measured by HPLC.

Statistics

Data in tables are expressed as mean±SD and in graphs as mean±SEM. A two-way ANOVA was used to evaluate the main effects of CS and hypoxia, as well as their interaction. *Post-hoc* pairwise comparisons were performed using the Student t-test.

Additional details on Methods are provided in the on-line supplementary material.

TABLE 1. PULMONARY HEMODYNAMICS AND RIGHT VENTRICLE HYPERTROPHY

					Two-way ANOVA main effects		
	Control	**CS**	**CH**	**CSCH**	**CS Exposure**	**Hypoxia**	**Interaction**
PAP at FiO_2 0.21, mmHg	6.4±1.7	8.0±0.7	8.6±2.6	10.5±1.8	0.028	0.005	0.843
PAP at FiO_2 0.12, mmHg	6.2±2.0	8.2±1.4	8.2±2.2	10.2±1.9	0.016	0.016	0.994
PAP at FiO_2 0.21, mmHg (recovery)	6.9±1.7	8.0±1.8	8.4±1.9	10.1±1.9	0.092	0.033	0.677
Mean systemic arterial pressure, mmHg	51.9±12.5	50.9±2.2	59.1±5.7	55.8±4.1	0.474	0.048	0.682
Fulton Index	0.30±0.01	0.32±0.03	0.34±0.05	0.37±0.05	0.063	<0.001	0.713

Values are mean±SD.
Definition of abbreviation: PAP: Mean pulmonary artery pressure.
Fulton Index = Right ventricle weight / (left ventricle weight + septum weight).

RESULTS

Survival and body weight

During the first 10 weeks, the mortality rate of animals exposed to CS was 45% whereas no animal died in the Control group. During the last 2 weeks, when hypoxia was added, one animal of each experimental group died, resulting in an additional mortality rate of 6.3% in CS, 6.3% in CH and 9.1% in CSCH groups. All the non-exposed animals completed the study. The main cause of death (88% of cases) was due to broncoconstriction during the exposure to CS. Animals that died during the study were excluded from the final analysis.

From week 4 the weight gain in the CS group decreased markedly. Hypoxia induced weight loss from the first week of exposure (week 11 of the study). As a result, at the end of the study, body weight in the three experimental groups was lower than in the Control group, showing the CSCH group the lowest value (supplemental Figure 1).

Pulmonary hemodynamics

Exposure to CS or to hypoxia raised the PAP, both under normoxic and hypoxic conditions (Table 1, Figure 1A). The increase of PAP in the CS and CH groups was of similar magnitude, and was much more pronounced in animals subjected to both stimuli (CSCH group) (Figure 1A). Interestingly, PAP did not change during acute hypoxic challenge in any of the experimental groups, reflecting a lack of hypoxic pulmonary vasoconstriction in this experimental model (Figure 1B). Exposure to hypoxia (CH and CSCH groups) was associated with an increase in systemic arterial pressure (Table 1, Figure 1C).

Right ventricular weight increased in animals exposed to hypoxia (Table 1) and increased further in animals exposed to hypoxia and CS (CSCH group) (Figure 1D).

At the end of the study, compared with the Control group, the hematocrit was 7% higher in the CS group, 5% in the CH group and 12% in the CSCH group (Supplemental Figure 2).

Mechanical properties and reactivity of pulmonary artery and aorta

Vascular distensibility. The distensibility of pulmonary arteries was significantly reduced in animals exposed to hypoxia, the effect being more pronounced in the CSCH group (Table 2 and supplemental Figure 3). Overall, the distensibility of the aorta was lower than that of pulmonary arteries and was similar in all groups.

Contractile responses. Table 2 shows the vascular responses to KCl and NE in the pulmonary artery. Contraction induced by KCl was greater in pulmonary arteries from animals exposed to CS or CH, compared with controls ($p<0.05$ each), being further increased when both stimuli were combined (Table 2 and supplemental Figure 4). Contraction induced by NE was greater in pulmonary arteries of animals exposed to hypoxia (CH and CSCH groups), whereas in the CS group it was similar to that in the controls (Table 2 and supplemental Figure 5). No differences were observed in the contractility of the aorta.

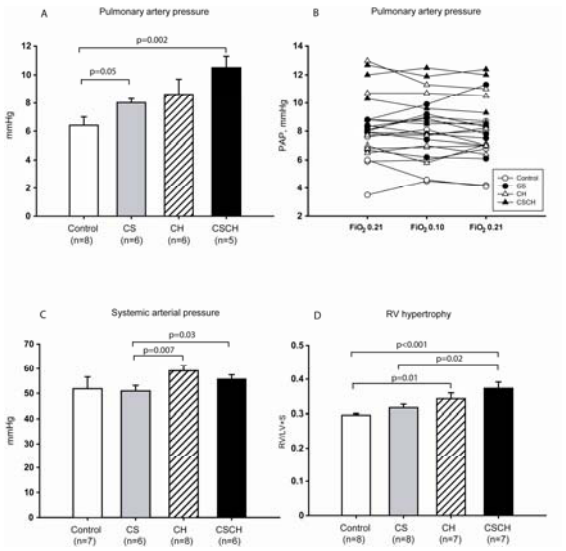

Figure 1. Pulmonary and systemic hemodynamics. (A) Mean pulmonary artery pressure (PAP) at baseline. Bars indicate mean ± SEM value in each experimental group. (B) Individual values of mean PAP in: normoxia (FiO_2 0.21), acute hypoxia (FiO_2 0.10) and recovery to normoxia (FiO_2 0.21). (C) Mean systemic arterial pressure at baseline. (D) Right ventricle (RV) hypertrophy, assessed as the ratio between the RV weight and the left ventricle weight plus the septum weight. CS: exposed to cigarette smoke; CH: exposed to chronic hypoxia; CSCH: exposed to cigarette smoke and chronic hypoxia.

TABLE 2. MECHANICAL PROPERTIES AND REACTIVITY OF PULMONARY ARTERY AND AORTA

					Two-way ANOVA main effects		
Pulmonary Artery	**Control (n=7)**	**CS (n=6)**	**CH (n=7)**	**CSCH (n=8)**	**CS Exposure**	**Hypoxia**	**Interaction**
Distensibility, $mmHg^{-1}*10^{-3}$	14.9±1.9	14.2±2.9	13.1±1.7	11.2±2.8	0.152	0.013	0.534
Contraction, mN							
KCl (60 mM)	20.3±3.2	31.9±4.1	30.5±5.5	33.8±8.5	0.002	0.008	0.059
NE ($0.2·10^{-6}$M)	5.5±2.9	6.6±3.9	10.8±4.3	9.0±3.0	0.815	0.007	0.284
Relaxation in response to, AUC:							
ADP % of change [a]	207.8±46.7	171.7±47.8	176.8±19.2	205.2±43.6	0.808	0.936	0.043
wall tension [b]	22.6±8.5	25.7±16.2	52.7±13.2	46.5±16.2	0.775	<0.001	0.406
ADP+L-NAME [b]	44.4±22.5	66.8±33.9	107.7±29.7	69.6±28.3	0.903	0.016	0.230
SNP [b]	18.4±8.1	23.6±12.3	35.4±8.5	32.1±13.1	0.820	0.005	0.316

Values are mean±SD.
Definition of abbreviations: KCl: potassium chloride, NE: Norepinephrine, AUC: area under the curve, ADP: Adenosine diphosphate; L-NAME: N^G-monomethyl-L-arginine; SNP: Sodium nitroprusside
Vascular response is shown as [a] percentage of change in tension from pre-contraction to NE and [b] absolute change in tension. The AUC values of ADP+L-NAME and SNP are shown as the delta change in the wall tension.

Endothelium-dependent vasorelaxation. Figure 2 shows the changes in wall tension of pulmonary artery and aorta induced by cumulative doses of ADP, after pre-contraction with NE. The initial wall tension, after NE pre-contraction, was higher in pulmonary arteries of animals exposed to hypoxia (CH and CSCH groups). As a result, when evaluating absolute values of wall tension, the area under the relaxation curve (AUC) in these two groups was larger than in the other two groups (Control and CS) (Table 2). To account for differences in initial wall tension we evaluated change in tension as percent of initial value. After performing this correction, no differences between groups in the AUC of endothelium-dependent responses were observed (Table 2).
The AUC in the aorta was similar in all groups (Figure 2B).

Pulmonary vascular remodeling
Wall thickness of pulmonary arteries was greater in the three experimental groups (CS, CH, CSCH), as compared with controls. No additive effects were observed when CS and hypoxia were combined (Figure 3A). In contrast, wall thickness in the aorta was not affected by any of the experimental conditions (Supplemental Table 1), although it was thinner in the CH group compared with the CS group (Figure 3B).
In animals exposed to hypoxia (CH and CSCH groups) the content of mucopolisaccharydes in main pulmonary artery was increased, whereas collagen was diminished, as compared with Control and CS groups. Elastin content was greater in the CSCH group compared with the Control group (Figure 4).

The proportion of small vessels showing positive immunoreactivity to SM-α-actin was higher in the 3 experimental groups, with a highly significant effect for CS exposure (Table 3). When arterioles were scored according to the degree of muscularization, it was apparent that in the CS and CSCH groups there was a decrease in the proportion of non-muscularized vessels and a concomitant increase in the proportion of fully-muscularized intrapulmonary vessels (Fig 5).

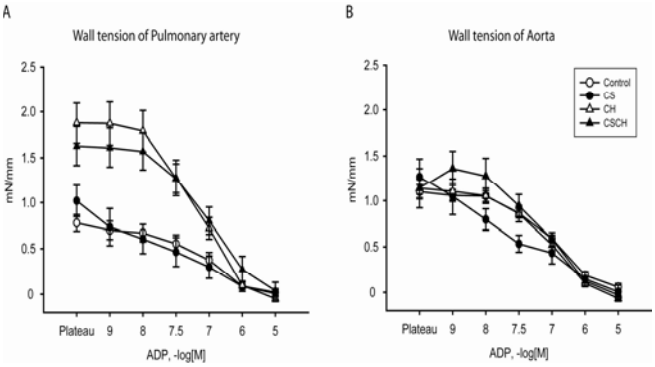

Figure 2. Vascular reactivity of main pulmonary artery and aorta. (A) Changes in wall tension of pre-contracted pulmonary artery rings, expressed in mN/mm of circumference, in response to cumulative doses of ADP. (B) Changes in wall tension of pre-contracted aorta rings, expressed in mN/mm of circumference, in response to cumulative doses of ADP. Values are mean ± SEM at each condition of ADP.

Animals exposed to hypoxia (CH and CSCH) showed a significantly greater proportion of small intrapulmonary vessels with double elastic laminas as compared with control animals (Table 3 and supplemental Figure 6)
Assessment of proliferating cells ($PCNA^+/PCNA^-$ nucleus ratio) in small intrapulmonary vessel walls revealed no differences between groups (Supplemental Figure 7). The total number of nuclei per vessel remained unchanged irrespective the type exposure.

Gene expression and activity of eNOS
The gene expression of eNOS was evaluated by real-time PCR in lung homogenates and normalized by

the expression of β-actin. Lung expression of eNOS was reduced in animals exposed to CS (p=0.028 for additive effect was observed when both stimuli were combined. Changes in PeNOS and eNOS protein expression were evaluated by western blot in lung homogenates. The ratio PeNOS/eNOS was calculated as a measure of the enzyme activity. No significant changes were observed in the eNOS activity in none of the 3 experimental groups, although a tendency to decrease was present in CS and CSCH groups (Supplemental Figure 8B).

Plasma chemistry of nitrites and nitrates, endothelin-1 and catecholamines

Nitrites and nitrates. Plasma levels of nitrites and nitrates decreased in animals exposed to CS, and tended to increase in those exposed to hypoxia (Table 4).

Endothelin-1. There was strong interaction between the effects of CS and hypoxia on plasma levels of ET-1. Whereas in normoxic animals ET-1 increased in those exposed to CS, CS reduced ET-1 levels in animals subjected to CH (Table 4).

Catecholamines. There were no significant differences between groups in the plasma levels of NE, epinephrine and serotonin (Table 4).

Correlations

In pulmonary arteries, the contraction to KCl correlated with the vessel wall thickness (r=0.45, p=0.02)**.**

The PAP was related to the percentage of SM-α-actin positive intrapulmonary vessels. As shown in Figure 6A, PAP increased to similar extent in animals exposed either to CS or CH, which showed a similar degree of muscularization, and increased further in those subjected to both exposures (CSCH group), which also showed the greatest number of muscularized vessels.

The number of small intrapulmonary vessels positive for smooth muscle SM-α-actin was inversely correlated with the RNA expression of eNOS in lung homogenates (Figure 6B).

Moreover, distensibility correlated positively with the content of mucopolysaccharide (p=0.013, R=0.470) and inversely with the content of collagen (p=0.013, R=-0.469).

CS effect in ANOVA) (Supplemental Figure 8A). No

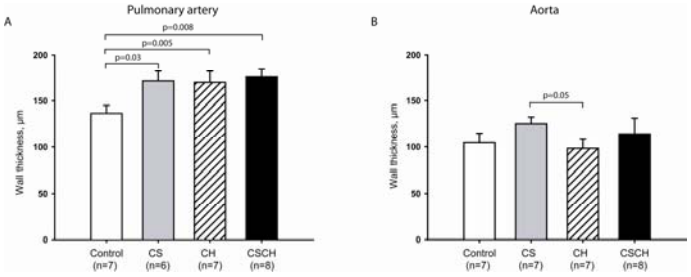

Figure 3. Morphometry of main pulmonary artery and aorta. Bars indicate mean ± SEM. Wall thickness of pulmonary artery (A) and aorta (B) in each experimental group.

DISCUSSION

Results of the present study show that both CS and CH produced a similar increase in PAP and RV hypertrophy, and that the combination of both agents had a synergistic effect on these alterations. Furthermore, CS and CH exerted different effects on the reactivity and mechanical properties of large pulmonary arteries and on the morphological characteristics of small intrapulmonary vessels.

We have previously hypothesized that pulmonary vascular changes that take place in COPD start at early disease stages, since they are apparent in patients with moderate disease severity and also in smokers with normal lung function[2;3]. Nevertheless, we acknowledge the pivotal role that hypoxia has in the development of pulmonary hypertension in this condition [22]. Given that COPD patients develop hypoxemia when airflow obstruction becomes severe, we hypothesized that the effects of hypoxemia on pulmonary vessels may add to the pre-existing effects of CS. In an attempt to mimic this sequence, in the present study we exposed guinea pigs to a hypoxic environment 10 weeks after initiating CS exposure. In addition, we evaluated the individual effects of CS and hypoxia on pulmonary and systemic vessels, as they have never been compared in the same animal model.

TABLE 3. MUSCULARIZATION OF INTRAPULMONARY ARTERIES

					Two-way ANOVA main effects		
	Control (n=8)	**CS (n=8)**	**CH (n=15)**	**CSCH (n=13)**	**CS Exposure**	**Hypoxia**	**Interaction**
Wall thickness, µm							
Aorta	105±28	126±19	98±26	114±49	0.148	0.486	0.838
Pulmonary artery	137±22	171±2	170±33	176 ±26	0.062	0.084	0.183
Small intrapulmonary vessels							
% of arteries α-actin^{+}	48.9±13.0	63.59±11.0	54.79±12.5	63.09±8.7	0.004	0.469	0.392
% of arteries with double elastic lamina	6.8±5.1	9.6±11.8	17.4±14.5	14.3±8.6	0.883	0.039	0.472

Values are mean±SD

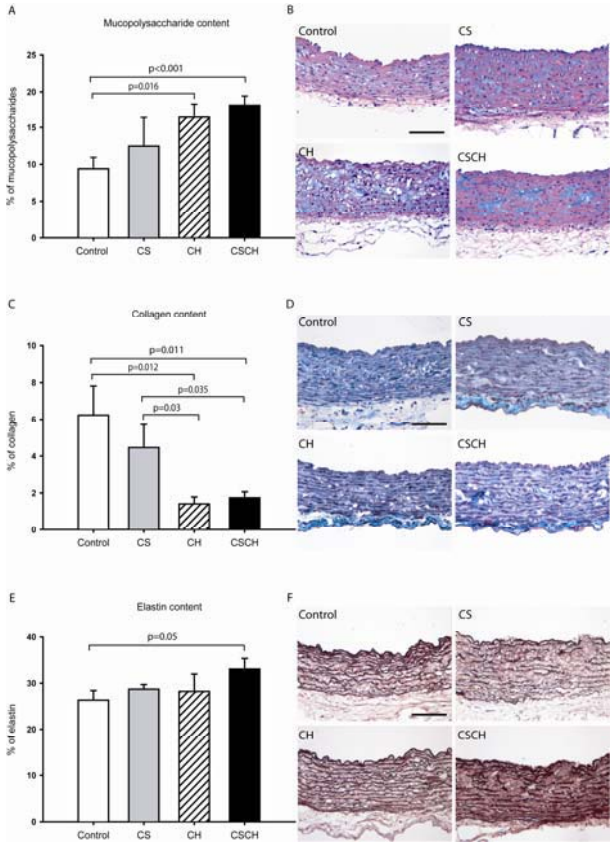

Figure 4. Extracellular matrix protein content in the main pulmonary artery. (A) Bar charts indicate the percentage of the area occupied by mucopolysaccharide in the total vessel area. (B) Representative microphotograph of Alcian blue staining in the 4 experimental groups. (C) Graph representation of the percentage of collagen in the pulmonary artery wall. Panel (D) shows representative images of Mason trichrome staining in each group of animals. (E) Elastin content shown as the percentage of the total vessel area. Panel (F) shows representative images of orcein staining in each experimental group.

Interestingly, CS and hypoxia exerted similar effects on PAP, right ventricular hypertrophy and pulmonary vessel remodeling, the effects on PAP and RV hypertrophy being further enhanced when both types of exposure were combined. On average, compared with the values observed in the Control group, CS exposure induced a 25% increase in PAP, exposure to hypoxia a 34% increase, and exposure to both factors a 64% increase. It is of note that the effect of 12 weeks of CS on PAP was of a similar magnitude as that induced by 2 weeks of hypoxia, thus emphasizing the prominent effect of CS exposure on pulmonary vessels, which is in agreement with the previous observation made by Yamamoto et al [10]. Yet, the current investigation demonstrates that the hemodynamic effects of CS exposure are of a similar magnitude to those produced by hypoxia and that the effects of both factors are further enhanced when they are combined.

The increase in PAP induced by CS or hypoxic exposures might be due to the effects of these agents on pulmonary vessel remodeling, distensibility or tone. Exposure to CS and/or to hypoxia resulted in an increased proportion of muscularized intrapulmonary vessels. Interestingly the morphological characteristics of muscularized arterioles were non-uniform and appeared to be related with the type of stimulus. Whereas CS exposure was strongly associated with an increased proportion of small vessels showing positive immunostaining to SM-α-actin, exposure to hypoxia was associated with a greater proportion showing double elastic laminas. These changes were not due to cell proliferation since the number of cells present in the vessel wall and the proportion of those showing positive immunoreactivity to the proliferating cell nuclear antigen (PCNA) did not differ between groups. Our findings are in agreement with those reported by King *et al* [23] who showed ultrastructural differences in smooth muscle cells in vessels of rats treated with monocrotaline and rats submitted to hypoxia. While arterioles of monocrotaline-treated rats contained immature smooth muscle cells with coarse peripheral myofilaments, bounded by thin indistinct elastic laminas, arteriolar smooth muscle cells of hypoxic rats were mature with fine myofilaments and bounded by electron dense laminas. It can be hypothesized, therefore, that hypoxia may have a stimulating effect on the production of elastin by mature smooth muscle cells, whereas CS may induce changes in cell phenotype with increased cytoplasmatic content of SM-α-actin.

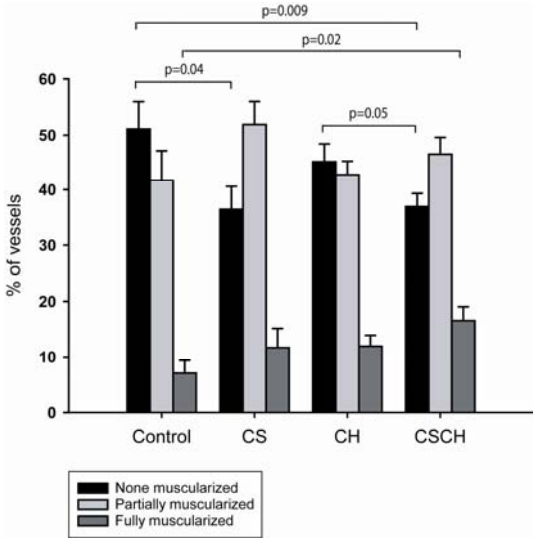

Figure 5. Classification of the intrapulmonary arteries according to their degree of muscularization. Bar charts show the % of non-, partially- and fully-muscularized vessels evaluated in lung sections by immunohistochemistry against SM-α-actin. Animals exposed to both cigarette smoke and chronic hypoxia (CSCH group) developed a greater number of fully-muscularized arteries. Values are mean ± SEM.

TABLE 4. BLOOD CHEMISTRY

					Two-way ANOVA main effects		
	Control (n=7)	CS (n=6)	CH (n=8)	CSCH (n=8)	CS Exposure	Hypoxia	Interaction
Levels in plasma:							
Nitrites and nitrates, mM	4.15±0.93	3.46±0.44	5.15±1.16	3.71±0.85	0.005	0.082	0.297
ET-1, pg/mL	1.02±0.10	1.12±0.23	1.56±0.64	0.75±0.32	0.022	0.580	0.005
Catecholamines, pmoles/mL of plasma:							
Norepinephrine	6.17±1.14	7.35±6.5	8.20±2.47	7.29±3.54	0.922	0.488	0.462
Epinephrine	4.02±1.16	5.09±2.66	6.44±5.59	8.05±5.71	0.464	0.149	0.83
Serotonin	2.66±0.67	2.31±2.1	7.40±8.16	2.94±2.84	0.229	0.183	0.303

Values are mean±SD
Definition of abbreviation: ET-1: endothelin-1. *P*-values < 0.05 were considered significant.

Exposure to hypoxia had greater effect on vessel distensibility than CS (Table 2), although guinea pigs subjected to both exposures showed the lowest distensibility (Table 2 and supplemental Figure 3). The cellular and molecular mechanisms responsible for pulmonary artery stiffening as a result of hypoxia are not well understood. Deposition of extracellular matrix in pulmonary arteries has been shown in animal models of hypoxia-induced pulmonary hypertension[24;25]. Interestingly, we observed a significant increase in the content of mucopolysaccharides in the wall of main pulmonary arteries, with a concomitant decrease of collagen, both in the CH and CSCH groups, as compared with the Control group. The content of both proteins correlated with the distensibility of pulmonary arteries, suggesting that an imbalance in extracellular matrix protein content may modulate the mechanical properties of the vessels wall.

The increase in PAP in the experimental groups could be also explained, at least in part, by an increase in vessel tone. In humans, exposure to hypoxia induces pulmonary vasoconstriction. However, in the present investigation we did not observe any change in PAP when animals were exposed to acute hypoxia, which is consistent with the lack of hypoxic pulmonary vasoconstriction in the guinea pig [26]. Accordingly, the increased PAP in the CH group might be explained by adjustments in the contractile/synthetic phenotype of SMC in response to reduced oxygen concentrations[27;28]. In this respect, it should be noted that *in vitro*, pulmonary arteries from animals exposed to hypoxia showed greater contraction to NE than controls (Table 2), likely reflecting changes in SMC adrenergic receptors that regulate vessel tone. Regretfully, we were unable to show a clear increase in the plasmatic levels of NE and epinephrine in animals exposed to hypoxia (Table 4), presumably due to the variability in these measurements and the reduced number of animals in each group. Overall, these results suggest that the effects of hypoxia on the pulmonary circulation exceed those produced by the stimulation of a sympathetic response mediated by peripheral chemoreceptors and baroreceptors in the systemic circulation.

The assessment of endothelium-dependent vasodilatation of pulmonary arteries was largely influenced by the pre-contraction induced by NE, since in arteries from animals exposed to CH the contraction to NE doubled that shown in arteries of the Control or CS-exposed groups (Figure 2). When ADP-induced relaxation was evaluated as the AUC of the absolute change in tension, values in animals exposed to hypoxia were greater than in the other two groups. However, we do not interpret such a difference as demonstrative of the impairment of endothelium-dependent relaxation, but as the result of different baseline tension after NE precontraction. To account for this difference we evaluated the change in tension as a percentage of the baseline value. In this case, we did not observe significant differences in the AUC of the relaxation curve among the groups (Table 2). Accordingly, we conclude that in the current investigation neither CS nor exposure to hypoxia produced endothelial dysfunction in pulmonary arteries. In a previous study we showed that exposure to CS for 3 and 6 months induced endothelial dysfunction in the guinea pig[13]. Differences between the two studies can be explained by the fact that CS exposure in the present study (4 cigarettes/day) was lower than that used in the previous study (7 cigarettes/day). We used a lower dose of CS because we anticipated greater mortality in animals exposed to a hypoxic environment for two weeks after being exposed to CS for 10 weeks, as indeed it occurred. The lack of impairment of endothelial function in the main pulmonary arteries contrasts with the observation of decreased expression of eNOS mRNA and protein activity in lung tissue and reduced plasma levels of nitrites/nitrates in the CS group. We speculate that this situation may represent an initial step that antecedes endothelial dysfunction as assessed *in vitro* in organ bath chambers. Interestingly, the lung expression of eNOS was inversely related to the SM-α-actin content in small intrapulmonary vessels, suggesting that vascular cells of these vessels may be a first target of CS, whereas large vessels may require more intense exposure to CS.

We evaluated ET-1 and serotonin levels in plasma because of its actions on SMC physiology and vascular tone. Levels of ET-1 were increased in CS and CH groups, in keeping with the increased levels observed in smokers and COPD patients[29;30]. However, no differences in ET-1 levels were observed in CSCH animals, suggesting that the synergistic effect on PAP does not appear to be mediated by ET-1 in plasma.

The greatest effects on PAP and RV hypertrophy were observed in guinea pigs subjected to combined CS and CH exposure. Since animals subjected to both factors showed similar vessel remodeling and contractile response to NE, we hypothesize that the greater hemodynamic effect observed in this group can be explained by the combined effects of CS on vessel remodeling and the greater reactivity induced by CH. This observation in the guinea pig is in keeping with the observations made in COPD in which patients with moderate disease severity show a similar degree of pulmonary vessel remodeling as patients with severe disease, whereas they differ markedly in PaO_2 and PAP[31]. Furthermore, in patients with COPD and chronic respiratory failure the degree of pulmonary vessel remodeling is not related to the presence of pulmonary hypertension or its severity[32]. Accordingly, our findings suggest that the presence of pulmonary hypertension in COPD, which is commonly associated with chronic respiratory failure, may be due to factors related to hypoxia that add to an underlying process of vessel remodeling produced by cigarette smoking. The different intensity of vessel remodeling and reactivity might explain the great variability in the relationship between PAP and PaO_2 observed in COPD[33].

Animals exposed to CS and CH showed greater hematocrit values, likely due to the additive effects of hypoxia and carboxyhemoglobin on red cell production. Enventually, the increase in blood viscosity resulting from increased red cell concentration could contribute to some degree to the increase in both PAP and SAP. The CSCH group was also the group with less weight gain. Presumably, the mechanisms responsible for lower weight gain could be related to the systemic effects induced either by CS or chronic hypoxia, akin to those observed in COPD. Body weight was unrelated to mortality probably because the principal cause of death was bronchoconstriction induced by CS.

Our study has limitations. Cardiac output was not measured due to technical difficulties for its assessment in our experimental setting. Accordingly we cannot disregard that sympathetic changes might underline some of the differences noticed in SAP. Further, the use of anesthesia during the acute hypoxic challenge might have attenuated hypoxic pulmonary vasoconstriction (HPV)[34]. Accordingly, we cannot completely exclude that the guinea pig may exhibit some degree of HPV, as it has been suggested by Thompson *et al* [35].

In summary, results of the present investigation show that in the guinea pig exposure to CS or to CH has similar effects on pulmonary hypertension and RV hypertrophy, and when the two factors are combined the hemodynamic effects are magnified. In animals exposed to CS, mechanisms underlying these hemodynamic changes appear to be related to the remodeling of small intrapulmonary vessels. Exposure to hypoxia modifies the mechanical properties of large pulmonary arteries, presumably by altering extracellular matrix deposition in the vessel wall, enhances their sensitivity to adrenergic agonists, and induces small vessel remodeling. Altogether, this indicates that hypoxemia represents a critical step in the progression of pulmonary vascular impairment that accelerates and, in some aspects amplifies, the initial effects of CS. These findings contribute to unravel the mechanisms underlying the development of pulmonary hypertension in COPD and to clarify the variability in the relationship between arterial oxygenation and pulmonary hypertension.

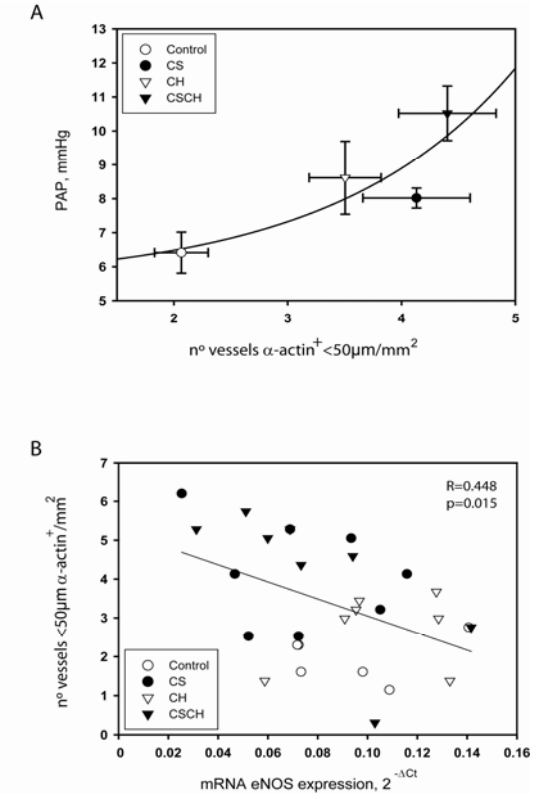

Figure 6. (A) Plot of pulmonary artery pressure (PAP) against the degree of vascular remodeling, expressed as the number of smooth muscle α-actin$^+$ vessels per mm^2 of area. The equation was fitted to a modified simple exponential curve. Each data point corresponds to the mean value ± SEM of each group. (B) Relationship between the RNA expression of eNOS in lung homogenates and the degree of muscularization of small intrapulmonary arteries. The lower expression of eNOS is related to a higher content of smooth muscle α-actin in arteries <50μm.

Acknowledgments

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Effects of Chronic Cigarette Smoke and Hypoxia Exposure on the Pulmonary Circulation in the Guinea Pig

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SUPPLEMENTARY MATERIAL

METHODS

Animals and management

Eighty-three male Hartley guinea pigs (Harlam Ibérica, Spain), with an average weight of 350 g, were divided randomly into four groups: one group was exposed to cigarette smoke (CS) for 12 weeks (n=33), a second group was kept in a normal atmosphere for 10 weeks and subsequently exposed to a FiO_2 of 0.12 for two weeks in a hypoxic chamber (Chronic Hypoxia; CH) (n=16), a third group was exposed to CS for 12 weeks and to hypoxia during the last two weeks (Cigarette Smoke and Chronic Hypoxia; CSCH) (n=18), and a control group (Control) was sham-exposed to CS and kept in a normal atmosphere (n=16).

All animals were housed in a light–dark cycle of 12 h, in a controlled temperature room, and were given a diet of standard chow and water supplemented with vitamin C (1 g/L; Roche Farma, Madrid, Spain) ad libitum. All procedures involving animals and their care were approved by the Ethics Committee of the University of Barcelona and the Institutional Committee of the University of Valladolid for Animal Care and Use, and were conducted according to institutional guidelines that are in compliance with national (Generalitat de Catalunya decree 214/1997, DOGC 2450) and international (Guide for the Care and Use of Laboratory Animals, National Institutes of Health, 85–23, 1985) laws and policies.

Cigarette smoke exposure

Guinea pigs were exposed to the smoke of four research cigarettes per day, 5 days a week for a period of 12 weeks using a nose-only system [1;2] (Protowerx Design Inc; Langley, British Columbia, Canada). Control animals were sham-exposed for the same period of time.

The number of cigarettes (2R4F; Kentucky University Research; Lexington, KY, USA; 11 mg tar, 0.8 mg nicotine per cigarette) per day was selected as a sub-maximal dose causing endothelial dysfunction as observed in preliminary studies [2]. This particular dose was selected with the intention of avoiding a high mortality rate as well as the possible concealment of synergistic effects of the two stimuli in the CSCH group.

Exposure to chronic hypoxia

Animals were placed in hypoxic glass chambers (125 [l] x 50 [h] x 50 [w] cm; 8 animals/chamber) that were continuously fluxed with a gas mixture (12% O_2 in N_2; PO_2 ≈85 mmHg; equivalent to ≈4300 m). Accumulation of CO_2 was prevented by the continuous flow of

the gas mixture and by a CO_2 absorbant (soda lime, Analema, Vorquímica S.L., Spain). Guinea pigs had free access to food and water, and remained in this atmosphere all the time apart for 30–40 min/day for routine cleaning, maintenance and for the smoking session.

Pulmonary hemodynamics

Half of the animals in each group were used to measure the pulmonary artery pressure (PAP). One hour before measuring the PAP, animals were placed in a normoxic environment and anesthetized by intraperitoneal administration of ketamine (50 mg/ml; 50 mg/kg. Pfizer Pharmaceuticals, Dun Laoghaire, Ireland) and xylazine (2%; 7 mg/kg. Bayer AG, Leverkusen, Germany). Guinea pigs were tracheotomized and immediately pump ventilated (Harvard Rodent Ventilator, Model 683, Harvard Apparatus, South Natick, Massachussetts) with a gas mixture (20% O_2, 80% N_2) at 40 breaths/min and a positive end-expiratory pressure of 2 cm H_2O. The PAP was measured in room air via an open chest cardiac puncture. A catheter (20 G Becton Dickinson) connected to a pressure transducer (Hewlett Packard 1280 Gould Statham, USA) was introduced into the right ventricle until it reached the pulmonary artery. We confirmed the position of the catheter by pulse tracing. The transducer was connected to an acquisition card (Power Lab 16SP; ADI Instruments; Castle Hill, Australia), which sent the signal to the computer for instant monitoring of pressures and storage for offline analysis. The right carotid artery was also isolated and cannulated to simultaneously measure the arterial systemic pressure in normoxic conditions. Then, a second measure of the PAP was performed in hypoxic conditions for 4 minutes by ventilating the animal with a FiO_2 of 0.10. Finally, the recovery profile was evaluated by again pumping air with a FiO_2 of 0.21.

At the end of the experiment the cardiopulmonary block was removed and the right ventricle and the left ventricle plus the septa were isolated and weighed to calculate the Fulton Index [3].

Vessel distensibility and endothelial function

Half of the animals in each group were used to measure distensibility and vascular reactivity. After 3 months of CS exposure and 24 h after the last exposure, the animals were anesthetized and the cardiopulmonary block was removed. A segment of the aorta and the main pulmonary artery were isolated,cleaned of fat and connective tissue and cut into 3-mm-long rings. The diameter and length of the artery rings were measured using a calibrated eyepiece. Two rings from the thoracic aorta and the left and right branches of the main pulmonary artery were placed in organ bath chambers (Panlab, Barcelona, Spain) filled with Krebs-Henseleit's (KH) buffer (containing (in mM) 118 NaCl,

24 $NaHCO_3$, 11.1 glucose, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$), bubbled with a gas mixture of 21% O_2 and 5% CO_2 (pH 7.35–7.45) and kept at 37 ºC using an outer water bath warmed by a recirculating heater. Ring preparations were attached to an isometric transducer (Panlab, Barcelona, Spain) at resting tension. Then the micrometer was adjusted to stretch the rings by 1 mm and the tension was recorded.

Distensibility. Vessel distensibility (Dis) was evaluated [4;5] according to the equation $Dis=\Delta D/\Delta P \cdot D$ (where ΔD is the difference in diameter before and after 1 mm of stretching, ΔP is the difference in pressure at the same points and D is the final diameter of the ring).

Endothelial function. Rings were equilibrated for 1 h under a resting tension of 20 mmHg for pulmonary arteries and 25 mmHg for aortic rings, as indicated by preliminary studies [2]. After a period of stabilization, the arteries were contracted with KCl (60 mM) to determine their viability and contractile capacity. The rings were then pre-incubated with indomethacin ($1x10^{-5}$ M; Sigma Aldrich, St Louis, USA) 30 min before the experiments in order to inhibit the synthesis of cyclo-oxygenase products. Indomethacin was kept in the solution throughout the experiment. Rings were contracted with norepinephrine (NE; 10^{-7} and $0.2x10^{-6}$ M; Sigma Aldrich) to obtain a stable plateau of tension. The endothelial function in both the aorta and the left branch of the pulmonary artery was evaluated by adding cumulative doses (10^{-9} to 10^{-5}M) of adenosine-5'-diphosphate (ADP, Boehringer GmbH, Mannheim, Germany) to pre-contracted artery rings. A dose-response curve to sodium nitroprusside (SNP; 10^{-10} to 10^{-5} M, Sigma Aldrich), an exogenous NO donor, was also obtained for the right branch of the pulmonary artery and aorta. All procedures were repeated in the presence of N^G-monomethyl-L-arginine (L-NAME; 10^{-3} M, Sigma Aldrich), an inhibitor of NO synthase (NOS), in order to corroborate the endothelial function assay performed with ADP. The recorded tension for each drug dose was expressed as artery wall tension and was calculated by dividing the recorded force (in mN) by twice the measured length of the pulmonary artery ring [6]. The endothelial function was expressed as the difference between the wall tension achieved for each dose of vasodilator and the resting wall tension. The area under the curve (AUC) was calculated from the delta change in the wall tension response curve to different vasodilator (ADP or SNP) doses for each artery using specific software (Sigmaplot 10.0, Systat Software Inc, San José, CA, USA). Each curve was evaluated by an observer without knowledge of the smoke exposure status.

Macroscopic and microscopic morphologic studies

After the organ bath studies, all artery rings were fixed in 4% formaldehyde and cryo-embedded at an optimal cutting temperature (O.C.T). Morphometric studies were performed in 4-μm elastin-Van Gienson stained aorta and main pulmonary artery sections. The wall thickness of each artery was measured as the mean distance between the inner and the outer elastic lamina in H&E stained sections using an image analysis system (Leica Qwin).

The extracellular matrix protein content was evaluated in sections of main pulmonary arteries stained with Alcian blue for mucopolysaccharides, Mason trichrome for collagen and orcein for elastin. The area of each protein was calculated by an image analysis system (Image-Pro Plus 4.5) and related to the total wall vessel area. Results were expressed as a %.

Explanted lungs were inflated with 4% formaldehyde at a constant pressure of 25 cm H_2O for 24 h, and then embedded in paraffin. Histological examination was performed in 4-μm lung sections stained with hematoxylin-eosin (H&E). Intrapulmonary vessels were analyzed in lung tissue sections stained with orcein. One hundred vessels with an external diameter <50 μm were evaluated to assess the number of double elastic lamina arterioles. Results were expressed as the number of arteries with double elastic lamina/mm^2.

Immunohistochemical studies

The expression of α-actin in pulmonary vessels (<50 μm) was assessed via immunohistochemistry using anti-α-actin antibody (Dako, Glostrup, Denmark) on formalin-fixed lung slides. Briefly, samples were deparaffined and incubated with primary antibody (dilution 1/1000) for 1 hour followed by 1h of incubation with a secondary biotinilated antibody. Then an avidin–biotin reaction was performed to amplify the signal and 3,3'-diaminobenzidine (DAB+ chromogen, DakoCytomation, Carpinteria, California) staining was used to show positive vessels. Slides were counterstained with H&E staining. Twenty randomly selected fields per slide were photographed and evaluated. Arteries were scored according whether they were fully-, partially- or non-muscularized. The immunoreaction to α-actin was quantified as the % of fully-, partially- or non-muscularized arteries respect to the total number of arteries and the % of muscularized arteries irrespective the degree of muscularization.

Assessment of cell Proliferation

Immunohistochemistry assay against proliferating cell nuclear antibody (PCNA; mouse monoclonal [PC10], Abcam, Cambridge, UK) was performed in paraffin lung slices. Samples were deparaffined and incubated with anti-PCNA antibody (dilution 1/5000) for 1 hour 30min. Then amplification of the signal was performed by peroxidase labeled polymer-HRP (Dako EnVision, Carpinteria, CA.) for 40 minutes and 3,3'-diaminobenzidine (DAB+ chromogen, DakoCytomation, Carpinteria, CA) staining was used to show positive vessels. Slides were counterstained with H&E staining. A total number of 25 vessels <50μm were evaluated in each slide. The number of positive and negative nuclei for PCNA in the vessel wall of intrapulmonary arteries was counted. The proportion of proliferating nuclei was expressed as the ratio between $PCNA^{+}/PCNA^{-}$ nuclei per vessel. The total number of nuclei (both, positive and negative for PCNA) was also counted in each intrapulmonary vessel.

Real-time PCR

Total RNA was extracted from lung tissue using an RNeasy Microkit (Qiagen GmbH, Hilden, Germany). For reverse transcription, 1.5 mg of total RNA was retrotranscribed using a high-capacity cDNA Archive kit (Applied Biosystems). Quantification of eNOS was assessed via real-time PCR using SYBR Green I chemistry (SensiMix (dT) DNA Kit, Quantance Ltd, Ballards Lane, London). Normalization of gene expression levels was performed using β-actin as the endogenous housekeeping gene. Primers were designed as previously described [2]. Amplification was performed on a Chromo4 thermocycler (MJ Research, BioRad, Hercules, CA), and each sample was run in duplicate. The primer sequences for eNOS were 3'-AGCCAACGCGGTGAAGATC-5' and 5'-TTAGCCATCACCGTGCCC-3' and for β-actin 3'-ATATCGCTGCGCTCGTTGTC-5' and 5'-AACGATGCCGTGCTCAATG-3'. The results were normalized to ß-actin expression levels and relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method [9].

Western blot analysis

Total protein from lung tissue was subjected to 10% SDS-PAGE and separated proteins were transferred onto polyvinylidene difluoride membranes (GE Healthcare Limited, Amersham Place, Little Chalfont, Buckinghamshire, UK). The membranes were incubated sequentially with the following antibodies: Anti-phosphorilated-eNOS (ser1177) (Cell Signaling), Anti-eNOS/NOS Type III (BD Transduction Laboratories) and β-actin (Cell Signaling). The activity of eNOS was calculated as the ratio of PeNOS/eNOS.

Plasma chemistry of vasoactive agents

Plasma nitrites and nitrates. Plasma was obtained after 15 min centrifugation of complete EDTA-blood at 2000 g and 4 ºC. The supernatant was assayed immediately or stored at –80 ºC. A method based on the Griess reaction was used to measure nitrites and nitrates [7]. In the first step nitrates were transformed in nitrites by the nitrate reductase (6.03*10–3 U/μL) in the presence of NADPH (550 μM). In the second step total nitrites were reacted with a Griess solution (Naphthoethylendiamine chlorhydrate 0.2%/ sulphanilamide 2% in H_3PO_4 at 5%, 1:1) generating a blue product. This gave a maximum peak of optical density at 540 nm that could be read using an ELISA reader at the same time as the calibration curves of the $NaNO_2$ standard.

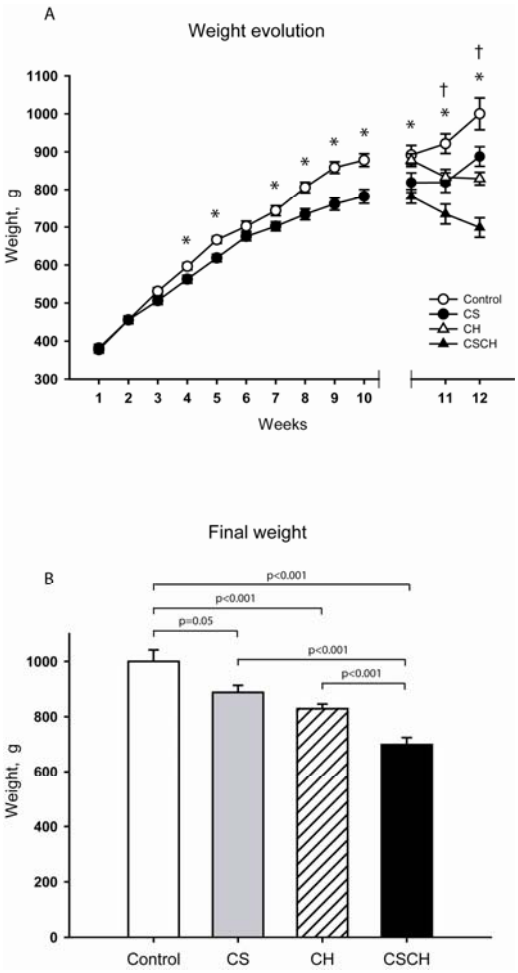
Catecholamines (CA) and serotonin (5-HT) determination in plasma. Citrated-blood was centrifuged to obtain the platelet-rich plasma (PRP). PRP was again centrifuged and supernatant plasma was withdrawn into tubes containing 60 mg/ml sodium metabisulfite and frozen at –80 ºC until plasma CA and plasma 5-HT analysis was performed. An aliquot of supernatant plasma (500 μl) was adsorbed in Waters Oasis-HLB cartridges (Waters, Milford, MA, USA) and eluted with 0.5 ml of citric acid (26.7 mM) and 2.5% methanol (pH = 2.9) for HPLC-ED analysis. Chromatographic conditions and quantification procedures have been described previously [8], except for the mobile phase, which consisted of a mixture of NaH_2PO_4 (150 mM) and MeOH (6%) pH (3.6).

Endothelin-1. Endothelin-1 levels in plasma were determined using a Biomedica ELISA assay (Biomedica Medizinprodukte; Vienna, Austria).

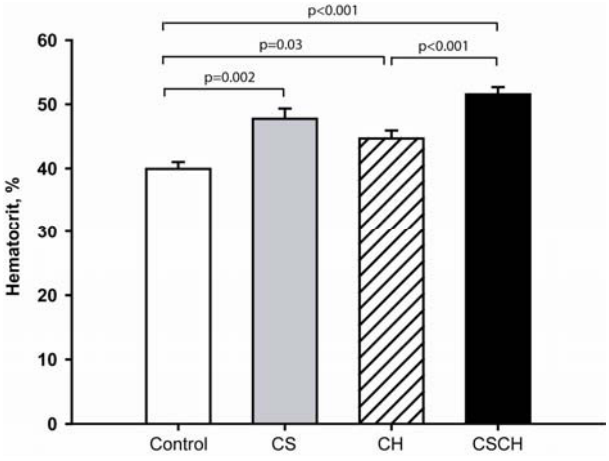
Statistics

Data in tables are expressed as mean±SD and in graphs as mean±SEM. Two-way ANOVAs were performed to evaluate the main effects of CS and hypoxia and their interaction. *Post-hoc* pairwise comparisons were performed using the Student t-test. The area under the curve (AUC) was determined for each curve using a logistic curve-fitting equation. *P*-values < 0.05 were considered significant.

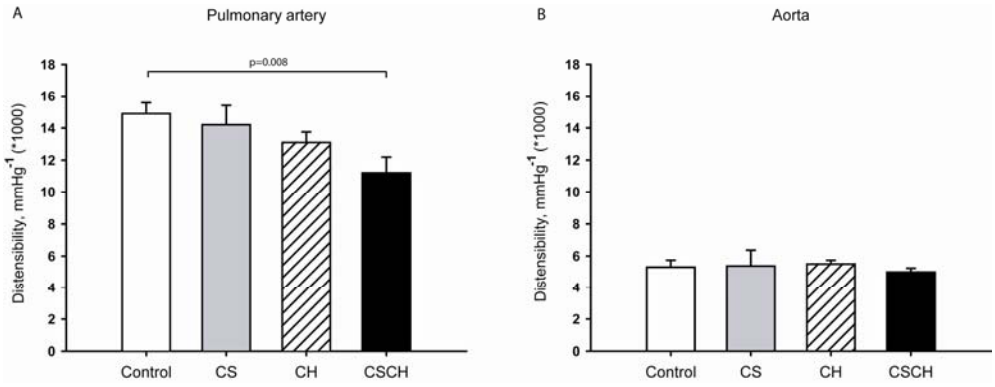
RESULTS



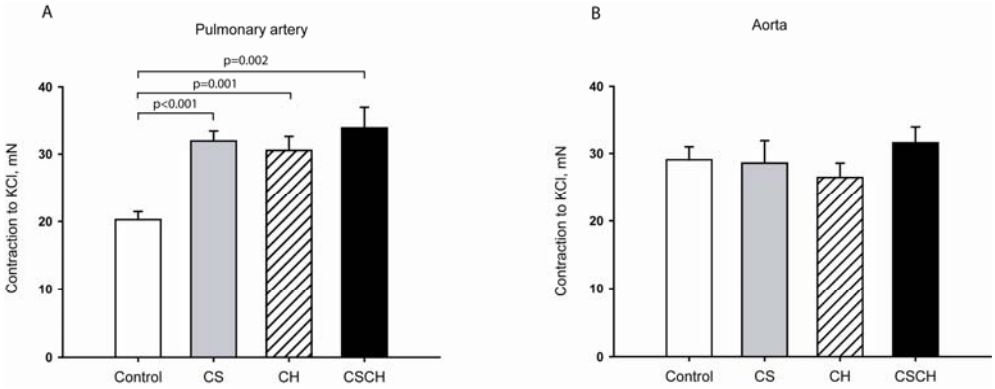
Supplemental Figure 1. (A) Weight evolution of the four experimental groups. Initially, animals were exposed for ten weeks either to cigarette smoke (CS) or air (sham exposed animals). During this period of time CS exposure induced a decreased in the weight gain that was present from the fourth week on. Then, half of the animals of the CS and Control groups were additionally exposed to chronic hypoxia (CH). In these animals hypoxia produced a weight loss from the first week of exposure. ($p<0.05$: *all groups vs Control, † all groups vs animals exposed to CS and CH (CSCH). (B) Weight of the four groups at the end of the study. Values are mean ± SEM.



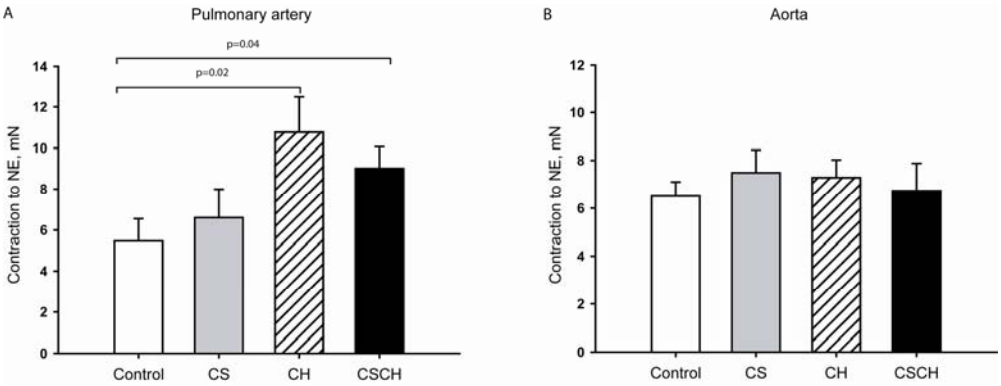
Supplemental Figure 2. Hematocrit values at the end of the study. All groups showed an increase in hematocrit, compared with the Control group, being markedly increased in animals exposed to both agents (CSCH group). Values are mean ± SEM.



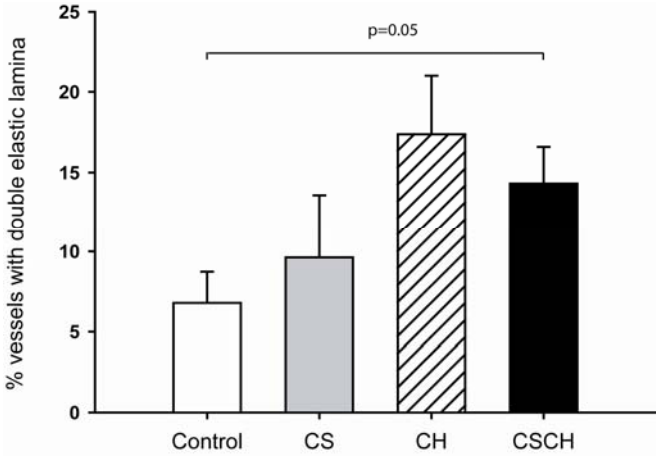
Supplemental Figure 3. Distensibility of pulmonary artery (A) and aorta (B). Distensibility was calculated from each ring as the change of pressure between rest and after 1mm stretching. The pulmonary arteries in the CSCH group were stiffer as compared with controls. Distensibility of the aorta rings was not different between groups. Values are mean ± SEM.



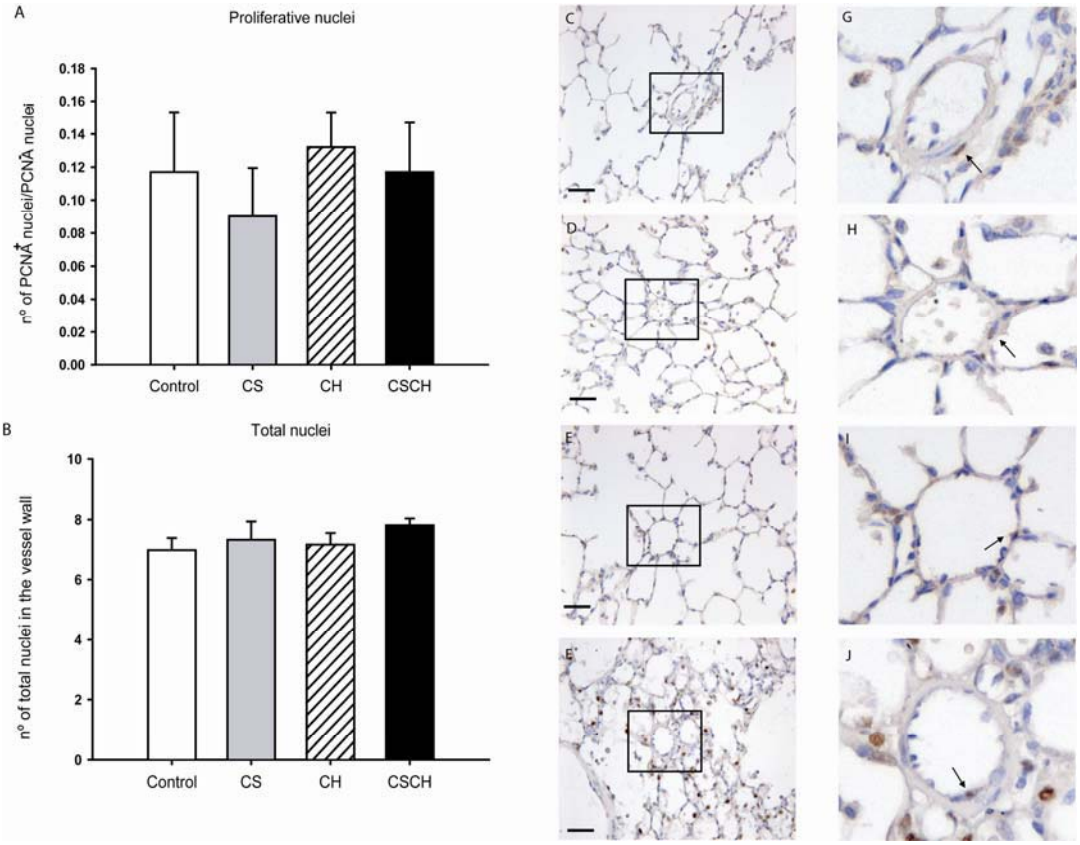
Supplemental Figure 4. Contractility of pulmonary artery and aorta induced by addition to KCl (60mM). (A) Pulmonary arteries of all experimental groups showed greater contraction to KCl as compared with controls, whereas (B) no changes were observed in rings of aorta. Values are mean ± SEM.



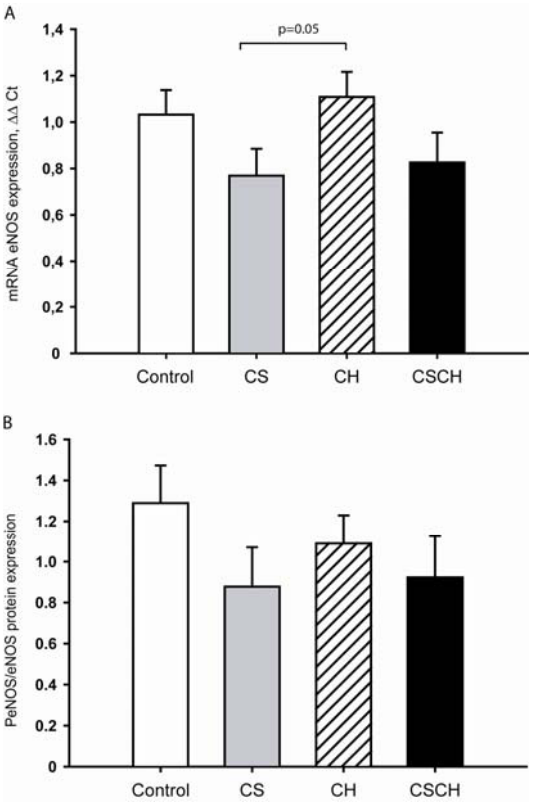
Supplemental Figure 5. Maximal contraction to NE in pulmonary arteries and aorta. (A) Pulmonary arteries of animals exposed to hypoxia (CH and CSCH groups) showed marked reactivity to NE compared with the Control group. (B) No differences in the contractile response to NE were observed in the aorta. Values are mean ± SEM.



Supplemental Figure 6. Muscularization of small intrapulmonary arteries was evaluated as the presence of double elastic lamina in arteries <50µm. Guinea pigs exposed to hypoxia (CH and CSCH) showed an increased in the percentage of vessels with double elastic lamina as compared with controls. The % of vessels in the CS group was placed between Control and CH groups. *p<0.05 vs Control. Values are mean ± SEM.



Supplemental Figure 7. (A) Ratio between the number of proliferative nuclei positive for PCNA and non-proliferative nuclei (negative for PCNA) in the vessel wall of small intrapulmonary arteries (<50µm). (B) Number of total nuclei per artery measured in vessel wall of small intrapulmonary arteries (<50µm). C, D, E and F Microphotographies of a small intrapulmonary artery with a nucleus positive for PCNA (arrow) in lung sections of a Control, CS, CH and CSCH groups respectively. Bar chart represents 50µm. G, H, I and J magnification of photographs C, D, E and F respectively.



Supplemental Figure 8. (A) eNOS mRNA expression was evaluated by RT-PCR in lung homogenates. Animals exposed to CS (CS and CSCH groups) showed lower expression of eNOS compared with the other groups. (B) The bar charts show the mean ± SEM ratio of Phosphorylated eNOS (PeNOS)/eNOS protein measured in lung homogenates by western blot. No differences were noticed between groups although a trend to diminish was observed in CS animals (CS and CSCH) when the ratio PeNOS/eNOS was calculated.

Supplemental Table 1. Mechanical properties and reactivity of aorta

					Two-way ANOVA main effects		
Aorta	**Control (n=7)**	**CS (n=7)**	**CH (n=7)**	**CSCH (n=8)**	**CS Exposure**	**Hypoxia**	**Interaction**
Distensibility, $mmHg^{-1}*10^{-3}$	5.3±1.3	5.4±2.4	5.5±0.6	45.0±0.7	0.677	0.857	0.562
Contraction, mN							
KCl (60 mM)	29.1±5.1	28.6±9.4	26.5±5.6	31.6±6.6	0.373	0.944	0.282
NE ($0.2\cdot10^{-6}$M)	6.5±1.6	7.5±2.7	7.3±1.9	6.7±3.3	0.816	0.995	0.410
Relaxation in response to, AUC:							
ADP	30.7±5.2	36.6±22.2	32.5±8.2	40.0±14.5	0.218	0.630	0.878
ADP+L-NAME	72.2±35.6	77.7±30.5	118.5±41.2	120.8±70.1	0.834	0.026	0.934
SNP	24.7±9.4	28.1±8.7	29.6±11.5	28.3±12.3	0.80	0.517	0.555

Values are mean±SD.

Definition of abbreviations: KCl: potassium chloride, NE: Norepinephrine, AUC: area under the curve, ADP: Adenosine diphosphate; L-NAME: N^G-monomethyl-L-arginine; SNP: Sodium nitroprusside.

Vascular response is shown as absolute change in tension. The AUC values are shown as the delta change in the wall tension.

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Resum del segon article:

Effects of cigarette smoke and hypoxia on the pulmonary circulation in the guinea pig

Estudis previs han demostrat que tant l'exposició a fum de tabac com la hipòxia crònica produeixen canvis funcionals i estructurals en la circulació pulmonar. La hipòtesi d'estudi és que la combinació d'ambdós factors té un paper crític en la patogènesis de la HP a la MPOC. L'objectiu de l'estudi va ser avaluar els efectes del fum de tabac i la hipòxia crònica, com a factors individuals o en combinació, sobre la circulació pulmonar en el cobai. Per tal de dur a terme un estudi que reproduís la cronologia de fets que es donen a la MPOC, els animals es van dividir en 4 grups: control, exposat a fum de tabac durant 3 mesos (CS), només exposat a hipòxia durant les dues darreres setmanes d'aquests 3 mesos (CH), i exposat a fum de tabac durant 3 mesos combinant amb l'exposició a hipòxia durant les dues darreres setmanes d'aquests 3 mesos (CSCH). Al finalitzar l'estudi es va mesurar la PAP en tots els animals així com el pes del ventricle dret respecte el pes de ventricle esquerre més el septe (índex de Fulton). Mitjançant tincions amb orceïna i immunohistoquímica per α-actina es va avaluar la formació de doble làmina elàstica i la muscularització dels vasos intrapulmonars respectivament. També es va avaluar la morfometria i composició de l'artèria pulmonar principal així com la funció endotelial d'aquestes mitjançant bany d'òrgans, A més es va avaluar l'expressió d'eNOS a pulmó.

Els resultats d'aquest segon estudi són:

1.- Hemodinàmica pulmonar

Tant la hipòxia com el fum de tabac van augmentar la PAP en similar magnitud. No obstant, la combinació d'ambdós factors va tenir un efecte sinèrgic sobre l'hemodinàmica pulmonar fent que la PAP incrementi i superi els valors individuals. Aquestes dades son corroborades pel increment en la relació del pes del ventricle dret respecte el pes del ventricle esquerre + septe. Aquesta relació indica el grau d'hipertròfia del ventricle dret degut al increment de

la PAP. Els animals dels grups CS i CH mostren un increment en el pes del ventricle dret superior al grup control mentre que el grup CSCH presenta un efecte sinèrgic mostrant els valors més elevats.

2.- Funció endotelial i reactivitat vascular de les artèries pulmonars i sistèmiques

2.1.- La resposta vascular a dosis acumulatives d'ADP es va avaluar mitjançant bany d'òrgans a les branques dreta i esquerra de l'artèria pulmonar i a l'artèria aorta. No es van observar diferències significatives en les corbes dosi-resposta entre els 4 grups experimentals.

2.2.- Tot i no observar canvis en resposta vasodilatadora si es va observar una resposta contràctil a NE més marcada en les artèries dels cobais exposats a hipòxia que va condicionar el punt de partida (basal) en el moment del inici d'avaluació de la funció endotelial.

2.3.- Tant el tabac com la hipòxia van induir un increment en la resposta contràctil a KCl en els anells d'artèria pulmonar.

2.4.- Les artèries pulmonars dels animals exposats a hipòxia van mostrar una menor distensibilitat que els controls, sent més pronunciada aquesta disminució en els animals que van ser exposats a fum de tabac i a hipòxia de forma combinada.

2.5.- No es van observar canvis en cap dels paràmetres avaluats a l'artèria aorta.

3.- Morfometria i composició dels vasos pulmonars

3.1.- Posteriorment al bany d'òrgans es van recollir les artèries i es van realitzar estudis morfomètrics per tal de determinar el gruix de l'artèria pulmonar i d'aorta. Mitjançant anàlisi de imatges es va comprovar que les artèries dels 3 grups experimentals mostraven

un increment en el gruix de la paret del vas respecte el grup control sense que s'observés un efecte sinèrgic entre el tabac i la hipòxia.

3.2.- En les mateixes seccions d'artèria pulmonar i aorta es van realitzar diverses tincions amb la finalitat de caracteritzar el component de matriu extracel·lular del que estan formades les artèries. L'anàlisi de les artèries tenyides amb blau alcià va revelar que els animals exposats a hipòxia (CH i CSCH) tenien un contingut de mucopolisacàrids més elevat que els altres grups. En canvi, el contingut de col·làgena en aquests mateixos grups es veia disminuït tal com va demostrar la tinció amb tricròmic de Mason. La proporció d'elastina avaluada en seccions tenyides amb orceïna no va variar entre grups però sí va augmentar en el grup exposat a tots dos estímuls, tabac i hipòxia (CSCH). Tant el percentatge de mucopolisacàrids com el de col·làgena van correlacionar negativa i positivament respectivament, amb la distensibilitat de l'artèria pulmonar. Per tant, la proporció de proteïnes de la matriu extracel·lular es veu alterada principalment per la exposició a hipòxia i afecta a les propietats elàstiques de l'artèria convertint-les en artèries més rígides o menys distensibles.

4.- Expressió de mediadors vasoactius sintetitzats per l'endoteli

4.1.- Els resultats de l'expressió d'eNOS són similars als obtinguts en el primer estudi, ja que també s'observa una disminució en la seva expressió en els animals exposats a fum de tabac. A més, no hi ha un efecte additiu de la hipòxia sobre l'efecte causat pel tabac de manera que no hi ha diferències amb el grup exposat a tabac i hipòxia. Ja que una disminució en l'mRNA no implica una reducció de l'activitat de l'enzim es va avaluar per western blot la relació entre la proteïna fosforilada (forma activa, PeNOS) i la proteïna total, és a dir PeNOS/eNOS, que indica l'activitat de l'enzim. En aquest cas no vam trobar diferències significatives entre grups tot i que s'observa una tendència a disminuir en els grups exposats a fum de tabac (CS i CSCH).

4.2.- El contingut d'altres indicadors de la funcionalitat de l'endoteli com nitrits/nitrats, ET i catecolamines van ser avaluats en mostres de plasma. Els nivells de nitrits i nitrats, productes derivats de la via del NO, van disminuir en els animals exposats a fum de tabac. En canvi el increment en els nivells plasmàtics d'ET va ser més evident en el grup exposat a hipòxia, tot i que en combinació amb el fum de tabac va causar una disminució marcada dels nivells d'ET. No es van observar canvis en els nivells de catecolamines.

5.- Muscularització dels vasos intrapulmonars

El remodelat vascular es va avaluar en vasos intrapulmonars <50µm fent front a dues característiques:

5.1.- Muscularització de vasos. En seccions de pulmó on s'havia realitzat una immunohistoquímica per α-actina, es va classificar el grau de muscularització en tres nivells: no muscularitzat, parcialment muscularitzat o totalment muscularitzat atenent al percentatge de paret del vas amb marcatge positiu per α-actina. En els animals exposats a tabac i tabac amb hipòxia es va observar una disminució del percentatge de vasos no musculatirzats a expenses de l'aparició de vasos totalment muscularitzats. Aquest increment en el percentatge total de vasos α-actina positius va correlacionar positivament amb la PAP i negativament amb l'expressió de mRNA. Per tal de saber si aquest increment en el percentatge de vasos muscularitzats era degut a un increment en la proliferació cel·lular es va quantificar el nombre de nuclis proliferants per vas identificats pel marcatge positiu a PCNA, un marcador de proliferació cel·lular. No es van observar diferències en la relació de nuclis proliferants respecte nuclis quiescents en cap dels grups experimentals, descartant així la relació entre la muscularització dels vasos i la proliferació cel·lular.

5.2.- La formació de doble làmina elàstica. La hipòxia va demostrar tenir un paper més destacat en la formació d'una nova làmina elàstica ja que els grups CH i CSCH van mostrar un increment en el percentatge de vasos amb doble làmina respecte el grup control.

ANNEX DE RESULTATS

En el transcurs d'aquesta tesi Doctoral, la doctoranda ha participat en altres projectes de recerca que han donat lloc a la publicació de dos articles, els quals es presenten a continuació. Aquests treballs comparteixen el model animal d'exposició a fum de tabac i tenen com a objectiu avaluar els efectes sistèmics induïts per aquest factor. La participació de la doctoranda en aquests articles ha estat la realització del treball experimental, sent primera autora a règim compartit en un d'ells i co-autora a l'altre. La rellevància d'aquests estudis recau en la validació del model animal, primer objectiu que es planteja en aquesta tesi Doctoral.

Tercer estudi:

Cigarette smoke-induced oxidative stress: a role in COPD skeletal muscle dysfunction.

Esther Barreiro, Víctor I. Peinado, Juan B. Galdiz, Elisabet Ferrer, Judith Marin-Corral, Francisco Sánchez, Joaquim Gea, Joan Albert Barberà.

American Journal of Respiratory and Critical Care Medicine. 2010,Aug 15;182(4):477-88

Factor de impacte: 10.69

Quart estudi:

Cigarette smoking exacerbates nonalcoholic fatty liver disease in obese rats.

Lorenzo Azzalini-Elisabet Ferrer, Leandra N. Ramalho, Montserrat Moreno, Marlene Domínguez, Jordi Colmenero, Víctor I. Peinado, Joan Albert Barberà, Vicente Arroyo, Pere Ginès, Joan Caballería, Ramón Bataller.

Hepatology. 2010 May;51(5):1567-76.

Factor de impacte: 10.84

Tercer article:

Cigarette smoke-induced oxidative stress: a role in COPD skeletal muscle dysfucntion

Esther Barreiro, Víctor I. Peinado, Juan B. Galdiz, Elisabet Ferrer, Judith Marin-Corral, Francisco Sánchez, Joaquim Gea, Joan Albert Barberà.

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Cigarette Smoke–induced Oxidative Stress

A Role in Chronic Obstructive Pulmonary Disease Skeletal Muscle Dysfunction

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Rationale: **Inflammation and oxidative stress contribute to muscle dysfunction in patients with chronic obstructive pulmonary disease (COPD). Oxidants contained in cigarette smoke (CS) induce adverse effects on tissues through oxidative phenomena.**

Objectives: **To explore oxidative stress and inflammation in quadriceps of human smokers and in diaphragm and limb muscles of guinea pigs chronically exposed to CS.**

Methods: **Muscle function, protein oxidation and nitration, antioxidants, oxidized proteins, inflammation, creatine kinase activity, and lung and muscle structures were investigated in vastus lateralis of smokers, patients with COPD, and healthy control subjects and in diaphragm and gastrocnemius of CS-exposed guinea pigs at 3, 4, and 6 months.**

Measurements and Main Results: **Compared with control subjects, quadriceps muscle force was mildly but significantly reduced in smokers; protein oxidation levels were increased in quadriceps of smokers and patients with COPD, and in respiratory and limb muscles of CS-exposed animals; glycolytic enzymes, creatine kinase, carbonic anydrase-3, and contractile proteins were significantly more carbonylated in quadriceps of smokers and patients with COPD, and in respiratory and limb muscles of CS-exposed guinea pigs. Chronic CS exposure induced no significant rise in muscle inflammation in either smokers or rodents. Muscle creatine kinase activity was reduced only in patients with COPD and in both diaphragm and gastrocnemius of CS-exposed animals. Guinea pigs developed bronchiolar abnormalities at 4 months of exposure and thereafter.**

Conclusions: **CS exerts direct oxidative modifications on muscle proteins, without inducing any significant rise in muscle inflammation. The oxidative damage to muscle proteins, which precedes the characteristic respiratory changes, may contribute to muscle loss and dysfunction in smokers and patients with COPD.**

Keywords: cigarette smoke; guinea pigs; healthy smokers; muscle inflammation and oxidative stress; quadriceps muscle function

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Oxidative stress is a proposed contributor to chronic obstructive pulmonary disease (COPD) muscle dysfunction. Oxidants contained in cigarette smoke induce adverse effects on tissues through oxidative modifications of key biological structures. It remains to be elucidated whether chronic cigarette smoking induces direct oxidative damage in skeletal muscles.

What This Study Adds to the Field

Chronic cigarette smoking exerts direct oxidative modifications on muscle proteins, without inducing significant rise in either molecular or cellular muscle inflammation. Importantly, the oxidative damage to specific muscle proteins, which precedes the characteristic respiratory changes, may contribute to muscle mass loss and dysfunction in smokers and patients with COPD.

It is generally accepted that the large number of oxidants contained in cigarette smoke (CS) induces adverse effects on tissues through oxidative damage of key biological structures. In addition, CS-induced activation of inflammatory cells may also contribute to enhanced oxidant production in tissues. For instance, lipid peroxidation (1, 2), protein and thiol oxidation (3, 4), and oxidized DNA (5) levels were shown to be increased in the blood of smokers (1–5) and in several organs of animals chronically exposed to CS (5). Moreover, smoking is also a recognized risk factor for many chronic conditions such as dyslipidemia, glucose intolerance (6), and nutritional abnormalities characterized by anorexia, weight loss, and reduced brown and white adipose tissues (7, 8).

Highly prevalent conditions such as chronic obstructive pulmonary disease (COPD) are frequently associated with muscle loss and skeletal muscle dysfunction. These systemic manifestations have a considerable impact on the exercise tolerance and quality of life of the patients, and are also associated with increased mortality (9). Systemic and local oxidative stress, among other factors, has been suggested as a contributor to this process of muscle dysfunction and wasting in COPD (10). Moreover, the spillover of oxidants and inflammatory molecules from the lungs is another potential mechanism of muscle dysfunction in COPD. However, it could be reasoned that CS per se may also exert deleterious effects on skeletal muscles. In this regard, smokers have been shown to exhibit lower peripheral muscle fatigue resistance than nonsmokers (11). Moreover, in spontaneously hypertensive rats exposed to CS, proportions and sizes of muscle fibers were indeed altered in soleus and extensor digitorum longus (12, 13). Also, the vastus lateralis muscle of

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smokers was shown to exhibit a reduction in the content of constitutive nitric oxide synthases together with a smaller size of the slow-twitch fibers (14).

Despite this progress, it remains to be elucidated whether CS induces direct oxidative damage within skeletal muscle fiber structures. In this regard, transient and repeated bouts of reduction–oxidation (redox) imbalance induced by chronic CS exposure may oxidize key proteins involved in muscle metabolism or function, eventually contributing to the muscle dysfunction of patients with COPD. In the current investigation, two different approaches were used: (*1*) limb muscles of current smokers free of lung or cardiovascular disease were analyzed together with muscles of patients with severe COPD; and (*2*) guinea pigs, which develop lesions in their airways similar to those documented in human smokers (15–17), were exposed to chronic CS exposure. On this basis, our objectives were to selectively explore redox balance in lower limb muscles of both human smokers and patients with severe COPD, and in both diaphragm and limb muscles of guinea pigs exposed to CS for 3, 4, and 6 months. Furthermore, the nature and function of the muscle proteins exhibiting the greatest levels of oxidation as well as inflammatory events were also determined in these muscles. Some of the results of these studies have been previously reported in the form of an abstract (18, 19).

METHODS

See the online supplement for additional information.

Human Study Subjects

This is a hospital-based study in which a group of nine white, male, current smokers with normal spirometry were recruited from the smoking cessation clinic together with 10 healthy male, age-matched control subjects and 10 stable patients with severe COPD (20). Asymptomatic smokers were defined as individuals with a smoking history of more than 20 pack-years and who exhibited a postbronchodilator ratio of FEV_1 to FVC greater than 0.7 (20). The current investigation was designed in accordance with both the ethical standards on human experimentation in our institution and the World Medical Association guidelines for research on human beings. Approval was obtained from the institutional ethics committee on human investigation (Hospital de Cruces, Barakaldo, Spain). Informed written consent was obtained from all individuals.

Nutritional and Functional Assessment

Nutritional evaluation included determination of body mass index and fat-free mass index by bioelectrical impedance (21). Forced spirometry was performed according to standard procedures (22). Quadriceps strength was evaluated in smokers, patients, and control subjects by isometric maximal voluntary contraction (QMVC) as formerly described (23).

Muscle biopsies

Muscle samples of smokers, patients with COPD, and control subjects were obtained from the vastus lateralis by open muscle biopsy as previously described (24–26).

Animal Experiments

Experimental groups. Groups of seven male Hartley guinea pigs were exposed to the smoke of seven commercial cigarettes (24 h, 5 d/wk) for periods of 3, 4, and 6 months (15–17, 27). Corresponding control animals underwent the same procedures except for CS exposure. Twenty-four hours after the end of each experimental period, diaphragm, gastrocnemius, and lungs were obtained from all animals. This was a controlled study designed in accordance with the institutional ethics standards and the Helsinki Convention for the use and care of animals. All experiments were approved by the institutional Animal Research Committee at Hospital Clinic (Barcelona).

Muscle Biology Analyses

Immunoblotting of one-dimensional electrophoresis. The effects of reactive oxygen and nitrogen species (ROS and RNS, respectively) on muscle proteins were evaluated according to methodologies previously published (24, 28–31).

Identification of carbonylated and tyrosine-nitrated muscle proteins: two-dimensional electrophoresis. Carbonylated and nitrated proteins were separated and identified in the muscles as published elsewhere (25, 30, 32, 33).

Identification of carbonylated and tyrosine nitrated muscle proteins: mass spectrometry. Identification of carbonylated and nitrated proteins was conducted in the proteomics laboratory according to previously published procedures (25, 30, 32, 33).

Creatine kinase activity assay. Total muscle creatine kinase activity was measured in all muscles as previously published (25, 32, 33).

Cytokines. Protein levels of the cytokines tumor necrosis factor (TNF)-α and IL-6 were quantified in all muscles as published elsewhere (26).

Muscle inflammatory cells. As previously published, inflammatory cell counts were determined immunohistochemically in all muscles (14, 34).

Muscle fiber counts and morphometry. Morphometric analyses were conducted in all muscle as published elsewhere (24, 29, 33).

Morphometric Studies in Lung Tissue

In the guinea pigs, the number of bronchiolar goblet cells was evaluated in paraffin-embedded lung sections counterstained with hematoxylin–eosin and alcian blue. The degree of emphysema was assessed by measuring the mean distance between alveolar septa.

Statistical Analysis

Results are presented as means (SD). In each experimental model, comparisons of physiological and biological variables among the different study groups were analyzed by one-way analysis of variance. Tukey's *post hoc* analysis was used to adjust for multiple comparisons.

RESULTS

Clinical Characteristics

Human studies. As shown in Table 1, age, body mass index, and fat-free mass index did not significantly differ among the three study groups of subjects. Lung function parameters were significantly reduced in patients with COPD compared with either smokers or healthy control subjects, and all patients had severe COPD (Table 1). Interestingly, QMVC was mildly but significantly reduced in the smokers compared with the healthy control subjects. As expected, QMVC was also significantly decreased in the patients with severe COPD compared with either smokers or healthy control subjects (Table 1).

TABLE 1. ANTHROPOMETRIC CHARACTERISTICS AND RESPIRATORY AND MUSCLE FUNCTIONS OF HUMAN STUDY SUBJECTS

	Control Subjects (*n* = *10*)	Smokers (*n* = *9*)	COPD (*n* = *10*)
Age, yr	56 (6)	53 (9)	58 (3)
BMI, kg/m^2	26.7 (4.0)	27.4 (5.1)	26.5 (4.2)
FFMI, kg/m^2	20.0 (2.4)	18.1 (2.6)	18.6 (2.9)
FEV_1, % pred	94 (13)	89 (5)	30 (6)*†
FVC, % pred	91 (11)	93 (9)	75 (11)‡§
FEV_1/FVC, %	79 (7)	76 (10)	32 (8)*†
QMVC, kg	38.50 (1.7)	36.78 (1.5)‖	28.20 (1.31)*†

Definition of abbreviations: % pred = percentage of the predicted value; BMI = body mass index; COPD = chronic obstructive pulmonary disease; FFMI = fat-free mass index; QMVC = quadriceps maximal voluntary contraction.

Values are expressed as means (SD).

* $P \leq 0.001$, between patients with COPD and healthy control subjects.

† $P \leq 0.001$, between patients with COPD and healthy smokers.

‡ $P \leq 0.01$, between patients with COPD and healthy control subjects.

§ $P \leq 0.05$, between patients with COPD and healthy smokers.

‖ $P \leq 0.05$, between healthy smokers and healthy control subjects.

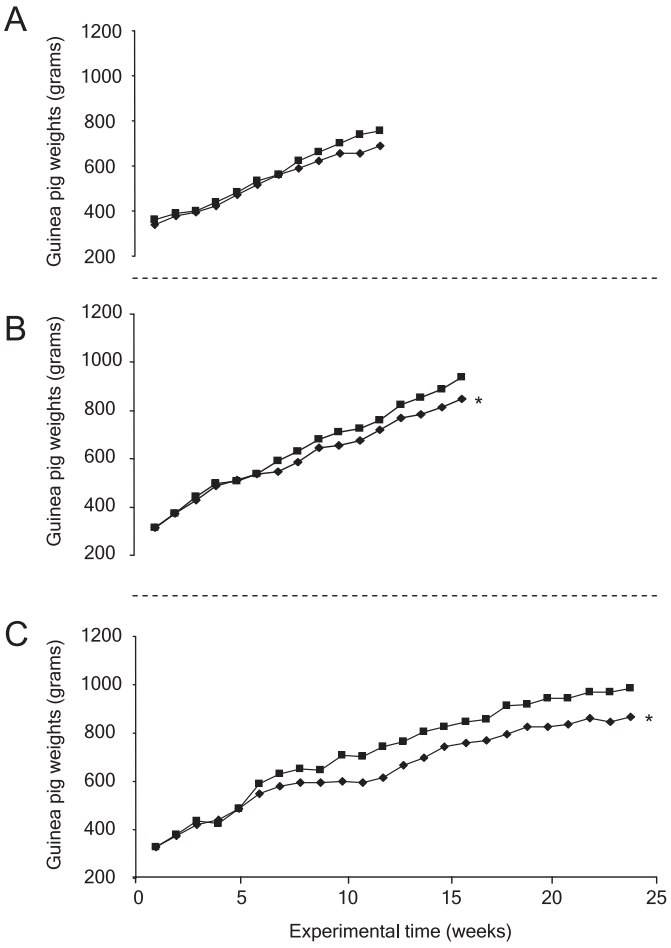

Figure 1. (*A*) Rate of body weight gain of cigarette smoke (CS)–exposed guinea pigs (*solid diamonds*) and corresponding control animals (*solid squares*) over 3 months. No significant differences were observed in the rate of body weight gain between CS-exposed and control guinea pigs at 3 months. (*B*) Rate of body weight gain of CS-exposed guinea pigs (*solid diamonds*) and corresponding control animals (*solid squares*) over 4 months. The rate of body weight gain was significantly reduced (*$P < 0.05$) in the CS-exposed guinea pigs compared with the control subjects at 4 months. (*C*) Rate of body weight gain of CS-exposed guinea pigs (*solid diamonds*) and corresponding control animals (*solid squares*) over 6 months. The rate of body weight gain was significantly decreased (*$P < 0.05$) in the CS-exposed guinea pigs compared with control animals at 6 months.

Animal studies. Guinea pigs exposed to CS for 3 months did not experience any significant reduction in their body weight gain compared with corresponding control subjects (Figure 1A). Importantly, guinea pigs exposed to CS for 4 and 6 months exhibited a significant decrease in their body weight gain compared with the corresponding control animals (Figures 1B and 1C, respectively).

Molecular Markers of Muscle Redox Balance and Oxidized Proteins

Human studies. Total protein carbonylation levels were significantly greater in the vastus lateralis of both smokers and patients with COPD than in the control subjects (Figure 2A). Muscle protein carbonylation levels were, in turn, significantly higher in the limb muscles of patients with COPD than in those of healthy smokers (Figure 2A). Several glycolytic enzymes, creatine kinase, ATP synthase, carbonic anhydrase-3, and actin were identified to be consistently carbonylated in the quadriceps of smokers, patients with severe COPD, and healthy control subjects (Figure 2B and Table 2; and *see* Table E1 in the online supplement). Interestingly, the enzymes enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), creatine kinase, ATP synthase, and carbonic anhydrase-3 exhibited significantly greater levels of carbonylation in the vastus lateralis of smokers and patients with severe COPD than in healthy control subjects (Figure 2C). Importantly, among both smokers and patients with severe COPD, a significant inverse relationship was found between muscle protein carbonylation levels and QMVC (Figure 2D). Levels of malondialdehyde (MDA)–protein adducts, were significantly increased in the quadriceps of both smokers and patients with COPD compared with control subjects (Figure 2E).

Total protein tyrosine nitration levels were significantly greater only in the limb muscles of patients with COPD, but not in smokers, than in control subjects (Table 3). Protein content of the mitochondrial enzyme manganese-superoxide dismutase (Mn-SOD) was significantly increased in the vastus lateralis of both patients with COPD and smokers compared with control subjects (Table 3), whereas muscle catalase levels did not differ among the study groups (Table 3). Compared with healthy control subjects, creatine kinase activity was significantly reduced only in the vastus lateralis of the patients with severe COPD, but not in the smokers (Table 3).

Animal studies. Chronic CS exposure induced a significant increase in reactive carbonyls in both diaphragm and gastrocnemius muscles of guinea pigs after 3, 4, and 6 months of exposure compared with corresponding control animals (Figures 3A and 3B, respectively). In respiratory and limb muscles of the guinea pigs, enzymes involved in glycolysis, creatine kinase, ATP synthase, actin, and tropomyosin were shown to be carbonylated in both CS-exposed and control animals (Figure 3C, Table 2, and Table E2). Carbonylation levels of the enzyme creatine kinase were significantly higher in the diaphragm of CS-exposed guinea pigs at 3 and 6 months of exposure than in the corresponding muscles of control subjects (Figure 3D). Moreover, proteins such as enolase, aldolase, GAPDH, creatine kinase, actin, and tropomyosin displayed greater carbonylation levels in the gastrocnemius of guinea pigs exposed to CS for 3 and 6 months compared with corresponding control muscles (Figure 3E). Interestingly, chronic exposure to CS also induced a significant rise in MDA–protein adducts in the diaphragm and gastrocnemius of guinea pigs exposed to CS for 3, 4, and 6 months compared with control rodents (Figures 3F and 3G, respectively).

In CS-exposed animals, muscle protein tyrosine nitration levels were greater for all time cohorts, except for the diaphragm, in which increased protein nitration levels did not reach statistical significance after 3 months of exposure (Figures 4A–4C). Enzymes involved in glycolysis, creatine kinase, and actin were also shown to be tyrosine nitrated in both CS-exposed and control animals (Table 2 and Table E3). Importantly, enolase, aldolase, and creatine kinase exhibited significantly greater levels of tyrosine nitration in the diaphragm of CS-exposed guinea pigs at 3 and 6 months than in control subjects (Figure 4D). Furthermore, GAPDH and creatine kinase also showed significantly higher levels of tyrosine nitration in the gastrocnemius of CS-exposed rodents at 3 and 6 months than in corresponding control muscles (Figure 4E).

Mn-SOD protein content did not differ significantly between CS-exposed guinea pigs and control animals in any of the muscles (Figures 5A and 5B). Interestingly, only the gastrocnemius from rodents exposed to CS for 4 and 6 months exhibited a significant increase in catalase compared with control animals (Figures 5C

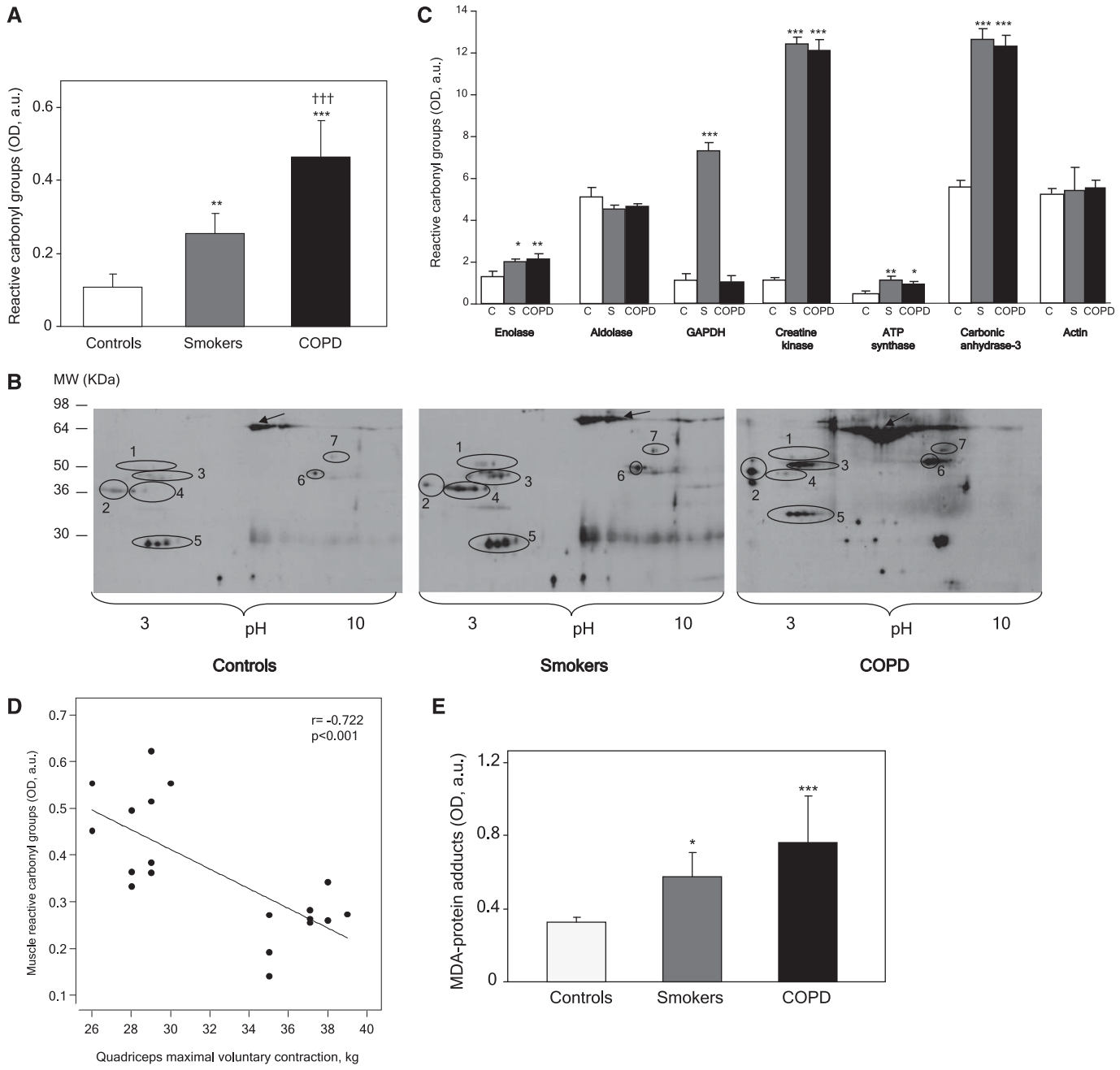

Figure 2. (*A*) Mean values and standard deviation (optical density [OD] values expressed as arbitrary units [a.u.]) of total reactive carbonyl groups were significantly higher in the quadriceps of both patients with chronic obstructive pulmonary disease (COPD) (***$P < 0.001$) and healthy smokers (**$P < 0.01$) than in control subjects. Moreover, levels of reactive carbonyls were significantly increased in the vastus lateralis of patients with COPD than in smokers (†††$P < 0.001$). (*B*) Representative two-dimensional immunoblots corresponding to the detection of carbonylated proteins in crude muscle homogenates of vastus lateralis of a healthy control subject (*left*), a smoker (*middle*), and a patient with severe COPD (*right*). β-Enolase (*1*), fructose biphosphate aldolase A (*2*), creatine kinase (*3*), glyceraldehyde-3-phosphate dehydrogenase (*4*), carbonic anhydrase-3 (*5*), actin (*6*), and ATP synthase (*7*) were consistently oxidized in the vastus lateralis of the three study groups. Albumin was also carbonylated in the muscles of both control and cachectic rats (*arrow* in each panel). (*C*) Mean values and standard deviation of total reactive carbonyls (OD values expressed as arbitrary units) of each identified protein in limb muscles of smokers, patients with COPD, and healthy control subjects. Note that levels of reactive carbonyls of several muscle proteins (enolase, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], creatine kinase, ATP synthase, and carbonic anhydrase-3) were significantly greater in the vastus lateralis of the smokers (S) and patients with severe COPD (COPD) than in control subjects (C). Statistical significance is expressed as follows: smokers (S) versus control individuals (C): *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. (*D*) Among all the smokers and patients with COPD, muscle protein carbonylation levels, expressed as OD values expressed as arbitrary units, inversely correlated with quadriceps maximal voluntary contraction. (*E*) Mean values and standard deviation (OD values expressed as arbitrary units) of total malondialdehyde (MDA)–protein adducts were significantly greater in the quadriceps of both patients with COPD (***$P < 0.001$) and healthy smokers (*$P < 0.05$) than in control subjects.

TABLE 2. IDENTIFIED OXIDIZED AND NITRATED PROTEINS IN SKELETAL MUSCLES OF HUMANS AND GUINEA PIGS

	β-Enolase	Aldolase	Triose-phosphate Isomerase-1	GAPDH	Creatine Kinase	ATP Synthase	Carbonic Anhydrase-3	Actin	Tropomyosin
Identified Carbonylated roteins									
Humans									
Quadriceps muscle									
Nonsmokers	+	+		+	+	+	+	+	
Smokers	+	+		+	+	+	+	+	
Patients with severe COPD	+	+		+	+	+	+	+	
Guinea pigs									
Diaphragm									
Control subjects	+	+			+	+		+	
CS exposed	+	+			+	+		+	
Gastrocnemius									
Control subjects	+	+	+	+	+	+		+	+
CS exposed	+	+	+	+	+	+		+	+
Identified Nitrated Proteins									
Guinea pigs									
Diaphragm									
Control subjects	+	+	+		+			+	
CS exposed	+	+	+		+			+	
Gastrocnemius									
Control subjects	+	+	+	+	+			+	
CS exposed	+	+	+	+	+			+	

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; CS = cigarette smoke; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

and 5D). Interestingly, creatine kinase activity levels were significantly decreased in both diaphragm and gastrocnemius muscles of CS-exposed guinea pigs at 4 and 6 months compared with their respective control subjects (Figures 6A and 6B).

Muscle Inflammatory Cells and Cytokines

Human studies. Muscle levels of the cytokines IL-6 and TNF-α were not significantly modified in any of the three study groups (Table 4). Levels of inflammatory cells, although low in all muscles, were significantly greater in the vastus lateralis of patients with severe COPD compared with either smokers or healthy control subjects (Table 4).

Animal studies. Chronic exposure to CS did not have any significant effects on muscle levels of the cytokines IL-6 and TNF-α and those of inflammatory cells (leukocytes and macrophages) in guinea pigs at any time (Table 5).

Muscle Fiber Structure

Human studies. Proportions of type I fibers were significantly reduced, whereas those of type II fibers were significantly increased in the vastus lateralis muscles of patients with COPD compared with either smokers or healthy control subjects (Table 3). The proportions of quadriceps muscle fibers did not significantly differ between smokers and control subjects (Table 3). The size of quadriceps type I or type II fibers did not significantly differ among the three study groups (Table 3).

Animal studies. Compared with control rodents, the diaphragm of guinea pigs exposed to CS for 6 months exhibited a significant decrease in the proportions of type I fibers, whereas those of type II fibers exhibited a significant rise in the same animals (Table 6). No significant differences were observed in muscle fiber size between exposed and nonexposed animals in any time cohort (Table 6).

TABLE 3. MUSCLE OXIDATIVE STRESS, CREATINE KINASE ACTIVITY, AND FIBER PHENOTYPE IN HUMAN STUDY SUBJECTS

	Control Subjects ($n = 10$)	Smokers ($n = 9$)	COPD ($n = 10$)
Redox markers			
Protein nitration, a.u.	0.61 (0.09)	0.78 (0.22)	0.99 (0.32)*
Mn-SOD, a.u.	0.14 (0.06)	0.23 (0.07)*	0.22 (0.07)*
Catalase, a.u.	0.15 (0.04)	0.16 (0.03)	0.18 (0.08)
Enzyme activity			
Creatine kinase activity, U/L	662.1 (148.3)	664.8 (108.06)	454.0 (67.4)†‡
Muscle fiber type, %			
Type I fibers	42 (7)	38 (8)	20 (5)§‖
Type II fibers	58 (7)	62 (8)	80 (5)§‖
Muscle fiber size (CSA), μm^2			
Cross-sectional area, type I fibers	1,907 (463)	2,046 (480)	2,149 (221)
Cross-sectional area, type II fibers	2,014 (654)	1,868 (365)	2,119 (389)

Definition of abbreviations: a.u. = arbitrary units; CSA = cross-sectional area; Mn-SOD, manganese superoxide dismutase.
Values are expressed as means (SD).
* $P \leq 0.05$, between either healthy smokers or patients with COPD and healthy control subjects.
† $P \leq 0.01$, between patients with COPD and healthy control subjects.
‡ $P \leq 0.01$, between patients with COPD and healthy smokers.
§ $P \leq 0.001$, between patients with COPD and healthy control subjects.
‖ $P \leq 0.001$, between patients with COPD and healthy smokers.

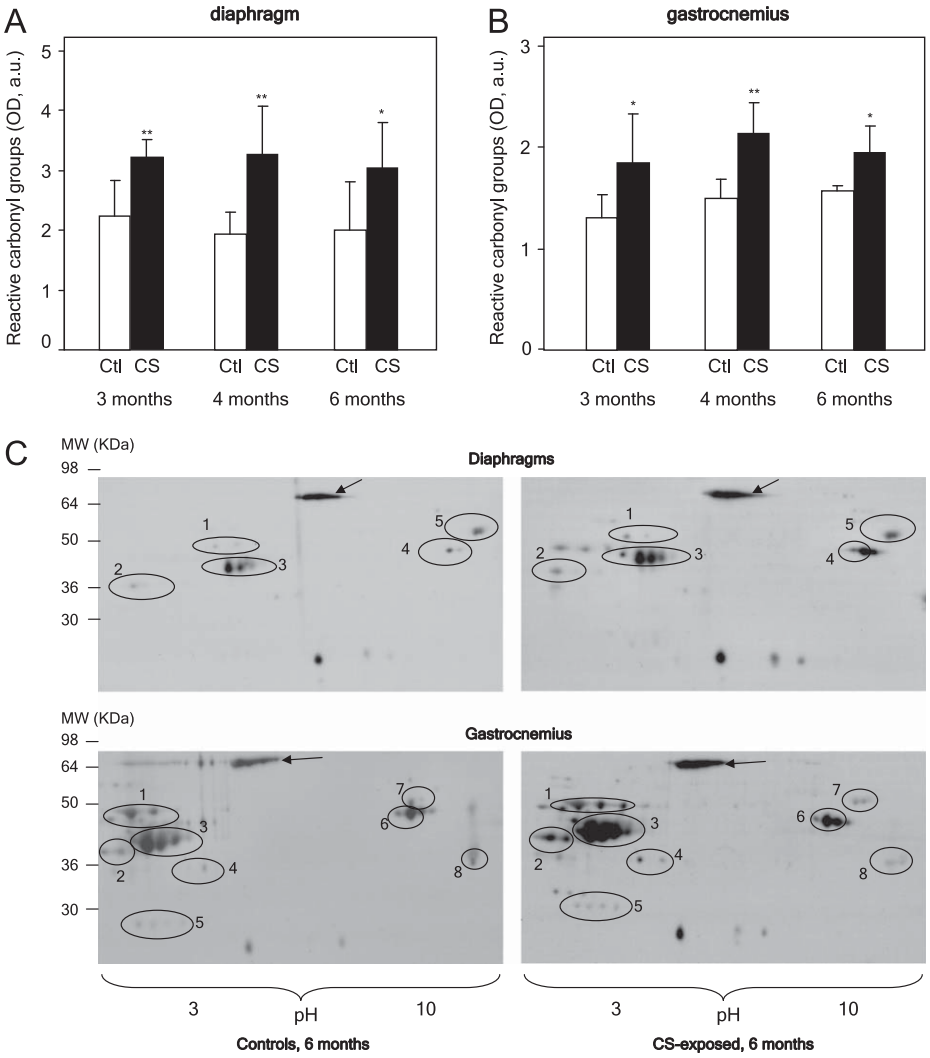

Figure 3. (*A*) Mean values and standard deviation (optical density [OD] values expressed as arbitrary units [a.u.]) of total reactive carbonyl groups were significantly higher in the diaphragm of guinea pigs exposed to cigarette smoke (CS) for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $*P < 0.05$ and $**P < 0.01$. (*B*) Mean values and standard deviation (OD values expressed as arbitrary units) of total reactive carbonyl groups were significantly greater in the gastrocnemius of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $*P < 0.05$ and $**P < 0.01$. (*C*) Representative two-dimensional immunoblots corresponding to the detection of carbonylated proteins in crude muscle homogenates of diaphragm (*top*) and gastrocnemius (*bottom*) of control and CS-exposed guinea pigs at 6 months (*left* and *right*, respectively). β-Enolase (*1*), fructose biphosphate aldolase A (*2*), creatine kinase (*3*), actin (*4*), and ATP synthase (*5*) were consistently oxidized in the diaphragm of both CS-exposed and control guinea pigs. β-Enolase (*1*), fructose biphosphate aldolase A (*2*), creatine kinase (*3*), glyceraldehyde-3-phosphate dehydrogenase (*4*), triosephosphate isomerase (*5*), actin (*6*), ATP synthase (*7*), and tropomyosin (*8*) were consistently oxidized in the gastrocnemius of both CS-exposed and control guinea pigs. Albumin was also carbonylated in the muscles of both control and CS-exposed rodents (*arrow* in each panel). (*D*) Mean values and standard deviation of total reactive carbonyls (OD values expressed as arbitrary units) of each identified protein in the diaphragm of CS-exposed guinea pigs (S) and control animals (C) at 3 and 6 months (*left* and *right*, respectively). Note that levels of reactive carbonyls in creatine kinase protein were significantly greater in the diaphragm of CS-exposed guinea pigs than in control animals at 3 and 6 months of exposure. Statistical significance is expressed as follows: CS-exposed (S) versus control animals (C): $**P < 0.01$ and $***P < 0.001$. (*E*) Mean values and standard deviation of total reactive carbonyls (OD values expressed as arbitrary units) of each identified protein in the gastrocnemius of cigarette smoke (CS)-exposed guinea pigs (S) and control animals (C) at 3 and 6 months (*left* and *right*, respectively). Note that levels of reactive carbonyls of several muscle proteins (enolase, aldolase, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], creatine kinase, actin, and tropomyosin) were significantly greater in the gastrocnemius of CS-exposed guinea pigs than in control animals. Statistical significance is expressed as follows: CS-exposed (S) versus control animals (C): $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. (*F*) Mean values and standard deviation (OD values expressed as arbitrary units) of total malondialdehyde (MDA)–protein adducts were significantly greater in the diaphragm of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. (*G*) Mean values and standard deviation (OD values expressed as arbitrary units) of total MDA–protein adducts were significantly greater in the gastrocnemius of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $**P < 0.01$ and $***P < 0.001$.

Changes in Lung Structure of Guinea Pigs

In the bronchioles of guinea pigs exposed to CS for 4 and 6 months, there was prominent goblet cell metaplasia with a four- to sevenfold increase in the number of goblet cells compared with nonexposed animals (Table 6). The alveolar space size, as measured by the nonlinear intercept, slightly increased with aging, but did not differ between CS-exposed and control animals (Table 6), indicating that CS-exposed rodents did not develop emphysema over the study period.

DISCUSSION

In skeletal muscles of both humans and guinea pigs chronically exposed to CS and of patients with COPD compared with control muscles, the following modifications were observed: (*1*) a mild but significant reduction in quadriceps muscle force in the healthy smokers, (*2*) an inverse relationship between muscle protein carbonylation levels and quadriceps force among smokers and patients with COPD, (*3*) increased protein oxidation in the quadriceps of smokers and patients with COPD as well as in diaphragm and gastrocnemius of CS-exposed animals, (*4*) a significant rise in protein nitration in both respiratory and limb muscles of CS-exposed rodents but not in human smokers, (*5*) a significant increase in oxidative modifications of proteins involved in glycolysis, energy production and distribution, carbon dioxide hydration, and muscle contraction in both humans and guinea pigs, (*6*) a CS exposure–induced, significant

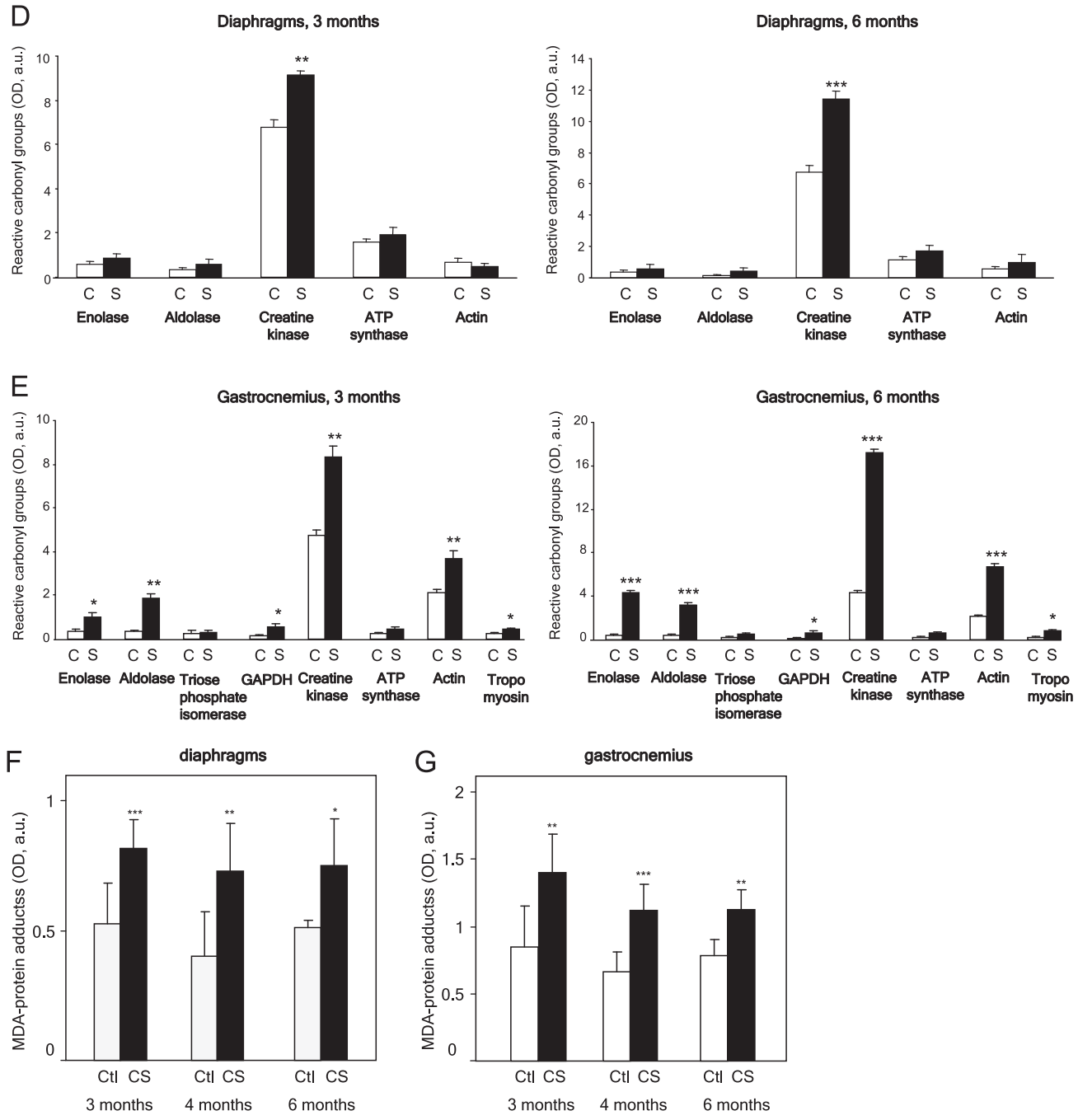

Figure 3. (*Continued*)

reduction in creatine kinase activity in both respiratory and limb muscles of guinea pigs, and (*7*) a lack of any significant effect on muscle inflammatory cell or cytokine levels subsequent to chronic exposure to CS.

Interestingly, in the present investigation, quadriceps muscle force was significantly reduced, although mildly, in healthy smokers compared with control subjects. This finding is in line with a previous study, in which healthy smokers exhibited greater peripheral muscle fatigue (11). Also, as previously reported (9, 26, 30), patients with severe COPD, independently of their muscle mass, exhibited a significant reduction in quadriceps muscle function (27%) compared with healthy control subjects and smokers. In addition, among the population of smokers and patients with severe COPD, muscle protein carbonylation levels were inversely correlated with quadriceps muscle force. This is in agreement with former studies from our group (26, 30), in which muscle protein oxidation was also shown to negatively correlate with quadriceps muscle function in patients with COPD.

The present investigation is the first to provide evidence of the posttranslational oxidative modifications induced by both ROS and RNS on muscle proteins in human smokers and in animals chronically exposed to CS. Interestingly, in agreement with our initial hypothesis, protein oxidation, as measured by either reactive carbonyls or MDA–protein adducts, was significantly increased in the muscles of both smokers and exposed guinea pigs. Increased levels of protein tyrosine nitration, a biological marker of excessive RNS production, reached

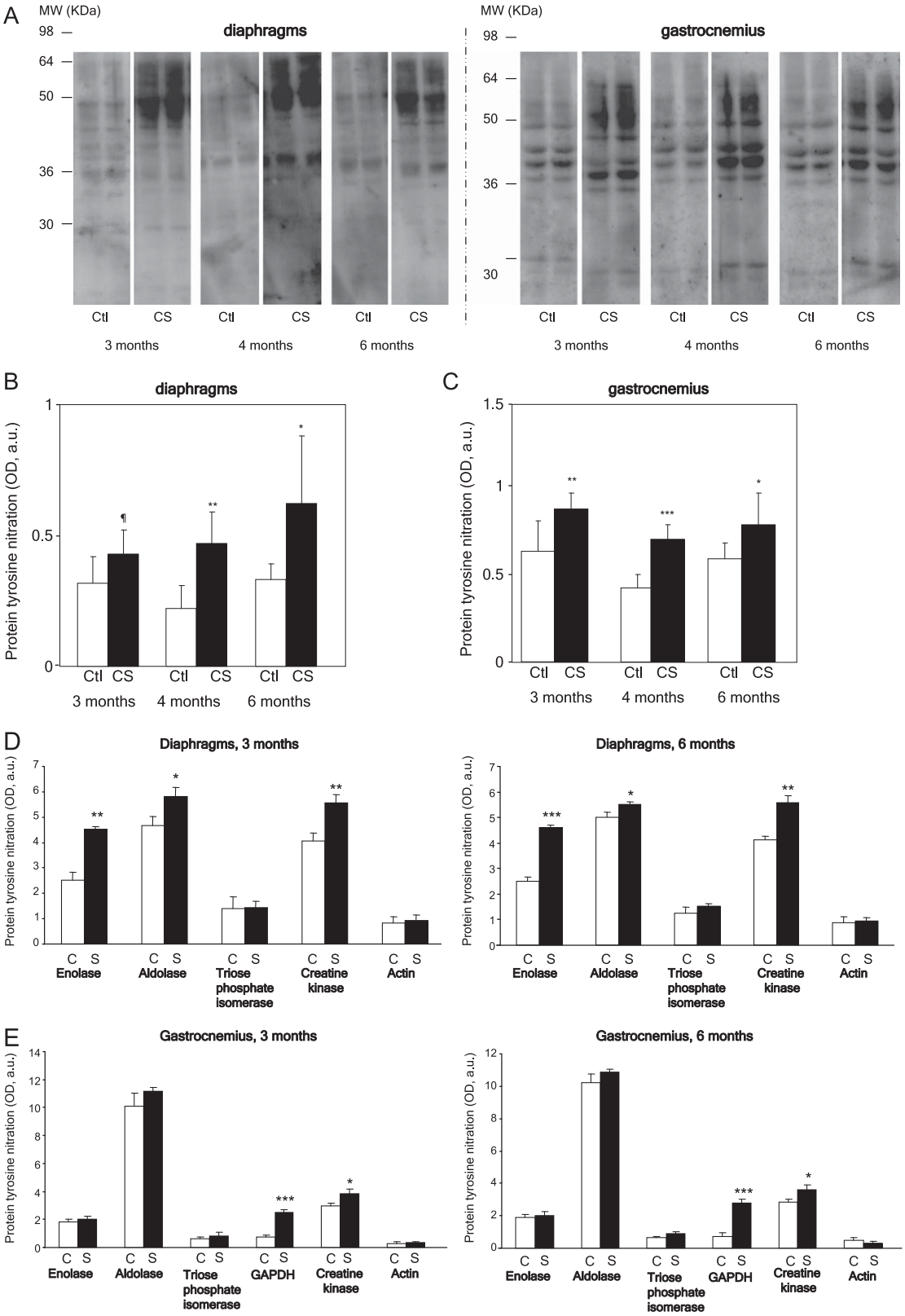

Figure 4. (*A*) Representative examples of protein tyrosine nitration immunoblots in the diaphragm and gastrocnemius muscles of cigarette smoke (CS)–exposed and control (Ctl) guinea pigs at 3, 4, and 6 months. Several tyrosine-nitrated proteins were detected. (*B*) Mean values and standard deviation (optical density [OD] values expressed as arbitrary units [a.u.]) of total protein nitration were significantly higher in the diaphragm of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $P = 0.070$, $^{*}P < 0.05$, and $^{**}P < 0.01$. (*C*) Mean values and standard deviation (OD values expressed as arbitrary units) of total protein nitration were significantly greater in the gastrocnemius of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (*D*) Mean values and standard deviation of total protein tyrosine nitration (OD values expressed as arbitrary units) of each identified protein in the diaphragm of CS-exposed guinea pigs (S) and control animals (C) at 3 and 6 months (*left* and *right*, respectively). Note that levels of nitrotyrosine formation of the proteins enolase, aldolase, and creatine kinase were significantly greater in the diaphragm of CS-exposed guinea pigs than in control animals. Statistical significance is expressed as follows: CS-exposed (S) versus control animals (C): $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (*E*) Mean values and standard deviation of total protein tyrosine nitration (OD values expressed as arbitrary units) of each identified protein in the gastrocnemius of CS-exposed guinea pigs (S) and control animals (C) at 3 and 6 months (*left* and *right*, respectively). Note that levels of nitrotyrosine formation of the proteins glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and creatine kinase were significantly greater in the gastrocnemius of CS-exposed guinea pigs than in control animals. Statistical significance is expressed as follows: CS-exposed (S) versus control animals (C): $^{*}P < 0.05$ and $^{***}P < 0.001$.

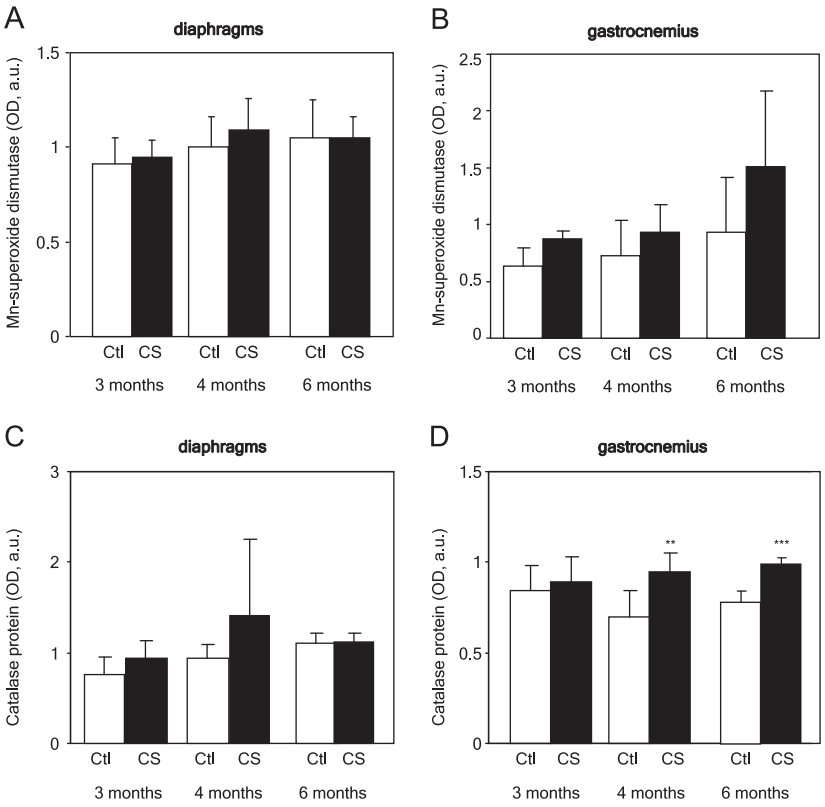

Figure 5. (*A*) Mean values and standard deviation (optical density [OD] values expressed as arbitrary units [a.u.]) of manganese (Mn)-superoxide dismutase protein did not significantly differ in the diaphragm of guinea pigs exposed to cigarette smoke (CS) for 3, 4, and 6 months compared with control muscles (Ctl). (*B*) Mean values and standard deviation (OD values expressed as arbitrary units) of Mn-superoxide dismutase protein did not significantly differ in the gastrocnemius of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). (*C*) Mean values and standard deviation (OD values expressed as arbitrary units) of catalase protein did not significantly differ in the diaphragm of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). (*D*) Mean values and standard deviation (OD values expressed as arbitrary units) of catalase protein were significantly greater in the gastrocnemius of guinea pigs exposed to CS for 4 and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $^{**}P < 0.01$, and $^{***}P < 0.001$.

statistical significance only in the diaphragm and gastrocnemius of CS-exposed animals and in the quadriceps of patients with severe COPD, but not in the vastus lateralis of human smokers. This is in keeping with a previous investigation in which increased protein tyrosine nitration was demonstrated in the vastus lateralis of patients with COPD and no differences were detected in the levels of several nitric oxide end-products, including nitrotyrosine, in the quadriceps of human smokers compared with control subjects (14).

Peroxynitrite, which is formed from the near-diffusion-limited reaction between nitric oxide and superoxide anions, accounts for most protein tyrosine nitration in skeletal muscles (28). In the current study, Mn-SOD protein content, but not catalase, was significantly increased in the quadriceps of both human smokers and patients with severe COPD compared with control subjects. In CS-exposed guinea pigs, however, muscle Mn-SOD levels did not differ from those in control animals, and chronic CS exposure induced a significant rise in total protein tyrosine nitration in the respiratory and limb muscles of guinea pigs. In view of these findings, it could be concluded that in patients with severe COPD and in the muscles of guinea pigs, greater production of RNS than in healthy smokers may have outcompeted with Mn-SOD for superoxide anion, eventually leading to the formation of significantly increased levels of protein tyrosine nitration within the vastus lateralis muscle.

Importantly, in the guinea pig model, the effects of oxidants on muscle proteins were observed in both respiratory and limb muscles, suggesting that chronic CS exposure probably exerted direct deleterious effects on all muscles of the exposed animals. Likewise, the significant increase in muscle protein oxidation observed in human smokers, free of lung or cardiovascular disease, is likely to be attributed to a direct action of ROS and RNS (aldehydes, peroxides, nitrogen oxides, and peroxyl radicals, among others) contained in CS. Moreover, the effects of oxidants on muscles occurred at an earlier stage than the effects observed in the respiratory system. These findings reinforce the concept that CS per se is likely to be involved in direct tissue toxicity in the skeletal muscles of CS-exposed guinea pigs, regardless of lung and bronchial alterations. In fact, our findings are in total agreement with previous investigations, in which a rise in various oxidative stress markers was demonstrated in the blood, lungs, and other organs of human smokers and

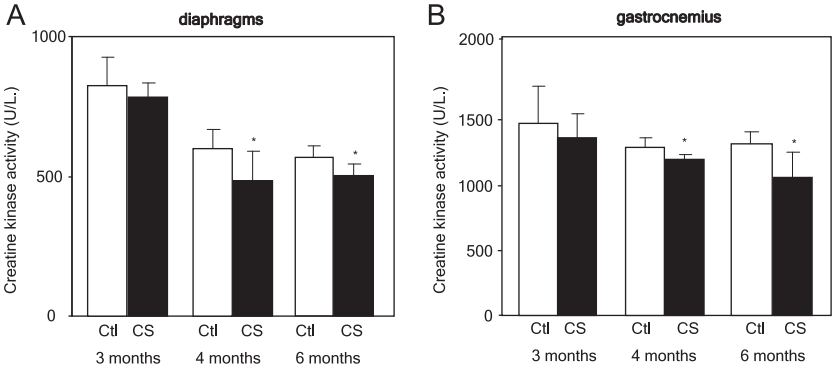

Figure 6. (*A*) Mean values and standard deviation (activity units [U]/L) of creatine kinase activity were significantly lower in the diaphragm of guinea pigs exposed to cigarette smoke (CS) for 4 and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $^{*}P < 0.05$. (*B*) Mean values and standard deviation (activity units [U]/L) of creatine kinase activity were significantly lower in the gastrocnemius of guinea pigs exposed to CS for 4 and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $^{*}P < 0.05$.

TABLE 4. MUSCLE INFLAMMATION IN HUMAN STUDY SUBJECTS

	Control Subjects (*n* = 10)	Smokers (*n* = 9)	COPD (*n* = 10)
IL-6, pg/ml	0.29 (0.19)	0.23 (0.12)	0.32 (0.30)
TNF-α, pg/ml	1.64 (0.37)	1.72 (0.33)	1.64 (0.24)
Total inflammatory cells, cells/mm^2	0.99 (0.60)	0.88 (0.51)	2.57 (1.70)*†

Definition of abbreviations: TNF = tumor necrosis factor.
Values are expressed as means (SD).
* $P \leq 0.05$ between patients with COPD and healthy control subjects.
† $P \leq 0.05$ between patients with COPD and healthy smokers.

animals chronically exposed to CS (1–7). In line with this, in a previous study (7), guinea pigs acutely exposed to CS also exhibited a significant increase in plasma lipid peroxidation together with a reduction in muscle glutathione levels immediately after the exposure.

It should also be mentioned that inflammatory events, that is, muscle proinflammatory cytokines and inflammatory cell infiltration, are not likely to contribute to muscle protein oxidation or nitration in any of the models of chronic exposure to CS analyzed in this investigation. In fact, only the vastus lateralis of patients with severe COPD exhibited a significant increase in inflammatory cell infiltration compared with either smokers or healthy control subjects. Nevertheless, this significant increase is likely to be of little biological relevance, because absolute levels were extremely low in all muscle specimens from both humans and rodents.

It is worth mentioning that in the current experimental setting, exposure to CS for 6 months was insufficient to induce pulmonary emphysema. In fact, bronchiole goblet cell metaplasia, but not lung morphometric modifications, was the only histological alteration found in the respiratory tract of CS-exposed guinea pigs. These findings are in line with a previous investigation from our group (7), but are in contrast with previously published studies in which guinea pigs were also chronically exposed to CS (15, 35, 36). Indeed, the length of CS exposure required to cause emphysema varies across animal species and is dependent on the methods of exposure and cigarette dose (15, 17). In keeping with this, in the present study, guinea pigs were exposed to a relatively moderate content of nicotine and other compounds in the cigarette smoke, as established by Diamond and colleagues (37). On this basis, differences in the dose of nicotine and other chemicals contained in CS, relatively high in some investigations (35–37) and moderate in others (7), might account for the discrepancies among studies regarding the development of emphysema in guinea pigs chronically exposed to CS. It should also be discussed that although chronic CS exposure did not induce lung destruction in the current study, it may have been sufficient to promote elastase-induced emphysema, as previously demonstrated (37).

In the present investigation, to understand the pathophysiological consequences of posttranslational oxidative modifications of the muscle proteins, the nature of the oxidatively modified proteins was identified. Importantly, this study is the first to show that highly abundant proteins involved in glycolysis, energy production and distribution, carbon dioxide hydration, and muscle contraction were significantly more oxidized in the quadriceps of human smokers and patients with severe COPD, and in the diaphragm and gastrocnemius of guinea pigs chronically exposed to CS. Interestingly, the line has been put forward that these specific proteins are prone to suffer oxidative modifications under certain experimental conditions. For instance, the diaphragm of endotoxemic rats exhibited increased oxidative modifications of glycolytic enzymes, creatine kinase, carbonic anhydrase-3, and contractile actin (32), as well as the diaphragm and vastus lateralis of patients with severe COPD (33). In the current investigation, creatine kinase and carbonic anhydrase-3 displayed the greatest oxidative modifications in the vastus lateralis of healthy smokers and patients with severe COPD. These findings are in agreement with previous studies from our group (25, 33), in which creatine kinase was also shown to be highly modified by oxidants and its activity significantly reduced in the muscles of patients with severe COPD. In the current investigation, creatine kinase activity was significantly reduced only in the vastus lateralis of patients with severe COPD, but not in smokers. It is likely that the amount of oxidants in muscles of the latter was still not sufficient to induce a significant decrease in the activity of this enzyme. On the other hand, chronic exposure to CS induced a significant decrease in creatine kinase activity in both the diaphragm and gastrocnemius muscles of guinea pigs at 4 and 6 months but not at 3 months. Modifications of the activity of creatine kinase may have relevant implications in muscle performance in response to chronic exposure to CS and in severe COPD. Clearly, future studies will shed light on the specific mechanisms whereby posttranslational oxidative modifications may lead to muscle protein loss and dysfunction in active smokers and patients with severe COPD.

Despite potential controversies with previous studies (7, 35, 36), in the present investigation CS was shown to influence body weight as demonstrated by the observed reduction in body weight gain in animals chronically exposed to CS for as little as 4 months. Although not specifically quantified, food intake between CS-exposed and control rodents was similar, even after 4 months. It should be noted that a reduction in body

TABLE 5. MUSCLE INFLAMMATION IN GUINEA PIGS AT VARIOUS PERIODS OF CIGARETTE SMOKE EXPOSURE

	3 mo		4 mo		6 mo	
	Control (*n* = 7)	CS Exposed (*n* = 7)	Control (*n* = 7)	CS Exposed (*n* = 7)	Control (*n* = 7)	CS Exposed (*n* = 7)
IL-6, pg/ml						
Diaphragm	7.80 (1.57)	9.42 (1.60)	5.47 (1.76)	7.79 (3.18)	5.38 (0.98)	5.22 (0.74)
Gastrocnemius	5.48 (1.21)	6.40 (1.70)	4.48 (0.76)	4.20 (1.05)	4.25 (0.34)	5.41 (1.96)
TNF-α, pg/ml						
Diaphragm	0.39 (0.17)	0.51 (0.23)	0.25 (0.05)	0.38 (0.29)	0.27 (0.10)	0.23 (0.05)
Gastrocnemius	0.38 (0.29)	0.38 (0.54)	0.30 (0.19)	0.25 (0.25)	0.21 (0.06)	0.24 (0.17)
Total inflammatory cells, cells/mm^2						
Diaphragm	0.99 (0.91)	1.45 (1.00)	0.45 (0.28)	0.75 (0.39)	0.31 (0.31)	0.46 (0.30)
Gastrocnemius	0.41 (0.24)	0.57 (0.18)	0.44 (0.22)	0.66 (0.51)	0.43 (0.58)	0.76 (0.02)

Definition of abbreviations: CS = cigarette smoke; TNF = tumor necrosis factor.
Values are expressed as means (SD).

TABLE 6. MUSCLE FIBER PHENOTYPE AND LUNG STRUCTURE IN GUINEA PIGS AT VARIOUS PERIODS OF CIGARETTE SMOKE EXPOSURE

	3 mo		4 mo		6 mo	
	Control ($n = 7$)	CS Exposed ($n = 7$)	Control ($n = 7$)	CS Exposed ($n = 7$)	Control ($n = 7$)	CS Exposed ($n = 7$)
Muscle fiber type, %						
Diaphragm, type I	37 (5)	34 (2)	29 (3)	30 (4)	35 (5)	30 (2)*
Gastrocnemius, type I	10 (3)	10 (4)	11 (5)	8 (3)	9 (3)	13 (6)
Diaphragm, type II	63 (5)	66 (2)	71 (3)	70 (4)	65 (5)	70 (2)*
Gastrocnemius, type II	90 (3)	90 (4)	89 (5)	92 (3)	91 (3)	87 (6)
Muscle fiber size (CSA), μm^2						
Diaphragm, type I	734 (143)	666 (304)	593 (146)	647 (195)	697 (192)	757 (134)
Gastrocnemius, type I	894 (256)	779 (198)	797 (200)	787 (212)	1,010 (399)	1,296 (582)
Diaphragm, type II	850 (135)	743 (127)	706 (204)	685 (148)	1,013 (130)	908 (203)
Gastrocnemius, type II	1,154 (325)	1,129 (247)	1,148 (228)	1,125 (246)	1,545 (253)	1,328 (248)
Lung structure						
Goblet cell metaplasia, cells/mm	0.4 (1.9)	0.4 (1.4)	1.0 (3.3)	4.3 (8.0)*	0.5 (1.6)	3.6 (6.3)*
Mean linear intercept, μm	60 (18)	61 (18)	74 (24)	72 (20)	75 (29)	72 (37)

Definition of abbreviations: CS = cigarette smoke; CSA = cross-sectional area.
Values are expressed as means (SD).
* $P \leq 0.05$, between CS-exposed and control guinea pigs.

weight gain, rather than the characteristic body weight and muscle mass loss of patients with COPD, was the outcome variable in the current study. On this basis, exploring whether the pathophysiological mechanisms leading to decreased body weight gain in CS-exposed animals share similarities with those involved in muscle mass loss and dysfunction in smokers and patients with COPD, which might even precede the pulmonary disease, warrants further attention in future studies.

In the current investigation, only the diaphragm, but not the limb muscles, of guinea pigs exposed to CS for 6 months exhibited a switch to a more glycolytic phenotype. Previous studies have yielded discrepant results regarding muscle phenotype changes in response to chronic CS exposure. In this regard, our findings are in agreement with those reported in earlier studies (38, 39), in which proportions of type I fibers were shown to be reduced in the quadriceps of smokers. More recently, Gosker and colleagues (40) have demonstrated that the soleus muscle of mice chronically exposed to CS also exhibited a reduction in the proportions of type IIa oxidative fibers compared with control muscles. Nonetheless, in another study (14), the size, but not the proportions, of type I and type IIa fibers was shown to be reduced in the quadriceps of smokers compared with control subjects. Interestingly, in an animal model of hypertensive rats exposed to CS, the soleus also exhibited a reduction in the percentage of type I fibers along with a decrease in the areas of all muscle fibers (12), whereas the extensor digitorum longus exhibited only a reduction in the size, but not in the proportions, of both oxidative and glycolytic fibers (13). In view of these results, it could be concluded that the predominance of an oxidative or glycolytic phenotype in a given muscle may account for the differences among studies related to CS-induced effects on muscle structure.

Study Limitations

See the online supplement for additional information.

A first limitation in the current investigation has to do with the relatively small number of healthy smokers, patients with COPD, and healthy control subjects studied. However, it should also be considered that muscle biopsies were obtained from current smokers free of lung or cardiovascular disease, from healthy control subjects, and from patients with severe and very severe COPD. In addition, the experimental model used, that is, guinea pigs chronically exposed to CS, also helped elucidate the oxidative phenomena directly induced by CS on skeletal muscle proteins in two different compartments, the respiratory and limb muscles.

A second limitation has to do with the lack of functional data concerning either the respiratory or limb muscles of guinea pigs. An initial step in this field of investigation was to explore the specificity of the oxidative phenomena of skeletal muscle proteins as well as their differential regulation in response to chronic exposure to CS in two different models: human and animal studies. On the other hand, it should also be taken into account that peripheral muscle function was, indeed, evaluated in smokers, patients with severe COPD, and healthy control subjects in the present investigation.

A third limitation is related to the nature of the identified proteins by means of two-dimensional electrophoresis and proteomics analyses in the muscle homogenates from both humans and guinea pigs. It is likely that less abundant muscle proteins or proteins of larger sizes may not have been detected in this system. Future investigations will be designed to explore whether proteins of specific muscle compartments and/or higher molecular weights could also be modified by ROS and RNS in response to chronic CS exposure.

Conclusion

In the present study, it is demonstrated for the first time that CS exerts a mild but significant reduction in quadriceps muscle force together with direct oxidative modifications of specific muscle proteins, without inducing any significant rise in muscle inflammation. The posttranslational oxidative alterations of the muscle proteins may negatively influence their function, for example, creatine kinase activity, eventually rendering the modified proteins more susceptible to increased protein breakdown, which in turn would lead to muscle loss and dysfunction in smokers and patients with COPD. In the animal model, CS-induced oxidative stress occurred in the muscles as early as 3 months after exposure. Importantly, this event preceded the characteristic bronchiolar and parenchymal changes induced by CS in the lungs, suggesting a direct toxic effect of CS on skeletal muscle proteins.

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Cigarette smoke-induced Oxidative Stress: a Role in COPD Skeletal Muscle Dysfunction

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ONLINE DATA SUPPLEMENT

METHODS

Smokers, COPD patients, and healthy controls

This is a hospital-based study in which a group of nine male current smokers with normal spirometry were recruited from the smoking cessation clinic together with 10 healthy male age-matched nonsmoking controls. Asymptomatic smokers in this study were defined as individuals with a smoking history >20 pack-years who exhibited a post-bronchodilator ratio of forced expiratory volume in one second (FEV_1) to forced vital capacity (FVC) greater than 0.7 (1). Furthermore, 10 male patients with stable severe chronic obstructive pulmonary disease (COPD) were recruited on an out-patient basis from the COPD clinic (1). All 3 groups of individuals were Caucasian, and were simultaneously participating in the project of the European Network for Investigating the Global Mechanisms of Muscle Abnormalities (ENIGMA) in COPD, specifically designed to investigate the mechanisms involved in muscle dysfunction in COPD. Moreover, 8 COPD patients were also involved in another previously published study aimed at investigating the effects of magnetic stimulation training on quadriceps redox balance (2). Exclusion criteria for smokers, COPD patients, and control subjects included chronic respiratory or cardiovascular disorders, limiting osteoarticular condition, chronic metabolic diseases, suspected para-neoplastic or myopathic syndromes, and/or treatment with drugs known to alter muscle structure and/or function including oral corticosteroids. Smokers, COPD patients, and healthy controls were qualified as sedentary after being specifically inquired about whether they were conducting any regular outdoor physical activity, going regularly to the gymnasium, or participating in any specific training program.

The current investigation was designed in accordance with both the ethical standards on human experimentation in our institution and the World Medical Association guidelines (Helsinki Declaration of 1975, as revised in 1983) for research on human beings. Approval was obtained from the institutional Ethics Committee on Human Investigation (*Hospital de Cruces,* Barakaldo). Informed written consent was obtained from all individuals.

Nutritional and Functional Assessment

Nutritional evaluation included body mass index (BMI) and determination of the fat-free mass index (FFMI) by bioelectrical impedance (3). Forced spirometry was performed using standard procedures and reference values by Roca *et al* (4) were used.

Quadriceps strength was evaluated in both patients and controls by isometric maximum voluntary contraction (QMVC) of the dominant lower limb as formerly described (5). Patients were seated with both trunk and thigh fixed on a rigid support of an exercise platform (Domyos HGH 050, Decathlon, Lille, France). The highest value from three brief reproducible maneuvers (<5% variability among them) was accepted as the MVC.

Muscle biopsies

Muscle samples of both current smokers, COPD patients, and nonsmoking controls were obtained from the quadriceps (vastus lateralis) by open muscle biopsy as described previously (2,6-9). Samples were 30-50 mg size in average. Muscle specimens were immediately frozen in liquid nitrogen and stored at –80ºC for further analysis or immersed in an alcohol-formol bath for 2h to be thereafter embedded in paraffin. All subjects were prevented from doing any potentially exhausting physical exercise 10 to 14 days before coming to the hospital to undergo the surgical procedures.

Animal experiments

Experimental groups. Groups of 7 male Hartley guinea pigs (300 g, initial weight, 1 month of age approximately) were exposed to the smoke of 7 commercial cigarettes (11 mg tar, 0.8 mg nicotine per cigarette)/24h, 5 days/week for periods of 3, 4, and 6 months using a nose-only inhalation system (10-13). Corresponding control animals followed the same procedures except for the cigarette smoke (CS) exposure. In both smoking and control animals, food was supplied *ad libitum* and water was supplemented with vitamin C (1g/L, Roche Farma, S.A., Madrid, Spain). No differences in food intake were observed between CS-exposed and control guinea pigs. Body weights were determined weekly throughout the entire experimental period. Twenty-four hours after the end of each experimental period, diaphragm, gastrocnemius, and lungs were obtained from all the animals under anesthesia. Sample specimens were immediately frozen in liquid nitrogen and stored at –80ºC for further analysis or immersed in an alcohol-formol bath for 2h to be thereafter embedded in paraffin. All animal experiments were conducted in the same center, at *Hospital Clinic.* This was a controlled study designed in accordance with both the ethical standards on animal experimentation in our institution (EU 609/86 CEE, *Real Decreto* 1201/05 BOE 252, Spain) and the Helsinki convention for the use and care of animals. All experiments were approved by the institutional Animal Research Committee (*Hospital Clinic*, Barcelona).

Morphometric studies in lung tissue

In the guinea pigs, the number of bronchiolar goblet cells was evaluated using paraffin-embedded lung sections counterstained with hematoxylin-eosin and Alcian blue. The degree of emphysema was assessed by measuring the mean distance between alveolar septa.

Muscle biology analyses

All muscle biology analyses corresponding to both human and animal experiments were conducted blind in the same laboratory by the same investigators, at *Hospital del Mar*-IMIM-*Universitat Pompeu Fabra* (Barcelona).

Detection of reactive carbonyls in muscle proteins. Changes in protein carbonylation in crude muscle homogenates were detected using the commercially available Oxyblot kit (Chemicon International Inc., Temecula, CA, USA). Carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone (DNP) by reaction with 2,4-dinitrophenylhydrazine (DNPH) according to the manufacturer's instructions. Briefly, 15 μg of protein were used per derivatization reaction. Proteins were then denatured by addition of 12% SDS. The samples were subsequently derivatized by adding 10 μl of 1X DNPH solution and incubated for 20 minutes. Finally, 7.5 μl of neutralization solution and 2-mercaptoethanol were added to the sample mixture. The specificity of reactive carbonyl measurements was confirmed by avoiding the derivatization process and by omission of the primary antibody, and incubation of the membranes only with secondary antibody [Goat anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated, from the Oxyblot kit, dilution: 1/300]. DNP-derivatized proteins were loaded onto 12% tris-glycine sodium dodecylsulfate polyacrylamide gels (SDS-PAGE) and separated by electrophoresis.

Formation of malondialdehyde (MDA)-protein adducts. Another mechanism of protein carbonylation is by the reaction of certain amino acids with unsaturated aldehydes generated during the peroxidation of polyunsaturated fatty acids. Lipid-derived aldehydes such as MDA can thus cause further cellular damage by binding to and modifying proteins, which lead to the formation of stable aldehyde-protein adducts. MDA reacts with lysine residues to form stable Schiff base adducts (14,15).

Immunoblotting of 1D electrophoresis. The effects of reactive oxygen and nitrogen species (ROS and RNS, respectively) on muscle proteins were evaluated according to methodologies published elsewhere (2,6,7,9,16-18). Frozen muscle samples from the vastus lateralis of smokers, COPD patients, and healthy control subjects along with diaphragm and

gastrocnemius muscle specimens from both CS-exposed and control guinea pigs were homogenized in a buffer containing HEPES 50 mM, NaCl 150 mM, NaF 100 mM, Na pyrophosphate 10 mM, EDTA 5 mM, Triton-X 0.5%, leupeptin 2 μg/ml, PMSF 100 μg/ml, aprotinin 2 μg/ml and pepstatin A 10 μg/ml. Samples were then centrifuged at 1,000 g for 30 min. The pellet was discarded and the supernatant was designated as a crude cytoplasmic homogenate. Protein levels in each homogenate were spectrophotometrically determined with the Bradford technique using triplicates in each case and bovine serum albumin (BSA) as the standard (Bio-Rad protein reagent, Bio-Rad Inc., Hercules, CA, USA). The final protein concentration in each sample was calculated from at least two Bradford measurements that were almost identical. Equal amounts of total protein from crude muscle homogenates were always loaded (20 μg per sample/lane) onto the gels, as well as identical sample volumes/lanes.

Three completely independent sets of experiments were conducted: i) vastus lateralis from current smokers, COPD patients, and control subjects, ii) diaphragms from CS-exposed and control guinea pigs, and iii) gastrocnemius from CS-exposed and control guinea pigs. For the purpose of comparisons among the different groups of either humans or guinea pigs, sample specimens were always run together and kept in the same order. Proteins were then separated by electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes, blocked with non-fat milk and incubated overnight with selective antibodies. The following antibodies were used to detect indices of protein carbonylation and nitration and antioxidant mechanisms: anti-2,4- DNP moiety antibody (Rabbit anti-DNP antibody, from the Oxyblot kit, Chemicon International Inc., Temecula, CA, USA, dilution: 1/150), anti-MDA-protein adducts antibody (Academy Bio-Medical Company, Inc., Houston, TX, USA, dilution: 1/4000), anti-3-nitrotyrosine antibody (Cayman Chemical Inc., Ann Arbor, MI, USA, dilution: 1/1000), anti-Mn superoxide dismutase (SOD) antibody (StressGen, Victoria, BC, Canada, dilution: 1/5000), and anti-catalase antibody (Calbiochem, San Diego, CA, USA, dilution: 1/2000). Tissue homogenates obtained from rat brain mitochondria and rat erythrocytes were used as positive controls of the enzymes Mn-SOD and catalase, respectively. Specific proteins from all samples were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and a chemiluminescence kit. For each of the antigens, samples from the different groups were always detected in the same film under identical exposure times. The specificity of the different antibodies was confirmed by omission of the primary antibody, and incubation of the membranes only with secondary antibodies. Blots were scanned with an imaging

densitometer and optical densities of specific proteins were quantified with Diversity Database 2.1.1 (BioRad, Philadelphia, PA, USA). Values of total reactive carbonyl groups, total MDA-protein adducts, and total protein tyrosine nitration levels in a given sample were calculated by addition of optical densities (arbitrary units) of individual protein bands in each case. Final optical densities obtained in each specific group of subjects corresponded to the mean values of the different samples (lanes) of each of the antigens studied. To validate equal protein loading among various lanes, SDS-PAGE gels were stained with Coomassie Blue (Figures E1 and E2).

Identification of carbonylated and tyrosine nitrated muscle proteins. 2-dimensional electrophoresis. Carbonylated proteins were separated and identified in the vastus lateralis of the current smokers, COPD patients, and healthy controls following procedures previously published studies, in which oxidatively modified proteins were documented in the vastus lateralis and diaphragms of patients with severe COPD (7,9,18). Moreover, in the diaphragms and gastrocnemius of CS-exposed and control guinea pigs at 3 and 6 months (2 extreme cohorts), carbonylated and nitrated proteins were also identified following identical methodologies (7,9,18). Briefly, 4 volumes of 10 mM DNPH were first added to crude muscle homogenates (400 μg protein/sample) and incubated for 30 min at room temperature in order to specifically identify carbonylated proteins. The reaction was stopped by adding the neutralization solution. In the experiments in which protein nitrated proteins were identified, muscle samples were not derivatized. In this case, muscle crude homogenates were directly subjected to the experimental procedures required for 2-dimensional electrophoresis. In both cases, crude muscle homogenates (400 μg protein/sample) were prepared for 2D-electrophoresis with the 2D Clean up kit (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer's instructions. The samples were then incubated for 15 min on ice, centrifuged for 5 min at 13,000g and the pellets were then washed three times and centrifuged at 13,000g for 5 min. The pellets were re-suspended in 2D re-hydration buffer (8M urea, 2% CHAPS, 20 mM DTT, and 0.002% bromophenol blue). Each muscle sample was then separated into two portions (200 μg total each) and both portions underwent 2-D electrophoresis. First-dimensional protein separation was performed with the the Ettan IPGPhor 3 (GE Healthcare Biosciences AB, Uppsala, Sweden). Samples were applied to immobilized pH gradient strips (18-cm nonlinear pH 3-10, GE Healthcare Biosciences AB, Uppsala, Sweden) for 30 min at room temperature. The strips were then covered with mineral oil overnight and isoelectric focusing was performed at a maximum of 10,000 V/ h for up to a

total of 35,200 V-h. For the second dimension, the IPG strips were equilibrated at room temperature for 30 min in equilibration buffer (6M urea, 2% SDS, 50mM tris-HCl, 30% glycerol, and 0.002% bromophenol blue) to which 1% DTT was added prior to use. An additional 30 min equilibration period was then used with equilibration buffer to which 2.5% iodoacetamide was added. The strips were then embedded in 0.5 % agarose on the top of 30% acrylamide gels. The second dimension SDS/PAGE was performed for 5 h, 70 mA per gel at 250 V. One of the resulting 2D gels for each muscle sample was then stained with silver stain. Gels were fixed overnight in a fixation solution (50% acetic acid, 50% methanol), then rinsed twice in water, sensitized for 1min in 0.2% sodium thiosulfate, followed by rinsing in water and immersion for 30 min in a silver nitrate solution (2% silver nitrate). Gels were then rinsed twice in water and developed in a developer solution (20% sodium carbonate, 0.05% formaldehyde, 0.004% sodium thiosulfate). A stop solution (6% acetic acid) was then added for 15 min followed by rinsing with water for 5min. Gels were then stored in 1% acetic acid. The second gel derived from a given sample underwent electrophoretical transfer to PVDF membrane and immunoblotting with anti-DNP antibody or anti-nitrotyrosine antibody as described above. Gels and PVDF membranes were imaged with a digital camera and aligned (Adobe Photoshop 8.0.1, San Jose, CA, USA) so as to identify positive carbonylated or nitrated protein spots on the gels. Optical densities (arbitrary units) of the immunoblot spots corresponding to each oxidatively modified protein were measured in each of the muscles of smokers, COPD patients, and healthy controls as well as in diaphragms and gastrocnemius of CS-exposed and control guinea pigs using Quantity One analysis software 4.6.5 (BioRad, Philadelphia, PA, USA).

Identification of carbonylated and tyrosine nitrated muscle proteins. Mass spectrometry. Identification of carbonylated and nitrated proteins was conducted in the Proteomics Laboratory at *Universitat Pompeu Fabra* following the quality criteria established by *ProteoRed* standards (*Instituto Nacional de Proteómica*, Spain) and procedures previously published (7,9,18-20). Protein carbonylated spots from silver-stained gels were manually excised for in-gel digestion in a 96-well ZipPlate placed in a Multiscreen vacuum manifold (Millipore, Billerica, ME, USA). Proteins were reduced, alkylated, and digested with sequence grade trypsin (Promega, Madison, WI, USA). Peptides were eluted with 15-25 µL of 0.1% TFA in 50% ACN. 2.5 µL of tryptic digest were deposited onto Mass·Spec·Turbo 192 type 1 peptide chips pre-spoted with CHCA (Qiagen, Germantown, MD, USA) and left for 3 min for peptide adsorption. Then each spot was washed for 5 s with 1 µl of finishing solution (Qiagen, Germantown, MD, USA) and left until dryness. Matrix assisted laser

desorption/ionization time-of-flight (MALDI-TOF) MS was performed in a Voyager DE-STR instrument (Applied Biosystems, Foster City, CA, USA) using a 337-nm nitrogen laser and operating in the reflector mode, with an accelerating voltage of 20 kV. Samples were analyzed in the m/z 800-3000 range and were calibrated externally using a standard peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA, USA). Peptides from trypsin autolysis were used for the internal calibration. Protein identification from MALDI-TOF results was done with the MASCOT search engine (Matrix Science, London, UK) using human proteins available in the SwissProt database as well in the NCBI non-redundant database for the identification of the guinea pig proteins. Moreover, the following parameters were used for database searches: one missed cleavage allowed, plus Cys carbamidomethylation as fixed and Met oxidation as variable modifications selected, respectively.

*Creatine kinase activity assay. I*n all muscle specimens from both human and animal studies, total muscle creatine kinase activity was measured with a commercially available kit (Diagnostic Chemicals Ltd., Charlottetown, PEI, Canada) according to the manufacturer's instructions and previously published studies (7,18,19). Briefly, the assay is based on the conversion of creatine phosphate and adenosine diphosphate (ADP) by creatine kinase to creatine and adenosine triphosphate (ATP). The ATP and D-glucose are then converted to ADP and D-glucose-6-phosphate by hexokinase. Glucose-6-phosphate dehydrogenase then reduces nicotinamide adenine dinucleotide to $NADPH^{+}$. The rate of increase in absorbance at 340 nm in a spectrophotometer due to the formation of $NADPH^{+}$ was directly proportional to the creatine kinase activity, expressed in U/L. The amount of total muscle protein used in the assay was identical for all the muscle samples from both human and animal studies. The intra-assay coefficient of variation was 0.35%, while the inter-assay coefficient was 2.3%.

Cytokine Enzyme-linked Immunosorbent Assay (ELISA). The protein expression of the cytokines TNF-alpha and IL-6 was quantified in the quadriceps of smokers, severe COPD, and healthy controls, as well as in the diaphragm and gastrocnemius of CS-exposed and control guinea pigs of all the time cohorts using specific sandwich ELISA kits [RayBiotech, Norcross, GA (human analyses) and Bender MedSystems, Vienna, Austria (guinea pig studies)] following similar previously published methodologies (8). Frozen muscle sample specimens were homogenized and protein concentration calculated as described above. For all the sample specimens, from either human or animal studies, equal amounts of total protein from muscle homogenates were always loaded in triplicates [15 μg (human muscles) and 50

μg (guinea pig muscles) in 200μL total volume each singlet for all the triplicates of all the study samples] onto the ELISA plates. All samples were incubated with the specific primary antibodies and were always run together in each assay. Before commencing the assay, samples and reagents were equilibrated to room temperature. A standard curve was always run with each assay run. Standards (200 μL) were performed as indicated by the manufacturer's instructions. Protocol was also followed according to the corresponding manufacturer's instructions for each cytokine. Absorbances were read at 450 nm using as a reference filter that of 655 nm. Intra-assay coefficients of variation for the different cytokines and studies ranged from 4.5% to 10%. Inter-assay coefficients of variation for the same cytokines ranged from 7.9% to 12%.

Muscle inflammatory cells. In order to evaluate the presence of inflammatory cells in the muscle fibers, immunohistochemical analyses were conducted in the vastus lateralis of the smokers, severe COPD patients, and healthy controls, as well as in diaphragms and gastrocnemius of CS-exposed and control guinea pigs of all the time-cohorts following similar methodologies published elsewhere (21,22). Briefly, on three-micrometer muscle paraffin-embedded sections, leukocytes (anti-CD45 antibody, clone 2B11 & PD7/26, Dako Cytomation Inc., Carpinteria, CA, USA) and macrophages (anti-CD68 antibody, clone PG-M1, Dako Cytomation Inc.) were identified following our regular immunohistochemical procedures. After incubation with the corresponding primary antibodies, slides were washed and incubated for 30 min with biotinylated universal secondary antibody followed by incubation (30 min) with horseradish peroxidase-conjugated streptavidin and diaminobenzidine (kit LSAB+HRP, Dako Cytomation Inc.) as a substrate. Sections of human tonsils were used as positive controls. Slides were counterstained with hematoxylin, dehydrated and mounted for conventional microscopy. Total numbers of both leukocytes and macrophages were counted in all muscle preparations from both human and animal studies under a light microscope (Olympus, Series BX61, Olympus Optical Co., Hamburg, Germany) coupled with an image-digitizing camera (Olympus, Series DP71, Olympus Optical Co.). Furthermore, the total area in each muscle section was also determined using the light microscope (Olympus, Series BX61), the image-digitizing camera (Olympus, Series DP7) and a morphometry program (NIH Image, version 1.60, Scion Corporation, Frederick, MD, USA). Results corresponding to inflammatory cell counts were expressed as the ratio of total inflammatory cell counts (both leukocytes and macrophages) to total muscle section area in mm^2. In this study, we chose as a reference the total muscle section area, and not the total volume, since the size (any of its 3 dimensions) of either leukocytes or macrophages was

bigger than the size of the muscle slides (3 micrometers).

Muscle fiber counts and morphometry. Morphometric analyses were carried out in the vastus lateralis of smokers, COPD patients, and healthy controls as well as in the diaphragms and gastrocnemius of CS-exposed and control guinea pigs of all time-cohorts. On three-micrometer muscle paraffin-embedded sections, myosin heavy chain-I and –II isoforms were identified using anti-myosin heavy chain-I (clone MHC, Biogenesis Inc., Poole, England, UK) and anti-myosin heavy chain-II antibodies (clone MY-32, Sigma, Saint Louis, MO), respectively, as published elsewhere (6,8,17). The cross-sectional area, mean least diameter, and proportions of type I and type II fibers were assessed using a light microscope (Olympus, Series BX50F3, Olympus Optical Co., Hamburg, Germany) coupled with an image-digitizing camera (Pixera Studio, version 1.0.4, Pixera Corporation, Los Gatos, CA, USA) and a morphometry program (NIH Image, version 1.60, Scion Corporation, Frederick, MD, USA). At least 100 fibers were measured and counted in each muscle specimen.

Statistical Analysis

Results are presented as mean (SD). Comparisons of physiological and biological variables among healthy controls, smokers, and COPD patients were analyzed using one-way analysis of variance. Tukey's *post hoc* analysis was used to adjust for multiple comparisons. Moreover, variations in the rate of body weight gain over time and in biological variables among the guinea pig groups were also explored using one-way analysis of variance and Tukey's *post hoc* analysis. The sample size chosen was based on previous studies based on either human or animal models (2,5-12,16,17,19-31).

DISCUSSION

Study Limitations

A first limitation in the current investigation has to do with the relatively small number of healthy smokers, COPD patients, and healthy controls studied. However, it should also be taken into consideration that muscle biopsies were obtained from current smokers free of lung or cardiovascular disease, from healthy control subjects, and from severe and very severe COPD patients. Additionally, as also mentioned above in the Methods section, the sample size of both human smokers and controls was calculated on the basis of previously published studies by our group and other investigators, where similar physiological and biological

approaches were used in both patients and control subjects (2,5-9,17,21,22,26,29-31). Hence, on this basis, and in view of the significant results encountered in the current investigation, it is possible to conclude that the relatively small number of smokers, patients, and controls included was sufficient for the purpose of the study. Furthermore, from an ethical point of view, the utilization of a larger population size than that required for the obtaining of significant results is not advisable. Besides, the utilization of an experimental model of guinea pigs chronically exposed to CS also helps with the understanding of the oxidative damage directly induced by CS on skeletal muscle proteins in two different compartments, the respiratory and limb muscles.

A second limitation has to do with the lack of functional data in either the respiratory or limb muscles of the guinea pigs. As abovementioned, an initial step in this field of investigation was to explore whether and to what extent chronic exposure to CS might induce any direct oxidative modifications on proteins of skeletal muscles of either human smokers or animals chronically exposed to CS as well as their differential regulation in response to chronic exposure to CS in two different models: human and animal studies. After confirmation of these finding, a second step will be the design of future investigations targeted to specifically assess the functional effects of increased protein carbonylation on muscle contractile properties and metabolism in an experimental model of chronic exposure to CS. On the other hand, it should also be taken into account that peripheral muscle function was, indeed, evaluated, in smokers, severe COPD patients, and healthy controls in the present investigation.

A third limitation is related to the nature of the identified proteins by means of 2-dimensional electrophoresis and proteomics analyses in the muscle homogenates from both humans and guinea pigs. It is likely that less abundant muscle proteins or proteins of larger sizes may have not been detected in this system. In this regard, in the current investigation, highly abundant proteins of relatively medium-to-small size were the ones exhibiting greater carbonylation levels within the target muscles. On the basis of the present study results, future investigations will be designed in order to explore whether proteins of specific muscle compartments (e.g. mitochondria, cytosol, and membrane) and/or larger molecular weights could also be modified by ROS and RNS in response to chronic CS exposure. As the corresponding methodological procedures require the utilization of relatively large amounts of frozen muscle specimens, such approaches could not be used in the present study.

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E Barreiro et al. Figure E1

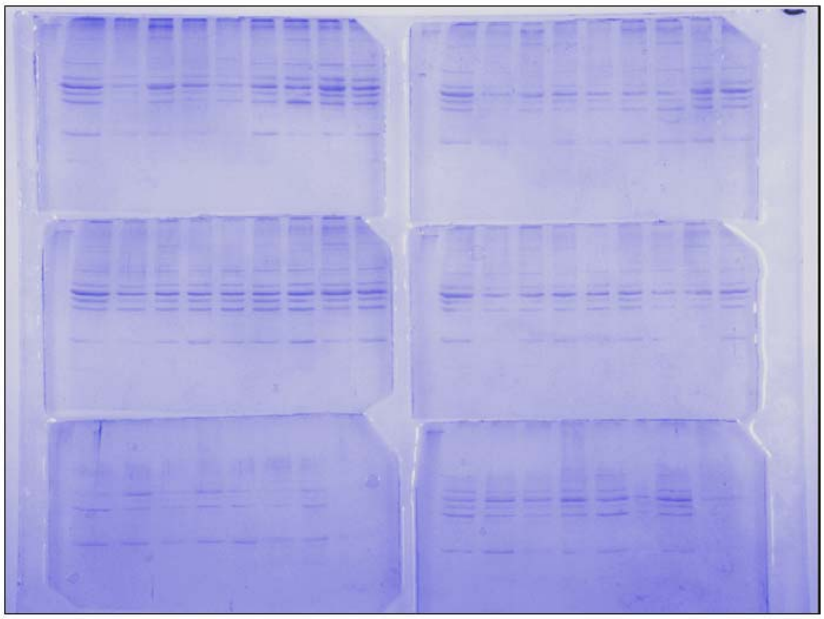

Figure legend: Representative examples of Coomassie brilliant blue stained gels in which human muscle samples were run. In each gel, first lane from left corresponds to the molecular weight marker.

E Barreiro et al. Figure E2

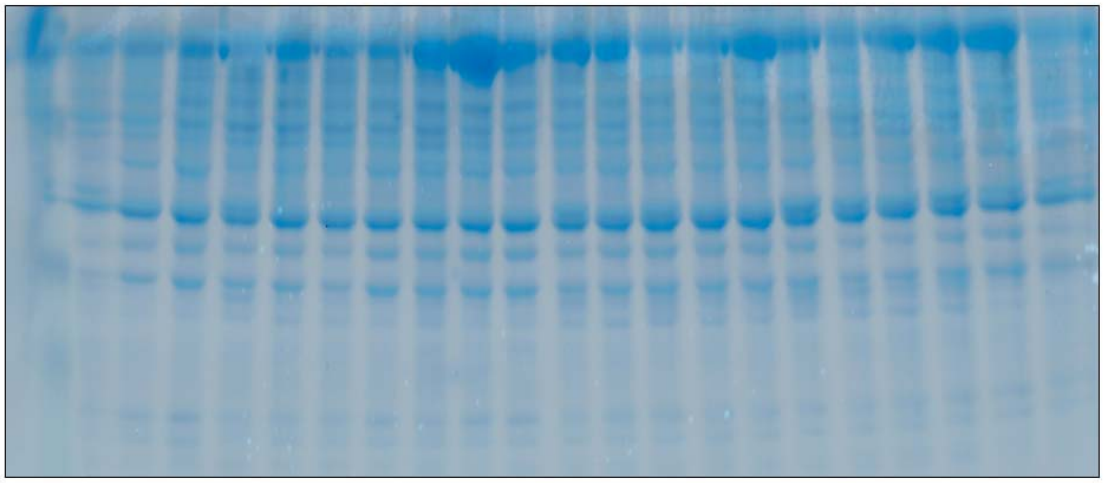

Figure legend: Representative example of a Coomassie brilliant blue stained gel in which muscle samples from guinea pigs were run. First lane from left corresponds to the molecular weight marker.

Table E1. Identified carbonylated proteins in the muscles of smokers, COPD patients, and nonsmoker controls

Study Subjects	Identified carbonylated proteins	Accession No.	Mass	MASCOT Score	Peptide Matched
Quadriceps Nonsmokers Controls	Beta-enolase	ENOB_HUMAN	47,299	96	13
	Fructose-biphosphate aldolase A	ALDOA_HUMAN	39,851	109	10
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	G3P_HUMAN	36,201	97	10
	Creatine kinase M-type	KCRM_HUMAN	43,302	171	15
	ATP synthase subunit beta, mitochondrial precursor	ATPB_HUMAN	56,525	214	19
	Carbonic anhydrase-3	CAH3_HUMAN	29,824	127	9
	Actin, alpha skeletal muscle	ACTS_HUMAN	42,366	117	13
Quadriceps Smokers	Beta-enolase	ENOB_HUMAN	47,299	165	20
	Fructose-biphosphate aldolase A	ALDOA_HUMAN	39,851	135	17
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	G3P_HUMAN	36,201	65	8
	Creatine kinase M-type	KCRM_HUMAN	43,302	189	17
	ATP synthase subunit beta, mitochondrial precursor	ATPB_HUMAN	56,525	156	22
	Carbonic anhydrase-3	CAH3_HUMAN	29,824	161	9
	Actin, alpha skeletal muscle	ACTS_HUMAN	42,366	105	11

Database: SwissProt 56.0
Protein scores greater than 56 are significant ($p<0.05$)

Table E2. Identified carbonylated proteins in the muscles of CS-exposed and control guinea pigs

Study Subjects	Identified carbonylated proteins	Accession No.	Mass	MASCOT Score	Peptide Matched
Diaphragms Control Guinea pigs	Enolase 3 beta muscle	gi/6679651	47,337	132	15
	Aldolase 1, A isoform	gi/6671539	39,787	134	15
	Creatine Kinase, muscle	gi/6671762	43,246	117	16
	ATP synthase beta subunit	gi/1374715	51,171	150	20
	Actin, alpha 1, skeletal muscle	gi/149043182	51,974	158	10
Diaphragms CS-exposed Guinea pigs	Enolase 3 beta muscle	gi/6679651	47,337	137	14
	Aldolase 1, A isoform	gi/6671539	39,787	154	16
	Creatine Kinase, muscle	gi/6671762	43,246	141	14
	ATP synthase beta subunit	gi/1374715	51,171	224	20
	Actin, alpha 1, skeletal muscle	gi/149043182	51,974	78	10
Gastrocnemius Control Guinea pigs	Enolase 3 beta muscle	gi/6679651	47,337	241	17
	Aldolase 1, A isoform	gi/6671539	39,787	189	18
	Triosephosphate isomerase 1	gi/6678413	27,038	140	12
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	gi/115502204	35,999	128	14
	Creatine Kinase, muscle	gi/6671762	43,246	166	16
	ATP synthase beta subunit	gi/1374715	51,171	226	21
	Actin, alpha 1, skeletal muscle	gi/149043182	51,974	88	16
	Tropomyosin 2 beta	gi/11875203	32,931	164	18
Gastrocnemius CS-exposed Guinea pigs	Enolase 3 beta muscle	gi/6679651	47,337	258	23
	Aldolase 1, A isoform	gi/6671539	39,787	186	17
	Triosephosphate isomerase 1	gi/6678413	27,038	144	12
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	gi/115502204	35,999	146	8
	Creatine Kinase, muscle	gi/6671762	43,246	184	18
	ATP synthase beta subunit	gi/1374715	51,171	158	20
	Actin, alpha 1, skeletal muscle	gi/149043182	51,974	158	11
	Tropomyosin 2 beta	gi/11875203	32,931	164	18

Database: NCBInr
Protein scores greater than 66 are significant ($p<0.05$)
Abbreviations: CS, cigarette smoke

Table E3. Identified nitrated proteins in the muscles of CS-exposed and control guinea pigs

Study Subjects	Identified nitrated proteins	Accession No.	Mass	Mascot Score	Peptide Matched
Diaphragms Control Guinea pigs	Enolase 3 beta muscle	gi/6679651	47,337	132	15
	Aldolase 1, A isoform	gi/6671539	39,787	134	15
	Triosephosphate isomerase 1	gi/6678413	27,038	140	12
	Creatine Kinase, muscle	gi/6671762	43,246	166	16
	Actin, alpha 1, skeletal muscle	gi/149043182	51,974	158	10
Diaphragms CS-exposed Guinea pigs	Enolase 3 beta muscle	gi/6679651	47,337	137	14
	Aldolase 1, A isoform	gi/6671539	39,787	154	16
	Triosephosphate isomerase 1	gi/6678413	27,038	144	10
	Creatine Kinase, muscle	gi/6671762	43,246	141	14
	Actin, alpha 1, skeletal muscle	gi/149043182	51,974	78	10
Gatrocnemius Control Guinea pigs	Enolase 3 beta muscle	gi/6679651	47,337	241	17
	Aldolase 1, A isoform	gi/6671539	39,787	189	18
	Triosephosphate isomerase 1	gi/6678413	27,038	140	12
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	gi/115502204	35,999	128	14
	Creatine Kinase, muscle	gi/6671762	43,246	166	16
	Actin, alpha 1, skeletal muscle	gi/149043182	51,974	88	11
Gastrocnemius CS-exposed Guinea pigs	Enolase 3 beta muscle	gi/6679651	47,337	258	23
	Aldolase 1, A isoform	gi/6671539	39,787	186	17
	Triosephosphate isomerase 1	gi/6678413	27,038	144	12
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	gi/115502204	35,999	146	8
	Creatine Kinase, muscle	gi/6671762	43,246	184	18
	Actin, alpha 1, skeletal muscle	gi/149043182	51,974	158	11

Database: NCBInr
Protein scores greater than 66 are significant ($p<0.05$).
Abbreviations: CS, cigarette smoke

Resum del tercer article:

Cigarette smoke-induced oxidative stress: a role in COPD skeletal muscle dysfunction.

El fum de tabac és una font rica en components oxidants capaços de produir efectes nocius en estructures biològiques claus. Aquests efectes es poden veure incrementats degut a la inflamació associada al consum de tabac donant lloc a peroxidació de lípids o a oxidació de proteïnes que contribueixen a la disfunció muscular en pacients amb MPOC. La hipòtesi d'aquest estudi és que el fum de tabac produeix estrés oxidatiu a nivell sistèmic que es veurà reflectit en els nivells de proteïnes oxidades en els músculs. En aquest estudi es va avaluar el balanç redox analitzant els nivells d'oxidació de proteïnes, antioxidants i creatina cinasa, així com la funció i l'estructura muscular del *vastus lateralis* de controls humans, fumadors i pacients amb MPOC. Per analitzar els efectes de les ROS sobre les proteïnes musculars i la identificació de les proteïnes carbonilades i nitrosilades es van realitzar tècniques d'electroforesis d'una i dues dimensions, respectivament. Les mateixes mesures es van repetir en el diafragma i el *grastrocnemius* de cobais exposats a fum de 7 cigarretes comercials, 5 dies/setmana durant 3, 4 i 6 mesos.

Els resultats d'aquest estudi són:

1.- Funció muscular

La força muscular dels subjectes fumadors va resultar lleugerament però significativament menor a la dels subjectes sans.

2.-Oxidació i carbonilació de proteïnes

Els nivells de proteïnes oxidades i carbonilades en el quàdriceps de fumadors i MPOC es veu incrementat respecte els controls, així com en el diafragma i *grastrocnemius* dels cobais exposats a fum de tabac. Les proteïnes oxidades són proteïnes implicades en la via de la glicòlisi, producció i distribució d'energia i contracció muscular tant en humans com en cobais. S'observa una relació inversa entre els nivells de proteïnes carbonilades a múscul i la força muscular dels fumadors i pacients MPOC.

3.- Nitració

S'observa un increment en la nitració de proteïnes en el diafragma i el gastrocnemius dels cobais exposats a fum de tabac. No obstant, aquestes diferències no es repeteixen en humans

4.- Inflamació

No s'observen canvis en els nivells d'inflamació (IL-6 i TNF-α) ni en humans ni en cobais. Només s'observa un lleuger increment en el nombre de cèl·lules inflamatòries en el múscul dels pacients MPOC.

5.- Estructura de la fibra muscular

La proporció de fibres tipus I va disminuir en el *vastus lateralis* dels pacients MPOC i en els diafragma de cobais exposats a fum de tabac durant 6 mesos comparat amb els seus respectius controls. Per contra, la proporció de fibres tipus II va augmentar en els mateixos individus i cobais.

No es van observar diferències en la mida de la fibra muscular del quàdriceps en humans ni en diafragma o *gastrocnemius* en cobais.

6.- Canvis en l'estructura pulmonar dels cobais

A partir de 4 mesos d'exposició a fum de tabac s'observa un increment en el nombre de cèl·lules caliciformes en els bronquis dels cobais comparat amb els controls.

No s'observa durant aquest període d'estudi presència d'emfisema pulmonar.

Quart article:

Cigarette smoking exacerbates nonalcoholic fatty liver disease in obese rats

Lorenzo Azzalini-Elisabet Ferrer, Leandra N. Ramalho, Montserrat Moreno, Marlene Domínguez, Jordi Colmenero, Víctor I. Peinado, Joan Albert Barberà, Vicente Arroyo, Pere Ginès, Joan Caballería, Ramón Bataller

Article publicat a **Hepatology.** 2010 May;51(5):1567-76.

Cigarette Smoking Exacerbates Nonalcoholic Fatty Liver Disease in Obese Rats

Lorenzo Azzalini,[1]* Elisabet Ferrer,[2]* Leandra N. Ramalho,[3] Montserrat Moreno,[1] Marlene Domínguez,[1] Jordi Colmenero,[1] Víctor I. Peinado,[2] Joan A. Barberà,[2] Vicente Arroyo,[1] Pere Ginès,[1] Joan Caballería,[1] and Ramón Bataller[1]

The prevalence of cigarette smoking (CS) is increased among obese subjects, who are susceptible to develop nonalcoholic fatty liver disease (NAFLD). We investigated the hepatic effects of CS in control and obese rats. Control and obese Zucker rats were divided into smokers and nonsmokers (n = 12 per group). Smoker rats were exposed to 2 cigarettes/day, 5 days/week for 4 weeks. The effects of CS were assessed by biochemical analysis, hepatic histological examination, immunohistochemistry, and gene expression analysis. Phosphorylation of AKT and extracellular signal-regulated kinase (ERK) and quantification of carbonylated proteins were assessed by western blotting. As expected, obese rats showed hypercholesterolemia, insulin resistance, and histological features of NAFLD. Smoking did not modify the lipidic or glucidic serum profiles. Smoking increased alanine aminotransferase serum levels and the degree of liver injury in obese rats, whereas it only induced minor changes in control rats. Importantly, CS increased the histological severity of NAFLD in obese rats. We also explored the potential mechanisms involved in the deleterious effects of CS. Smoking increased the degree of oxidative stress and hepatocellular apoptosis in obese rats, but not in controls. Similarly, smoking increased the hepatic expression of tissue inhibitor of metalloproteinase-1 and procollagen-alpha2(I) in obese rats, but not in controls. Finally, smoking regulated ERK and AKT phosphorylation. The deleterious effects of CS were not observed after a short exposure (5 days). *Conclusion:* CS causes oxidative stress and worsens the severity of NAFLD in obese rats. Further studies should assess whether this finding also occurs in patients with obesity and NAFLD. (HEPATOLOGY 2010;51:1567-1576.)

See Editorial on Page 1487

Cigarette smoking (CS) is considered a worldwide major cause of preventable morbidity and mortality.[1] The main clinical consequences of prolonged exposure to CS are chronic respiratory diseases, increased incidence of a variety of cancers, and increased risk of atherothrombotic clinical events such as myocardial infarction.[2] Hepatologists have traditionally paid scant attention to the deleterious effects of CS. This reflects the fact that smoking *per se* does not appear to cause liver injury and therefore is not considered a causative agent for chronic liver diseases.[3] However, there is in-

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CS, cigarette smoking; CYP2E1, cytochrome p450 2E1; DNP, 2,4-dinitrophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal-regulated kinase; ELISA, enzyme-linked immunosorbent assay; HDL, high-density lipoprotein; HNE, hydroxynonenal; ICAM, intercellular adhesion molecule; HOMA-IR, homeostasis model assessment of insulin resistance; IL, interleukin; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; NF-κB, nuclear factor-kappa B; PCR, polymerase chain reaction; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinases; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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creasing evidence that CS may negatively impact the incidence, severity, and clinical course of many types of chronic liver diseases.[4,5]

Chronic liver diseases are commonly characterized by continuous inflammation and oxidative stress in the hepatic parenchyma, which are two well-characterized systemic consequences of continuous exposure to CS. It is then plausible that prolonged exposure to cigarette smoke negatively impacts key pathogenic events implicated in chronic liver injury. In fact, epidemiologic studies suggest that CS could accelerate the progression of a variety of liver diseases such as hepatitis C[6,7] and primary biliary cirrhosis[8,9] and could represent a risk factor for hepatocellular carcinoma.[10,11] It is unknown whether CS also influences the severity of nonalcoholic fatty liver disease (NAFLD), the main cause of chronic liver injury in Western countries. Because the prevalence of CS is increased in obese people, who are at a risk of developing NAFLD, it is likely that CS may affect the clinical course of this entity.[12]

Nonalcoholic steatohepatitis (NASH) is a severe form of NAFLD characterized by inflammatory infiltrate and hepatocellular damage, with or without fibrosis. In a minority of patients this condition progresses to cirrhosis and endstage liver disease.[13,14] Both environmental and genetic factors seem to influence disease severity in patients with NAFLD.[15,16] Among environmental factors, alcohol intake has been shown to exacerbate the clinical course of patients with NASH.[17] We hypothesize that heavy exposure to CS could also exacerbate NAFLD. To test this hypothesis at the experimental level we used a well-characterized model of obesity-associated NAFLD in rats. Zucker rats naturally lack leptin receptor, thereby developing obesity due to increased food intake. These rats show features of insulin resistance and NAFLD, mimicking the typical findings in patients with obesity and fatty liver. To test the effects of CS we employed an experimental approach consisting of inhaling CS for 4 weeks. We investigated the effects of CS in both control and obese rats. Besides a detailed histological examination, we studied key events in the pathogenesis of NAFLD including insulin resistance, hepatic inflammation and fibrosis, as well as oxidative stress and apoptosis.

Materials and Methods

Animals. Obese (*fa/fa*) and control (*fa/+*) male Zucker rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were divided into four groups: obese smokers, obese nonsmokers, control smokers, and control nonsmokers. Twelve rats were studied per group. CS was administered as previously described.[18] Briefly, smoker rats were exposed with a nose-only system to first-hand smoke of two 2R4F cigarettes (Kentucky University Research, Lexington, KY) daily, 5 days a week, for 4 weeks. To further investigate if CS could also exert acute effects on the liver, we also conducted additional experiments using a short exposure (two 2R4F cigarettes daily for 5 consecutive days). In both cases a smoking device (Protowerx Design, Langley, BC, Canada) was used to puff the smoke of the cigarettes into the inhalation chambers where the rats were held still. Four rats were treated simultaneously. Each puff contained 20 mL of CS and each cigarette was smoked in 16 puffs, with intervals of 25 seconds. Nonsmoker rats underwent the same procedure but the cigarettes were not lighted. Animals were anesthetized with isoflurane (Abbott Laboratories, Madrid, Spain) and then sacrificed on the last day of the experiment, 2 hours after the last exposure to CS. Blood samples were obtained by transdiafragmatic cardiac puncture, collected in tubes with ethylenediaminetetraacetic acid (EDTA), then centrifuged at 3000 rpm for 10 minutes. Livers were collected and stored at −80°C. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals", prepared following the guidelines by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985) and the animal protocol was approved by the Ethics Committee of Animal Experimentation of the Universitat de Barcelona.

Plasma Biochemical Measurements. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, total cholesterol, high-density lipoprotein (HDL), and triglycerides serum levels were measured using standard enzymatic procedures by the Laboratory Medicine of the Hospital Clínic. Insulin levels were assessed by enzyme-linked immunosorbent assay (ELISA) (LINCO Research, St. Charles, MO).

RNA Isolation, Reverse Transcription, and Real-Time Quantitative Polymerase Chain Reaction (PCR). Total RNA was isolated from frozen liver samples using the RNeasy procedure as described by the manufacturer (Qiagen, Hilden, Germany). For complementary DNA (cDNA) synthesis, 0.5 μg of total RNA were retrotranscribed using MultiScribe (Applied Biosystems, Foster City, CA). cDNA templates were amplified by quantitative PCR using the TaqMan technology on an ABI Prism 7900 sequence detection system (Applied Biosystems). The Assay-On-Demand probes and primers for the quantification of 18S rRNA, procollagen α2(I), transforming growth factor beta-1 (TGF-β_1), tissue inhibitor of metalloproteinases-1 (TIMP-1), intercellular adhesion molecule-1 (ICAM-1), interleukin-6 (IL-6), tumor necrosis factor alpha

(TNF-α), vascular endothelial growth factor A (VEGF-A), cytochrome p450 2E1 (CYP2E1), and FAS ligand were provided by Applied Biosystems. All experiments were performed in duplicate and several negative controls were included. Results were normalized to 18S rRNA expression.

Histological Studies. Livers were fixed in 10% phosphate-buffered formalin for 24 hours at room temperature and then embedded in paraffin. Liver inflammation was assessed in 5-μm sections, which were stained with hematoxylin and eosin. Samples were blindly evaluated by an expert pathologist (L.N.R.). A necroinflammatory score was calculated including the following parameters: portal inflammation (0, absent; 1, mild; 2, moderate; and 3, severe mononuclear cell infiltration), lobular inflammation (0, absent; 1, mild; 2, moderate; and 3, severe polymorphonuclear cell infiltration), hepatocellular injury (0, absent; 1, rare microvesicular steatosis and occasional ballooning; 2, evident microvesicular steatosis and obvious ballooning; and 3, diffuse microvesicular steatosis, marked ballooning and frequent hepatocellular apoptosis or necrosis), portal necrosis (0, absent; 1, mild; 2, moderate; and 3, severe interface injury), and central necrosis (0, absent; 1, mild; 2, moderate; and 3, severe central necrosis and hemorrhagic areas). Samples were also assessed according to the scoring system for NASH proposed by Kleiner et al.,[19] which considers the sum of steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning (0-2) scores as the NAFLD Activity Score (NAS). Additional preparations were utilized to evaluate the amount of fibrosis by staining liver sections with 1% picro-Sirius red (Sirius Red F3B, Gurr BDH Chemicals, Poole, UK). Apoptosis was assessed using an *in situ* apoptosis detection kit (DeadEnd TUNEL, Promega, Madison, WI). The sections were also incubated with monoclonal anti-hydroxynonenal (HNE) antibody (1:500, A.G. Scientific, San Diego, CA); anti-nuclear factor-kappa B (NF-κB)-p65 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA); and anti-von Willebrand factor antibody (1:500; Dako, Carpinteria, CA) for 1 hour at room temperature. As negative controls, all specimens were incubated with an isotype-matched control antibody under identical conditions. The number of NF-κB-p65, von Willebrand factor and TUNEL-positive cells was evaluated in 30 randomly chosen high-power fields and mean values were obtained. For Sirius red and HNE, positive stained area was quantified using a morphometric analysis system using an optic microscope (Nikon Eclipse E600; Nikon, Tokyo, Japan) at 40$\times$ magnification. Images were imported using an image analysis software (AnalySIS, Soft Imaging System, Lakewood, CO) and automatically merged. The total positive area was calculated as the sum of the area of all positive pixels.

Western Blot. The levels of total and phosphorylated AKT and ERK expression, total and cleaved caspase 3, and phosphorylated pSMAD2 were assessed by western blot. The levels of oxydated proteins were assessed through detection of carbonyl groups.[20,21] Selective antibodies were used to detect total and phosphorylated AKT and ERK, total and cleaved caspase 3, and phosphorylated pSMAD2 (1:1000; Cell Signaling Technology, Beverly, MA), and carbonyl groups through derivatization to 2,4-dinitrophenylhydrazone (DNP) (anti-DNP moiety antibody, 1:150, Oxyblot kit, Chemicon International, Temecula, CA). Resulting blots were scanned with an ImageReader LAS-3000 imaging densitometer (Fujifilm, Tokyo, Japan), and optical densities of specific proteins were quantified with the ImageGauge software (Fujifilm). Ponceau red staining of crude homogenates on the membranes was used to determine equal loading/transfer across lanes.

Statistical Analysis. Statistical analysis was performed using SPPS 16 (Chicago, IL). Results are expressed as the mean $\pm$ standard deviation. The Kruskal-Wallis test and unpaired Mann-Whitney *U* test were performed as appropriate to determine statistical significance. Differences were considered significant if $P < 0.05$.

Results

Effects of Prolonged CS on Body Weight, Insulin Sensitivity, and Lipid Profile. Smoking was in general well tolerated by both control and obese rats and mortality was similar in all groups that were exposed to CS (10%). CS did not influence animals' body weight throughout the study. The mean weight of control and obese rats at baseline was 300.4 $\pm$ 10.3 g versus 371.8 $\pm$ 13.2 g, respectively ($P < 0.001$). The percentage of weight gain after CS exposure was similar in both control (18% versus 20%, nonsmokers versus smokers, respectively; $P =$ NS) and obese (19% versus 20%, nonsmokers versus smokers, respectively, $P =$ NS) rats. Because CS may affect metabolic parameters, we assessed its effects on insulin sensitivity and lipid profile. As expected, obese rats showed parameters indicative of insulin resistance, as assessed by homeostasis model assessment of insulin resistance (HOMA-IR), as well as hypercholesterolemia and hypertriglyceridemia (Table 1). CS did not modify insulin sensitivity nor lipid profile (including serum total cholesterol, HDL cholesterol, and serum triglycerides levels) in either control and obese rats.

Prolonged CS Exacerbates Liver Injury in Obese Rats. Obese rats exposed to CS showed a significant in-

Table 1. Effect of Prolonged Cigarette Smoking on Insulin Sensitivity and Lipid Profile in Control (fa/+) and Obese (fa/fa) Rats

	fa/+ Nonsmokers	fa/+ Smokers	fa/fa Nonsmokers	fa/fa Smokers
HOMA-IR (mmol · mIU · L^{-2})	11.9 ± 6.89	8.99 ± 5.75	123.75 ± 39.67*	127.53 ± 120.27**
Total cholesterol (mg/dL)	86 ± 6	84 ± 11	143 ± 6*	147 ± 7**
HDL-cholesterol (mg/dL)	32 ± 2	35 ± 2	63 ± 3*	65 ± 4**
Triglycerides (mg/dL)	66 ± 20	82 ± 42	316 ± 88*	285 ± 32**

Twelve rats were included per group. Results are expressed as mean ± standard deviation. *$P < 0.001$ vs. control (fa/+) nonsmoker rats; **$P < 0.05$ vs. control (fa/+) smoker rats. Abbreviations: HOMA-IR: homeostasis model assessment of insulin resistance. HDL: high-density lipoprotein.

crease in ALT serum levels, whereas this effect was not observed in control rats (Fig. 1A). We also assessed the effect of CS on liver histology, as detailed in Materials and Methods. CS was associated with an increase in the necroinflammatory score both in control and obese rats (Fig. 1B,C; Table 2). We next studied whether CS influences the severity of NAFLD in obese rats. For this purpose we used the NAFLD activity score proposed by Kleiner et al.,[19] which takes into account the degree of steatosis, hepatocellular ballooning, and lobular inflammation. As expected, control rats showed preserved liver histology, whereas obese rats showed signs of NAFLD. CS induced mild changes in control rats. In contrast, CS significantly increased the extent of hepatocellular ballooning and lobular inflammation, thereby increasing the NAFLD activity score in obese

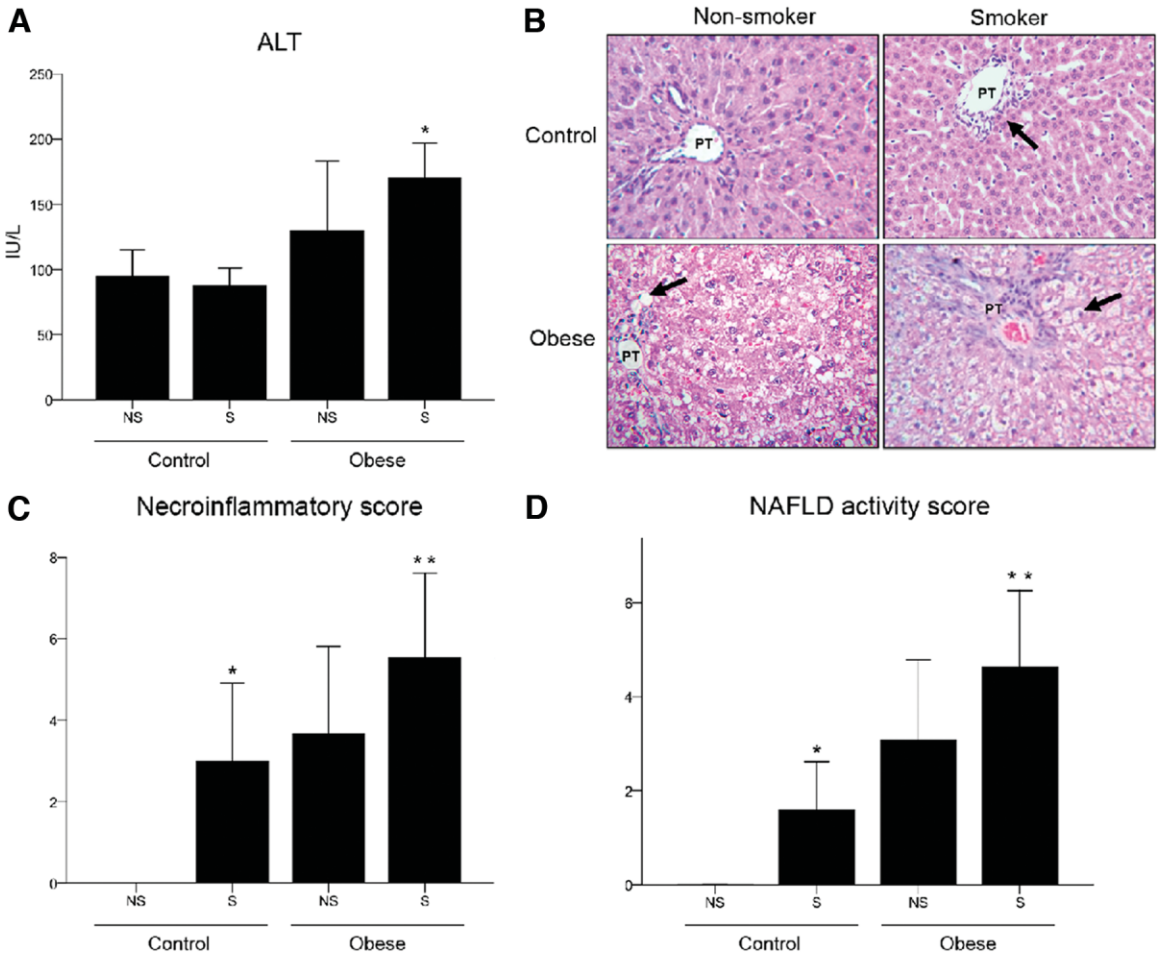

Fig. 1. (A) Effects of CS on serum aminotransferases in control and obese rats. Smoking increased ALT serum levels in obese, but not control rats, whereas it increased AST in both groups (not shown). *$P < 0.01$ versus obese nonsmoker rats. (B) Representative microphotographs of liver specimens from all groups of rats. Hematoxylin and eosin, 100× magnification. (C,D) Quantification of the degree of necroinflammatory injury using the necroinflammatory score and Kleiner et al.'s NAFLD activity score, as described in Materials and Methods. Arrows show inflammatory infiltrate, steatosis, and hepatocellular ballooning. CS induced increased liver damage in obese rats and induced mild changes in control rats. *$P < 0.001$ versus control nonsmoker rats; **$P < 0.05$ versus obese nonsmoker rats. NS: nonsmoker. S: smoker. PT: portal tract.

Table 2. Effect of Prolonged Cigarette Smoking on Histologic Necroinflammatory Score in Control (fa/+) and Obese (fa/fa) Rats

	fa/+ Nonsmokers	fa/+ Smokers	fa/fa Nonsmokers	fa/fa Smokers
Portal inflammation	0 ± 0	0.83 ± 0.39**	1.00 ± 0	1.00 ± 0
Lobular inflammation	0 ± 0	0.58 ± 0.51*	0.58 ± 0.51	1.27 ± 0.47§
Hepatocellular injury	0 ± 0	1.00 ± 0**	1.25 ± 0.45	2.00 ± 0§§
Portal necrosis	0 ± 0	0 ± 0	0 ± 0	0.36 ± 0.50#
Lobular necrosis	0 ± 0	0.58 ± 0.51*	0.83 ± 0.39	0.91 ± 0.30

Twelve rats were studied per group. Results are expressed as mean ± standard deviation. **$P < 0.001$ vs. control (fa/+) nonsmoker rats. *$P < 0.01$ vs. control (fa/+) nonsmoker rats. §$P < 0.01$ vs. obese (fa/fa) nonsmoker rats. §§$P < 0.001$ vs. obese (fa/fa) nonsmoker rats. #$P < 0.05$ vs. obese (fa/fa) nonsmoker rats. The criteria used to evaluate the necroinflammatory score are described in Materials and Methods.

rats (Fig. 1D). These results suggest that CS exacerbates NAFLD in obese rats.

Effects of Prolonged CS on Oxidative Stress. In order to discover the molecular mechanisms involved in the deleterious effects of CS in the liver, we first investigated the degree of oxidative stress, which is a pathogenic event induced by CS in other organs. For this purpose we used two different approaches: measurement of lipid peroxidation and protein carbonylation. CS was associated with a higher degree of oxidative stress in the liver of obese rats, as indicated by increased content of HNE protein adducts. This effect was not observed in control rats (Fig. 2A,B). The pro-oxidant effect of CS on obese rats was confirmed by detection of carbonylated proteins, a marker of oxidative stress (Fig. 2C). The pro-oxidant effect of CS was not associated with an up-regulation of CYP2E1, a key oxidant enzyme in the liver that metabolizes several components of CS (Table 3).

Mechanisms of the Deleterious Effects of CS in the Liver. To investigate the potential mechanisms of the effects of CS in the liver, we next studied whether it induces hepatocellular apoptosis. We found that CS increased hepatocellular apoptosis in obese but not in control rats (TUNEL, Fig. 3A,B). We also studied if CS modifies the pattern of NF-κB expression in the liver. We found increased p65 expression in nonparenchymal cells in the livers of obese and control rats exposed to CS (Fig. 3C,D). Collectively, these data suggest that CS may sensitize hepatocytes to cell death. Next, we assessed whether CS regulates intracellular pathways involved in hepatocellular death and cell defense against apoptosis (i.e., ERK and AKT, respectively). CS reduced the activation of AKT (Fig. 4A) and stimulated the phosphorylation of ERK (Fig. 4B), suggesting that it regulates several intracellular signaling pathways regulating hepatocyte resistance to cell death. In contrast, hepatic expression of Fas ligand, a proapoptotic factor, was not up-regulated by CS (Table 3). We next explored caspase 3 cleavage and SMAD2 phosphorylation, which mediates the proapoptotic effects of TGF-β_1.[22] Surprisingly, we found that smoking decreases the activation of caspase 3, as assessed by the ratio between cleaved caspase 3 and caspase 3, both in control and obese rats (Fig. 4C). This finding suggests that caspase 3 does not mediate the apoptotic effects of

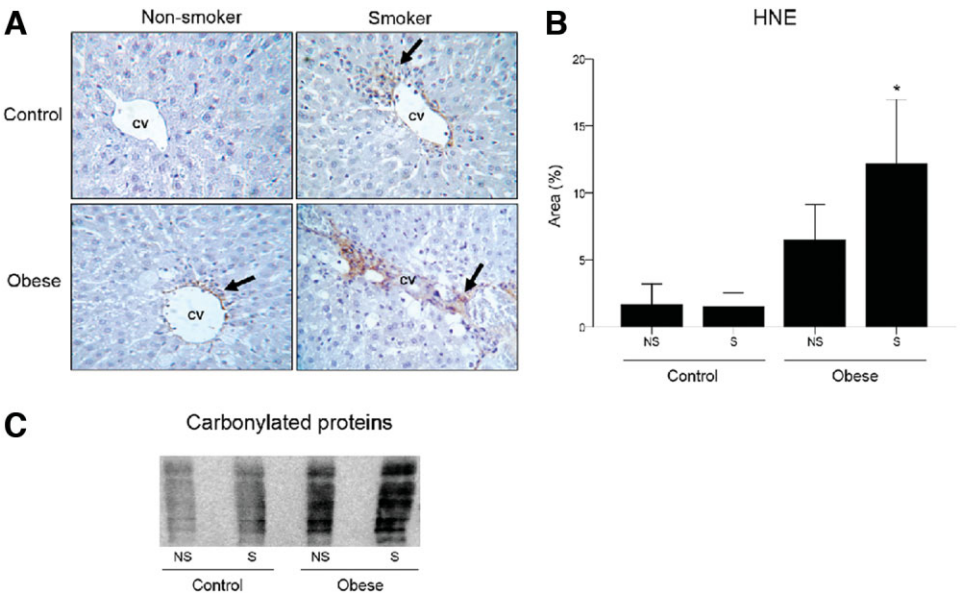

Fig. 2. (A) Representative microphotographs of liver specimens from all groups of rats; 200× magnification. Fixed liver sections were stained for HNE as described in Materials and Methods. Arrows show areas with positive HNE staining. (B) Quantification of HNE expression in liver specimens. CS markedly increased lipid peroxidation in obese rats, but not in control rats. *$P <$ 0.001 versus obese nonsmoker rats. (C) Expression of carbonylated proteins, as assessed by western blot (see Materials and Methods). CS increased carbonylation of cellular proteins both in control and in obese rats. NS: nonsmoker. S: smoker. CV: central vein.

Table 3. Effect of Prolonged Cigarette Smoking on Hepatic Gene Expression in Control (fa/+) and Obese (fa/fa) Rats

	fa/+ Nonsmokers	fa/+ Smokers	fa/fa Nonsmokers	fa/fa Smokers
Procollagen α2(I)	1.09 ± 0.45	1.55 ± 0.35*	1.36 ± 0.32	2.77 ± 1.03§
TGF-β_1	1.01 ± 0.17	0.62 ± 0.21*	0.88 ± 0.14	0.98 ± 0.50
TIMP-1	1.02 ± 0.21	0.99 ± 0.23	1.32 ± 0.33	2.14 ± 0.73§
ICAM-1	1.00 ± 0.29	0.70 ± 0.17	1.92 ± 0.56**	1.88 ± 0.89#
TNF-α	1.06 ± 0.42	0.57 ± 0.11**	0.80 ± 0.19	0.50 ± 0.26
CYP2E1	1.04 ± 0.29	1.01 ± 0.31	0.50 ± 0.23**	0.43 ± 0.20#
Fas ligand	1.02 ± 0.20	0.58 ± 0.13**	0.43 ± 0.09	0.30 ± 0.11
VEGF-A	1.11 ± 0.54	0.99 ± 0.52	0.87 ± 0.38	1.18 ± 0.68

Twelve rats were studied per group. Results are expressed as $2^{-\Delta\Delta Ct}$ (mean ± standard deviation). 18s rRNA was used as housekeeping gene. *$P < 0.05$ vs. control nonsmoker rats; §$P < 0.05$ vs. obese nonsmoker rats; **$P < 0.01$ vs. control nonsmoker rats; #$P < 0.01$ vs. control smoker rats. IL-6 expression resulted below the sensitivity threshold of the technique in most animals in the four groups. TGF-β_1: transforming growth factor β_1. TIMP-1: tissue inhibitor of metalloproteinases 1. ICAM-1: intercellular adhesion molecule 1. TNF-α: tumor necrosis factor α. CYP2E1: cytochrome p450 2E1. VEGF: vascular endothelial growth factor. IL-6: interleukin 6.

smoking in the liver. To investigate whether smoking affects SMAD2 phosphorylation we performed both western blot and immunohistochemistry studies. The western blot analysis of whole liver extracts showed that smoking does not stimulate SMAD2 phosphorylation (Fig. 4D). However, the detailed analysis of immunohistochemistry studies showed that smoking slightly increased the expression of phospho-SMAD2 in hepatocytes in both control and obese rats, especially in those located in pericentral areas (Supporting Fig. 3). Overall, these results suggest that smoking induces apoptosis through a caspase-independent mechanism.

We next investigated whether CS is associated with angiogenesis in the liver. We did not find neovasculariza-

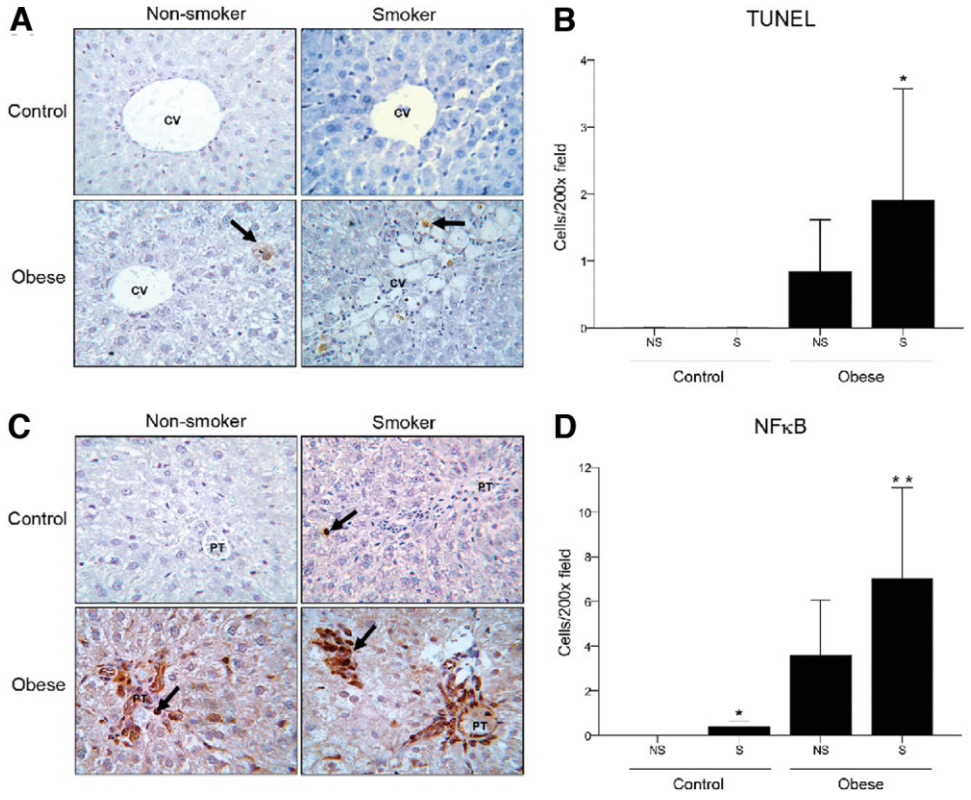

Fig. 3. (A) Representative microphotographs of liver specimens from all groups of rats; 200× magnification. Fixed liver sections were stained for TUNEL, as described in Materials and Methods. Arrows show cells with positive TUNEL staining. CS increased the amount of TUNEL-positive cells in obese rats, but not in control rats. (B) Quantification of TUNEL-positive cells per high-power field. CS produced an important increase in hepatocellular cell death in obese rats, but not in control rats (*$P < 0.001$ versus obese nonsmoker rats). (C) Representative microphotographs of liver specimens from all groups of rats; 200× magnification. Fixed liver sections were stained for the p65 subunit of NF-κB as described in Materials and Methods. Arrows show cells with positive p65 staining. CS increased the amount of p65-positive cells in obese rats, but not in control rats. (D) Quantification of p65-NF-κB-positive cells per high-power field. CS greatly increased the expression of p65 in obese rats. *$P < 0.05$ versus control nonsmoker rats; **$P < 0.05$ versus obese nonsmoker rats. NS: nonsmoker. S: smoker. CV: central vein, PT: portal tract.

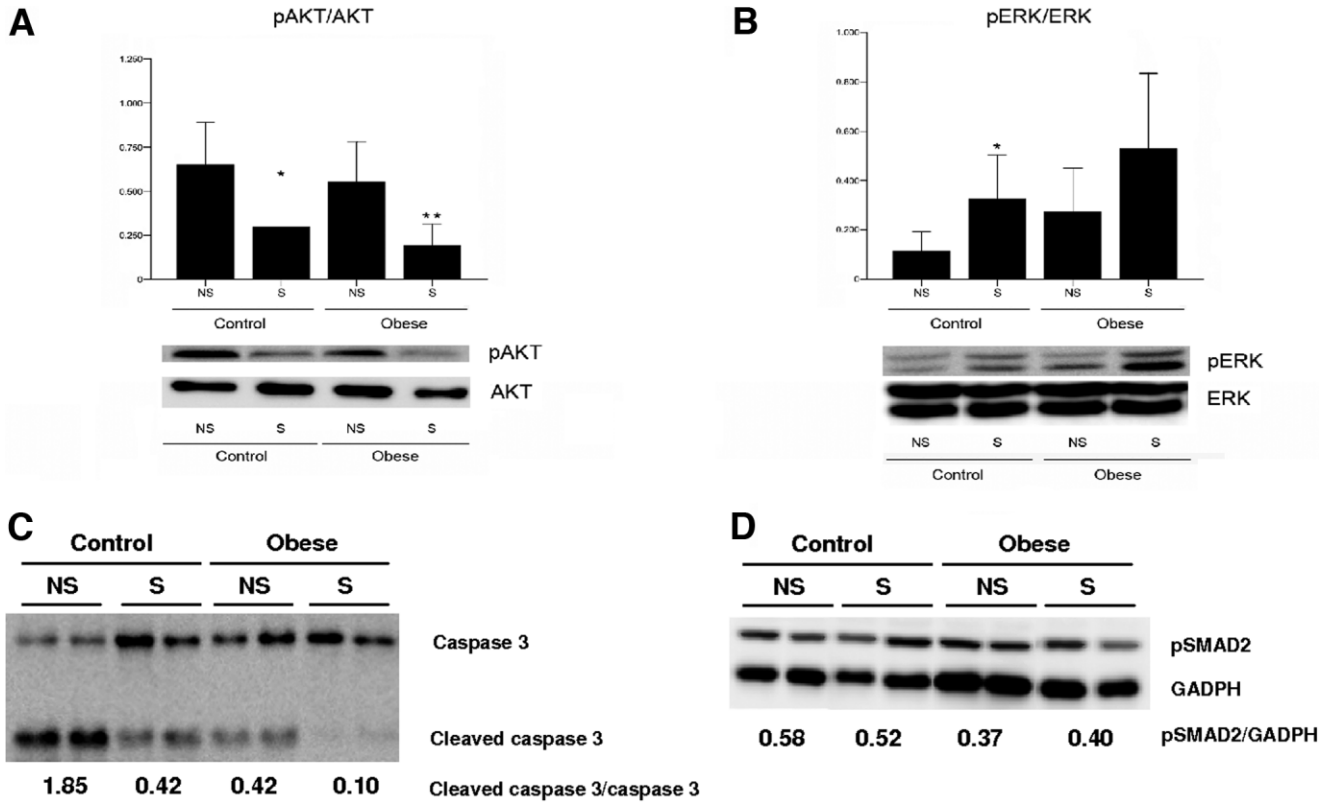

Fig. 4. (A,B) Effect of CS on AKT and ERK phosphorylation as assessed by western blot (see Materials and Methods). Results are expressed as the ratio of phosphorylated protein over total protein expression. CS induced a decrease in pAKT/AKT ratio and an increase in pERK/ERK ratio. $*P <$ 0.05 versus control nonsmoker rats; $**P < 0.01$ versus obese nonsmoker rats. Results are the mean with standard deviation of five independent experiments. (C,D) Effect of CS on caspase 3 and pSMAD2 as assessed by western blot (see Materials and Methods). Results are expressed as the ratio of cleaved-caspase/caspase and pSMAD2/GADPH, respectively. CS decreased caspase-3-driven apoptosis, whereas it caused little change in pSMAD2-induced apoptosis. NS: nonsmoker. S: smoker.

tion in any of the studied groups, as indicated by negative staining for von Willebrand factor (data not shown). Similarly, CS did not increase VEGF-A hepatic gene expression (Table 3).

Effect of Prolonged CS on the Expression of Inflammatory and Fibrogenic Genes in the Liver. We finally evaluated whether CS regulates the hepatic expression of proinflammatory and profibrogenic genes. CS did not increase the expression of genes involved in hepatic inflammation including ICAM-1 and TNF-α (Table 3). In contrast, CS increased the expression of key genes involved in hepatic fibrogenesis in obese rats, including procollagen α2(I) and TIMP-1, whereas TGF-β_1 was unaffected. This effect was not associated with the development of liver fibrosis, as assessed by quantification of Sirius red staining (not shown). These results show that CS induces profibrogenic gene expression in the liver, yet does not induce liver fibrosis *per se*.

Effects of Short Exposure to CS in Control and Obese Rats. We finally explore whether CS cause acute effects in the liver. For this purpose, control and obese rats were exposed to CS daily for 5 consecutive days. Smoking was in general well tolerated by both control and obese rats and no mortality was observed. Cigarette smoking did not modify ALT serum levels in obese rats, whereas it only induced minor changes in control rats (Supporting Fig. 1B). We next evaluated blindly all liver specimens stained with H&E to evaluate necroinflammatory changes. We found that smoking did not cause significant injury in control or obese rats (Supporting Fig. 1A; Table 4). As expected, Sirius red staining showed no sign of fibrosis in any of the studied groups (Supporting Fig. 2A). Similarly, acute cigarette exposure did not modify the hepatic expression of fibrogenic genes (Supporting Fig. 2B,C). Overall, these results indicate that smoking does not cause acute effects on the liver.

Discussion

The current study investigated the hepatic effects of CS in control and obese rats. We provide evidence that smoking induces hepatocellular apoptosis and oxidative stress in obese rats as compared to control animals, thus exacerbating NAFLD. These results are in keeping with previ-

Table 4. Effect of Short-Term Cigarette Smoking on Histologic Necroinflammatory Score in Control (fa/+) and Obese (fa/fa) Rats

	fa/+ Nonsmokers	fa/+ Smokers	fa/fa Nonsmokers	fa/fa Smokers
Portal inflammation	1.00 ± 0	1.00 ± 0	1.25 ± 0.50	1.00 ± 0
Lobular inflammation	0.50 ± 0.58	0.67 ± 0.50	1.50 ± 0.58	1.33 ± 0.50
Hepatocellular injury	1.25 ± 0.50	1.56 ± 0.53	2.00 ± 0	2.00 ± 0
Portal necrosis	0 ± 0	0 ± 0	0.75 ± 0.50	0.67 ± 0.50
Lobular necrosis	0.50 ± 0.58	0.78 ± 0.44	0.75 ± 0.50	0.78 ± 0.44

Twelve rats were studied per group. Results are expressed as mean ± standard deviation. No significant differences were found among smokers and nonsmokers in either control and obese rats. The criteria used to evaluate the necroinflammatory score are described in Materials and Methods.

ous data showing that CS exerts proinflammatory and oxidant effects in other organs, such as the kidney and the pancreas.[23-27] Here, we demonstrate that CS exacerbates liver damage in a genetic model of NAFLD. Our results suggest that CS could exert deleterious effects in the liver, acting as a cofactor favoring the progression of chronic liver diseases. Further studies in experimental and human liver injury models should confirm these findings.

We used a genetic model of obesity-induced NAFLD. We preferred this model over diet-induced obesity because CS can directly affect food intake and interfere with weight gain. To expose rats to CS, we used a well-accepted model for tobacco smoking exposure in rodents,[18,28] which we had used to study the systemic effects of CS exposure in guinea pigs.[29] We chose a 4-week period to expose rats to CS because we wanted to investigate a prolonged, rather than an acute, exposure. However, the length of CS exposure is obviously shorter than occurs in humans. This may explain the fact that we did not observe any change in insulin sensitivity nor in body weight in smoker rats, whereas these abnormalities are commonly found in smoker patients.[12,30] Therefore, we cannot rule out that longer exposures would have resulted in more pronounced effects on liver histology (i.e., fibrosis) or changes in the metabolic profile. Because heavy CS is associated with tissue hypoxia, we investigated whether hypoxia may have contributed to liver damage. Our data suggest that hypoxia was not involved, because the hepatic expression of parameters indicative of tissue hypoxia (i.e., VEGF-A gene expression and von Willebrand factor immunohistochemistry) were unaffected by smoking.

The main result of our study is that CS exacerbates NAFLD in obese rats. In particular, smoking induced hepatocellular ballooning and lobular inflammation in obese rats. To our knowledge, this is the first experimental study investigating the hepatic effects of CS in the setting of obesity. Although we observed some effects of CS in control rats, obese rats were more susceptible to the deleterious effects of CS. Of note, smoking was not associated with the development of significant fibrosis. This negative result can be due to two different reasons. First, rodents lacking leptin and/or leptin receptor do not develop significant fibrosis, revealing that leptin is required to develop collagen deposition in the liver. Second, we exposed rats to CS for 4 weeks and cannot rule out that longer exposures would have resulted in hepatic fibrosis. In fact, the finding that CS increases collagen synthesis at the mRNA level strongly suggests that it may exert profibrogenic effects in obese rats. It would be interesting to explore the fibrogenic effects of CS in other animal models of NAFLD that develop liver fibrosis.

Different mechanisms could have contributed to the deleterious effects of smoking in obese rats. First, we found that CS caused oxidative stress in obese rats. This finding could be relevant because oxidative stress is a well-characterized mechanism of injury in chronic liver diseases, including NAFLD.[31,32] In fact, antioxidant supplements have been proposed to treat patients with NAFLD.[33,34] We also found increased hepatocellular apoptosis in obese rats exposed to CS. This mechanism of cell death has been recently implicated in the pathogenesis of NASH in humans.[35] Surprisingly, the results of our study do not support a proinflammatory effect of CS in the liver, as indicated by the lack of effect on the expression of inflammatory genes. This negative result could be due to the relatively short exposure to CS and further studies with longer exposure should address this aspect. Finally, our study reveals some of the potential cell signaling pathways involved in the pathogenesis of smoking-induced liver injury. In particular, we showed that CS stimulates ERK phosphorylation and decreases AKT activation in both obese and control rats. ERK is induced after acute liver injury and regulates proliferation and biological actions in many liver cell types.[36] In hepatocytes, activation of ERK results in stimulation of DNA synthesis.[37] The AKT/PKB pathway is an important antiapoptotic factor in hepatocytes and exerts protective effects against cell death.[38] Interestingly, AKT-driven cell signaling is altered by CS in other organs.[39-42] Based on our results, the apoptotic effects of CS in the liver seem to be caspase-independent. Of note, the deleterious effects of CS in the liver were not observed after a short exposure (5 days). Because acute smoking exposure can cause deleterious effects in other organs and tissues (e.g. heart, lung,

and blood vessels), our results raise the possibility that the liver is particularly resistant to the acute effect of smoking.

Our results may have potential pathophysiological implications. First, we provide evidence that CS causes oxidative stress and apoptosis in the liver. This consequence may also play a role in the progression of other liver diseases, such as chronic hepatitis C and primary biliary cirrhosis. Second, we found that CS up-regulates the hepatic expression of genes involved in fibrogenesis such as procollagen α2(I) and TIMP-1. These effects could also contribute to accelerate liver fibrosis in patients with NAFLD. Further studies using different experimental models (e.g., methionine-choline deficient diet) should be performed. Third, we showed that CS impacts the severity of NAFLD, favoring the development of NASH. Because the development of NASH is a prerequisite for fibrosis progression in patients with NAFLD, it is possible that CS exerts a negative impact in the natural history of patients with NAFLD. Prospective clinical studies should investigate this assumption.

In summary, this study shows that CS exacerbates liver injury in a rat model of obesity-related fatty liver, in particular increasing hepatocellular apoptosis and oxidative stress. Further studies should investigate the hepatic effects of longer exposures to CS, as well as better delineate the cellular and molecular mechanisms involved. Importantly, further studies should investigate whether CS also impacts the natural history of patients with obesity-related NAFLD (i.e., development of NASH, more aggressive fibrosis progression, etc.).

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Resum del quart article:

Cigarette smoking exacerbates nonalcoholic fatty liver disease in obese rats

El fum de tabac és un agent tòxic que té especial incidència sobre el pulmó però que també s'associa a altres malalties com el càncer o cardiopaties. Noves evidències suggereixen que el tabac té un efecte deleteri en el fetge. L'estrès oxidatiu i el component inflamatori associats a fum de tabac són factors de gran rellevància que es podrien sumar a altres alteracions preexistents agreujant el desenvolupament d'un procés patològic ja iniciat. Basant-nos en aquests fets, hem aplicat el model d'exposició a fum de tabac en rates genèticament obeses. En aquest estudi es van formar 4 grups: un grup control, un grup de rates control exposades al fum de 2 cigarretes/dia, 5 dies a la setmana durant 4 setmanes, un grup de rates obeses exposades a tabac i un grup de rates obeses falsament exposades a fum de tabac. Per tal de realitzar un perfil lipídic dels animals es van recollir mostres de plasma al finalitzar l'estudi. En seccions de fetge es van fer tincions amb hematoxilina&eosina i immunoshistoquímiques per determinar el grau de lesió, inflamació i necrosis hepàtica. Mitjançant RT-PCR es va avaluar l'expressió gènica de marcadors pro-inflamatoris i angiogènics. Les vies implicades en els processos d'apoptosi es van avaluar mitjançant western blot.

Els resultats d'aquest quart treball són:

1.- Perfil lipídic

Tal com s'esperava, les rates obeses van mostrar resistència a la insulina i hipercolesterolèmia. El tabac no va influir en el perfil lipídic dels animals ni va induir pèrdua de pes.

2.- Dany hepàtic

L'exposició a fum de tabac en rates obeses va fer augmentar els nivells d'alanina aminotransferasa en sèrum així com el grau de lesió en l'estructura hepàtica, mentre que només va induir petits canvis en les rates controls.

3.- Estrés oxidatiu

El fum de tabac va augmentar l'estrès oxidatiu en el fetge de les rates obeses que no s'associa a un increment en la regulació del citocrom P450 (CYP2E1). També es va observar un increment en l'apoptosi hepatocel·lular en les rates obeses degut a l'exposició a fum de tabac mentre que no es van observar canvis en les rates controls. La inducció a l'apoptosi induïda pel fum de tabac no sembla estar regulada per la caspasa 3.

4.- Inflamació

L'exposició a fum de tabac no va incrementar l'expressió de gens implicats en la inflamació hepàtica com TNF-α i ICAM-1. Contràriament, el fum de tabac sí va incrementar l'expressió de gens implicats en la fibrogènesi hepàtica. Per tant, el tabac indueix l'expressió de gens profibrogènics però no indueix la fibrosi *per se.*

5.- Efectes aguts

No s'observen canvis en els paràmetres anteriorment mencionats en el fetge de rates obesses exposades a fum de tabac durant una setmana.

DISCUSSIÓ

La implicació del fum de tabac en la patogènesi de la MPOC és ben coneguda però actualment encara es desconeixen els mecanismes exactes pels quals es desenvolupa la malaltia. Encara menys estan establerts els mecanismes patogènics de les alteracions vasculars que s'associen a la malaltia. Aquesta tesi presenta certes claus per elucidar el paper de la hipòxia i sobretot del tabac sobre aquests alteracions vasculars. Amb aquest objectiu, hem desenvolupat en el nostre laboratori un model animal de MPOC a partir del qual hem intentat donar resposta a les varies qüestions:

1.- Validació del model experimental

La MPOC es caracteritza principalment per alteracions a la via aèria i al parènquima pulmonar. Aquestes alteracions, que en humans es presenten com a canvis morfològics als bronquis i bronquíols i com emfisema, es reprodueixen en el model de cobai exposat a fum de tabac. En els estudis efectuats hem observat la presència de metaplàsia de cèl·lules caliciformes als bronquis dels cobais després de 4 mesos d'exposició[146], mentre que l'emfisema apareix més tardanament, als 6 mesos d'iniciar-se l'exposició a tabac. El temps d'aparició d'aquestes alteracions de la via aèria és similar al dels estudis realitzats per Wright, que descriuen l'aparició de metaplàsia de cèl·lules secretores als 3 mesos[147] i la presència d'emfisema als 6 mesos d'exposició[148].

Contràriament al que observem en cobais, no hem sigut capaços de reproduir aquestes alteracions en rates exposades a fum de tabac durant els mateixos períodes de temps. Les rates mostren més resistència a l'aparició de canvis estructurals i dificulten l'estudi ja que requereixen períodes mes llargs d'exposició. Considerem doncs, que el model de cobai exposat a fum de tabac és un bon model de MPOC, ja que reprodueix les característiques anatomopatològiques més rellevants de la malaltia. Tant és així que la disponibilitat d'aquest model al nostre laboratori ha servit a altres grups com a eina experimental per al desenvolupament d'altres estudis tal com es mostra a l'annex de resultats d'aquesta tesi.

2.- Efectes del tabac sobre l'estructura vascular

A) Vasos pulmonars

Els canvis observats a les artèries pulmonars dels cobais exposats a fum de tabac es caracteritzen, morfològicament, per un increment en el gruix de la paret de l'artèria. Aquest engruiximent s'acompanya d'un canvi en la composició de les proteïnes de la matriu extracel·lular en la paret del vas. Les artèries pulmonars dels cobais exposats a fum de tabac presenten major proporció de mucopolisacàrids i menor proporció de col·làgena, canvis que afecten a les propietats elàstiques del vas. Per contra, la proporció d'elastina es manté estable independentment de l'exposició. Aquestes alteracions apareixen als 3 mesos d'exposició a fum de tabac i podrien condicionar la resposta vascular. L'origen dels canvis en la morfologia dels vasos principals podria estar vinculat amb els canvis observats en els vasos intrapulmonars ja que aquestes alteracions apareixen en el mateix moment en l'evolució de la patologia. Així, els cobais exposats a fum de tabac mostren un major nombre de vasos petits (<50µm) α-actina^{+}/desmina^{-}. Aquesta particularitat fenotípica indica la naturalesa poc madura de les cèl·lules musculars responsables del remodelat vascular. Un fet important a destacar és la proporció de vasos muscularitzats que s'origina en estadis inicials de l'exposició a tabac (3 mesos). En canvi, el procés de formació d'una segona làmina elàstica en els vasos intrapulmonars no apareix fins als 6 mesos. Altres estudis realitzats amb el mateix model demostren que l'inici de la muscularització es produeix durant les primeres setmanes d'exposició a fum de tabac, resultant en un increment en el nombre de vasos α-actina^{+} a les dues setmanes[149]. Aquests resultats indiquen l'acció directe del fum de tabac sobre la circulació pulmonar ja des de les primeres setmanes d'exposició. Wright descriu l'aparició de doble làmina elàstica en vasos intrapulmonars després d'un mes de ser exposats a fum de tabac[150], mentre que nosaltres ho observem als 6 mesos d'exposició. Aquestes diferències poden ser degudes en primera instància al nombre de cigarretes administrades, que fou superior a l'estudi de Wright, i en segon lloc a la metodologia d'avaluació. Independentment de les possibles discrepàncies

entre el temps d'aparició del remodelat vascular, és evident que els canvis en la circulació pulmonar són anteriors al desenvolupament d'emfisema pulmonar.

No podem atribuir la muscularització dels vasos intrapulmonars a una activació en la proliferació cel·lular ja que els estudis amb PCNA no mostren diferències entre els grups. No descartem, però, que amb períodes més llargs d'exposició pugui haver un increment en la proliferació cel·lular de la paret dels vasos intrapulmonars. No obstant, és possible que altres mecanismes com la hipertròfia de les cèl·lules musculars o el canvi de fenotip de les cèl·lules residents, estiguin incidint en l'increment del nombre de vasos muscularitzats. Actualment ha sorgit un nou concepte que proposa un mecanisme de desdiferenciació de cèl·lules endotelials a cèl·lules d'origen mesenquimal (*Endothelial to mesenchymal transition;* EnMT). Aquesta teoria justificaria el canvi de fenotip en l'absència de proliferació cel·lular. No obstant, són necessaris més estudis enfocats a aportar noves evidències de la presència d'aquest mecanisme.

B) Circulació sistèmica

Cap dels canvis vasculars observats a l'artèria pulmonar s'han reproduït a l'artèria aorta, fet que indica un major efecte del fum de tabac en l'espai pulmonar que en l'espai sistèmic. Tal com es menciona a la introducció, el fum de tabac està format per una fase gasosa i una particulada que per les seves característiques moleculars i el seu temps de vida poden estar afectant amb més intensitat els pulmons. De totes maneres, no descartem que exposicions més prolongades o concentracions més altes de fum de tabac puguin tenir efectes directes o secundaris sobre la circulació sistèmica. Afegit a això, és possible que els efectes sistèmics siguin més fàcilment detectats en vasos perifèrics de menor envergadura que l'artèria aorta. De fet, estudis realitzats per Ardite[146] i Barreiro[151] confirmen el fet de que el fum de tabac indueix un increment de la peroxidació lipídica a múscul com a conseqüència d'un desequilibri en el sistema redox. Aquest mateix desequilibri podria ser el causant de la pèrdua de pes observada en els animals fumadors i amb el temps afectar al sistema vascular perifèric.

3.- Canvis induïts pel tabac a la funció vascular

A) Vasos pulmonars

Hipertensió pulmonar. Estudis previs realitzats per Wright *et al.* demostren que en els cobais augmenta la pressió arterial pulmonar (PAP) a partir del primer mes d'exposició a fum de tabac[150] i que exposicions més prolongades (fins a 12 mesos) no resulten en un increment superior de la PAP. Aquest increment sostingut de la PAP no s'acompanya d'un increment de la resistència vascular pulmonar fins als 12 mesos d'exposició a fum de tabac, el que podria estar indicant un mecanisme de compensació de l'arbre vascular[150]. Així, els resultats obtinguts en el segon estudi d'aquesta tesi reforcen el coneixement de que l'exposició a fum de tabac augmenta la PAP i que el valor d'aquesta es correlaciona amb el gruix de la paret del ventricle dret. Existeixen diferents possibles causes implicades en el desenvolupament de la hipertensió pulmonar. En primer lloc, els canvis en la composició de les proteïnes de la matriu extracel·lular de la paret de l'artèria pulmonar poden afectar a les propietats mecàniques i físiques del vas donant lloc a una major rigidesa i menor distensibilitat. Aquest fet dificultaria l'adaptació de l'artèria a canvis de flux i oferiria més resistència al pas de la sang. En segon lloc, el canvi en l'estructura dels vasos intrapulmonars també podria jugar un paper important en l'aparició de la HP. De fet, existeix una relació positiva entre el nombre de vasos muscularitzats i la PAP. Tant la musculardització com la formació d'una doble làmina elàstica en vasos petits són alteracions que contribueixen a la resistència vascular pulmonar ja que redueixen el diàmetre de la llum del vas. En tercer lloc, hem demostrat una correlació inversa entre l'expressió d'eNOS i la musculardització de vasos intrapulmonars, el que podria estar indicant la implicació de l'endoteli en la presència d'HP. Aquests mecanismes no s'exclouen mútuament, és més, poden presentar-se de forma conjunta o fins i tot acompanyant l'acció d'altres mecanismes implicats en la HP.

Disfunció endotelial. Els resultats del primer estudi confirmen la hipòtesi del grup en tant que l'exposició a fum de tabac indueix disfunció endotelial en les artèries pulmonars de cobais. Aquest fet s'ha demostrat emprant la metodologia clàssica i ja establerta del bany

d'òrgans, on hem observat un desplaçament cap a la dreta de la corba dosi-resposta a ADP de les artèries pulmonars dels cobais exposats a fum de tabac. Aquest desplaçament indica el requeriment, en el grup d'animals fumadors, d'una major dosi de vasodilatador per obtenir el mateix percentatge de relaxació que el grup control. En els nostres estudis hem decidit utilitzar ADP després de comprovar que és el vasodilatador que millor resposta dóna en cobais. Tot i així existeixen certes diferències en el perfil de resposta de les artèries de cobai respecte les d'humà. Per exemple, els cobais responen a la màxima dosi d'ADP amb una relaxació del 100%, independentment de si els animals han estat exposats o no a fum de tabac. Per contra, en humans, un dels factors indicadors de la disfunció endotelial és el percentatge de relaxació màxima, ja que les artèries intrapulmonars dels pacients amb MPOC i dels fumadors amb funció pulmonar normal no són capaces de relaxar al 100%.

Per descartar la implicació de metabòlits derivats de la via del tromboxà, les artèries es van tractar amb indometacina prèviament al inici de l'estudi. D'aquesta manera assegurem la inhibició de la via de tromboxans que pugui afectar a la resposta vasodilatadora del vas. De la mateixa manera, per corroborar l'afectació del NO en la resposta vascular vam inhibir l'eNOS amb L-NAME. L'absència de resposta vasodilatadora corrobora la participació de la via del NO. Els resultats de l'expressió gènica d'eNOS apunten cap a aquesta mateixa direcció ja que l'expressió d'eNOS en cobais exposats a fum de tabac durant 3 i 6 mesos es troba disminuïda respecte el seu control, el que podria explicar el desplaçament a la dreta de la corba dosi-resposta.

Tot i demostrar la presència de disfunció endotelial, la metodologia té certes limitacions. La realització de l'estudi a dos temps diferents (3 i 6 mesos) ha permès reconstruir la seqüència d'alteracions que succeeixen a la circulació pulmonar, però alhora també ha mostrat un nou aspecte fins ara poc reportat a la literatura del cobai: l'efecte de la maduració sobre la funció de l'endoteli. Així, hem observat una menor resposta vasodilatadora i una menor expressió d'eNOS en els cobais controls als 6 mesos, fet que

ha reduït les diferències entre animals controls i fumadors a aquest temps. Desconeixem les causes exactes per les quals té lloc aquest fenomen, però creiem que podria estar relacionat amb un increment dels nivells de ROS que afectaria a la biodisponibilitat de NO i conseqüentment afavoriria la disfunció endotelial. Una altra limitació important és la reproductibilitat dels resultats en el segon estudi, en el qual no hem sigut capaços de demostrar presència de disfunció endotelial. Segurament aquest fet sigui degut a la menor dosi de tabac administrada als cobais, la qual cosa hauria reduït les diferències entre grups. De la mateixa manera, l'activitat d'eNOS es veu lleugerament disminuïda però no arriba a ser significativament diferent. Aquest resultat es podria interpretar com un pas previ a la disfunció endotelial que amb dosis més altes o exposicions més prolongades a fum de tabac podria arribar a la significació.

Contractilitat vascular pulmonar. La resposta de les artèries pulmonars a KCl no només ens ha servit per comprovar la viabilitat dels vasos en el bany d'òrgans sinó que també indica la capacitat màxima de resposta contràctil de l'artèria. En aquest sentit, les artèries pulmonars dels cobais fumadors responen amb més força que els controls. Aquest efecte està present als 3 mesos d'exposició a fum de tabac i augmenta als 6 mesos. És d'interés ressaltar que aquest increment en la capacitat contràctil correlaciona amb el gruix de la paret del vas, indicant una relació directe entre ambdós paràmetres.

Rigidesa vascular pulmonar. La distensibilitat pulmonar juga un paper important en la mecànica de la ventilació, de la mateixa manera que la distensibilitat vascular ho fa en l'hemodinàmica pulmonar. El concepte de distensibilitat ens dóna una idea de la capacitat de variació de volum que pot oferir un vas en resposta als canvis de pressió. Aquest és un aspecte important a tenir en compte a l'hora d'avaluar les possibles causes de la HP. El gruix de la paret del vas o la composició d'aquest són factors determinants de l'elasticitat del vas, així una variació en aquests factors podria condicionar la capacitat de resposta a canvis de pressió. Els cobais exposats a fum de tabac mostren una tendència a reduir la seva distensibilitat així com el contingut de col·làgena en la paret de l'artèria pulmonar. Per

contra, el contingut de mucopolisacàrids tendeix a augmentar en els animals exposats a fum de tabac.

B) Circulació sistèmica

No s'observen canvis a nivell sistèmic en la funció endotelial, la distensibilitat o la contractilitat. No obstant això, no descartem que en vasos sistèmics més petits existeixin canvis en aquests paràmetres.

4.- Efectes de la hipòxia.

A) Vasos pulmonars

Hipertensió pulmonar. És conegut que l'exposició aguda i crònica a hipòxia dóna lloc a un increment de la PAP[152], i de la mateixa manera que el fum de tabac el increment de pressió és sostingut i no augmenta amb exposicions prolongades. Nombroses publicacions descriuen la presència de HP amb diferents temps i graus d'exposició a hipòxia. No obstant, en el nostre coneixement, en cap cas s'ha comparat l'efecte de la hipòxia i el tabac sobre l'hemodinàmica pulmonar. En el segon estudi demostrem que, a les concentracions i temps d'exposició mencionats, la hipòxia i el tabac tenen efectes similars sobre la PAP. A més, la suma d'ambdós estímuls resulta en un efecte sinèrgic que incrementa encara més els valors de PAP. La hipertròfia del ventricle dret corrobora el increment de PAP en les mateixes proporcions.

Disfunció endotelial. Malauradament, en el segon estudi no hem demostrat presència de disfunció endotelial induïda pel tabac o per la hipòxia. Per tant, no podem comparar els efectes entre tabac i hipòxia a aquest nivell. Concordant amb aquests resultats, no obtenim diferències en l'activitat de l'eNOS entre grups. No obstant, observem una lleu reducció de l'activitat de la proteïna en les tres grups experimentals, especialment en els grups exposats a fum de tabac. Aquest podria ser l'indicador d'una futura lesió endotelial que podria agreujar-se amb exposicions més llargues o dosis més elevades de tabac o hipòxia. Els nivells de nitrits i nitrats en plasma estan reduïts en els animals exposats a tabac, el

que pot estar indicant una menor activitat de l'eNOS prèvia al desencadenament de la patologia.

Contractilitat vascular pulmonar. La reactivitat vascular en resposta a KCl en els animals sotmesos a hipòxia és similar a la dels cobais exposats a fum de tabac. La suma dels dos efectes incrementa la força de contracció de l'artèria pulmonar però no assoleix nivells prou elevats com per associar-ho a un efecte sinèrgic. Tot i això, el perfil de resposta apunta que tots dos efectes es sumen. La resposta a noradrenalina (NE) és, no obstant, diferent. La hipòxia exerceix un efecte important en la resposta contràctil mitjançada per la NE que no incrementa en afegir-hi l'estímul del tabac. Aquesta increment en la resposta contràctil en els cobais sotmesos a hipòxia podria ser deguda a un increment en el nombre de receptors de NE. Malauradament, aquest ha sigut un resultat inesperat motiu pel qual no es va avaluar la quantitat i/o afinitat per aquests receptors. Els nivells de NE i epinefrina en plasma en els cobais exposats a hipòxia estan elevats però la dispersió de les dades impedeix arribar a la significació. En conjunt, aquest resultat indica un increment del to vascular que podria estar jugant a favor de l'aparició de HP i en contra de la funció endotelial. De fet, l'anàlisi de la resposta endoteli-dependent ha estat difícil d'avaluar degut als diferents punts de partida dels 4 grups experimentals, ja que prèviament a l'estudi de vasorelaxació les artèries són pre-contretes amb NE.

Rigidesa vascular pulmonar. Un altre aspecte valorat en el segon estudi és la distensibilitat vascular. A diferència de la resposta a KCl o NE, la distensibilitat vascular no depèn de mediadors, receptors o canals de K^+, sinó de les propietats elàstiques del vas (i de l'activitat vegetativa). Els canvis en la composició de la paret vascular de l'artèria pulmonar estan relacionats amb la distensibilitat, mostrant major capacitat elàstica els vasos dels cobais controls i major rigidesa els vasos dels animals exposats a ambdós estímuls. Independentment, el tabac i la hipòxia no modifiquen la distensibilitat vascular, però al combinar-se tenen efecte sinèrgic.

Estructura vascular pulmonar. La hipòxia crònica té un efecte de similar magnitud que el tabac sobre l'engruiximent de la paret de l'artèria pulmonar. A més, la unió d'ambdós estímuls no resulta en una suma d'efectes, de tal manera que l'increment de l'àrea de la paret vascular és similar en els animals que reben un dels dos estímuls per separat o de forma combinada. Tal com s'ha comentat, el fum de tabac indueix canvis en la proporció del contingut de proteïnes de la matriu extracel·lular. En aquest aspecte la hipòxia actua en el mateix sentit però amb major intensitat, magnificant els efectes sobre la proporció de mucopolisacàrids i col·làgena. Igual que el tabac la hipòxia *per se* no té cap efecte sobre la proporció d'elastina en la paret del vas. Curiosament, l'acció conjunta de tabac i hipòxia mostra un efecte sinèrgic incrementant significativament el contingut d'elastina. De la mateixa manera, la combinació dels dos estímuls també resulta en un increment superior en la proporció de mucopolisacàrids i una disminució més marcada en la proporció de col·làgena.

Així com en l'artèria pulmonar els efectes de tabac i hipòxia actuen en un mateix sentit, en el remodelat dels vasos intrapulmonars les accions d'aquests dos factors són diferents. És evident que ambdós estímuls juguen un paper en les alteracions de l'estructura vascular, no obstant, el tabac té un efecte més present en la muscularització de vasos, mentre que la hipòxia actua amb més intensitat sobre el desenvolupament de dobles làmines elàstiques. Segons Hunter[153] la muscularització d'un vas es defineix per la presència d'una doble làmina elàstica, basant-se en el fet de que la làmina elàstica es desenvolupa a la vegada que ho fa la capa muscular. Per contra, els nostres resultats suggereixen que les característiques de la muscularització depenen del tipus d'estímul. De fet, estudis realitzats en rates sotmeses a hipòxia o tractades amb monocrotalina demostren que la ultraestructura de les cèl·lules musculars llises dels vasos pulmonars és diferent en ambdós casos[43]. Així, les cèl·lules musculars sotmeses a hipòxia presentaven un fenotip més madur amb fins miofilaments, mentre que les cèl·lules musculars sotmeses a monocrotalina mostraven un fenotip immadur amb miofilaments gruixuts. Per tant, és possible que la muscularització de vasos i la formació d'una segona làmina elàstica es

donin en diferent proporció i intensitat en funció de l'estímul inductor. En conjunt, aquests resultats mostren el paper del tabac i la hipòxia en el desenvolupament de la HP per mecanismes diferents i aparentment independents. Desconeixem quins són aquests mecanismes però podrien estar relacionats amb els canvis en el fenotip de la cèl·lula muscular. Finalment, la combinació dels dos estímuls resulta en un increment significatiu en el nombre de vasos totalment muscularitzats.

B) Circulació sistèmica

No es detecten efectes de la hipòxia en la composició i funcionalitat de l'artèria aorta, a excepció del gruix de la paret del vas dels animals exposats a hipòxia que resulta superior al gruix de l'aorta dels cobais exposats a fum de tabac. No considerem aquesta diferència com a un factor rellevant ja que independentment, cap dels dos grups presenta diferències respecte el grup control. A nivell d'hemodinàmica, succeeix el mateix. La pressió arterial sistèmica dels cobais hipòxics està per sobre de la dels cobais exposats a fum de tabac. No tenim una explicació clara d'aquest fenomen, però podria estar relacionat amb l'increment en els nivell d'ET a plasma, predisposant a aquests animals a una major tensió basal.

En conjunt, els resultats d'aquesta tesi corroboren la hipòtesi d'estudi plantejada i identifiquen el fum de tabac com a responsable de la lesió vascular a la MPOC. En segon lloc els resultats aporten la consolidació del model de cobai exposat a fum de tabac al nostre laboratori i la caracterització de les alteracions vasculars i pulmonars que del model se'n deriven. A més es descriuen les bondats i limitacions del model animal, coneixement necessari per al desenvolupament de nous projectes i futurs estudis. També s'estableix un ordre en la seqüència d'alteracions que tenen lloc des del inici fins als 6 mesos de l'exposició a fum de tabac. A més, es descriuen les diferències i similituds entre els efectes de la hipòxia i l'exposició a fum de tabac en la circulació pulmonar, fet que ajuda a entendre millor la patologia de la HP en la MPOC. Demostrem que el tabac i la hipòxia tenen efectes sinèrgics sobre la hemodinàmica pulmonar i que aquest sinergisme és degut als efectes individuals del tabac i la hipòxia sobre la muscularització i el dipòsit d'elastina

respectivament en els vasos intrapulmonars. A més, la hipereactivitat de les artèries a NE induïda per la hipòxia, la pèrdua de distensibilitat i l'alteració en el component proteic extracel·lular afavoreixen el increment en el to vascular. Per tant, l'aparició de la hipòxia marca un punt de inflexió en l'evolució de la MPOC agreujant l'hemodinàmica pulmonar.

CONCLUSIONS

A partir dels resultats obtinguts en els estudis anteriors s'extreuen les següents conclusions:

1.- El cobai exposat a fum de tabac es valida com un bon model experimental de MPOC ja que, a més d'induir metaplàsia de cèl·lules caliciformes i emfisema, reprodueix les alteracions vasculars pulmonars característiques d'aquesta malaltia.

2.- Els canvis en l'estructura de l'artèria pulmonar principal i dels vasos intrapulmonars ocorren en paral·lel i són anteriors a l'aparició d'emfisema.

3.- L'exposició a fum de tabac indueix canvis estructurals en l'artèria pulmonar principal com l'engruiximent de la paret del vas i canvis en la composició de proteïnes de la matriu extracel·lular.

4.- En els vasos intrapulmonars el fum de tabac indueix l'aparició de cèl·lules musculars llises amb muscularització completa de petits vasos als 6 mesos.

5.- El fum de tabac indueix disfunció endotelial de forma selectiva a les artèries pulmonars que es manifesta després de 3 mesos d'exposició.

6.- L'expressió d'eNOS disminueix degut a l'exposició a fum de tabac. L'activitat de la proteïna segueix el mateix perfil d'expressió.

7.- El fum de tabac i la hipòxia produeixen efectes similars sobre l'hemodinàmica pulmonar i tenen efectes sinèrgics quan es combinen ambdós estímuls.

8.- El fum de tabac i la hipòxia indueixen la presència de remodelat vascular. L'exposició a fum de tabac s'associa a muscularització de vasos intrapulmonars mentre que la hipòxia promou la formació d'una doble làmina elàstica.

9.- La hipòxia amplifica els efectes inicials del fum de tabac per la qual causa representa un punt d'inflexió en l'evolució de les alteracions vasculars pulmonars associades a la MPOC.

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ABREVIACIONS

AA	Arachinoid Acid
AC	Adenylate Cyclase
ADP	Adenosine triphosphate
AMPc	Cyclic Adenosine Monophosphate
ATP	Adenosine Triphosphate
AKT1	Activin Receptor-like Kinase type 1
BAL	Bronchoalveolar lavage
BMPR2	Bone Morphometric Protein Receptor type 2
COHb	Carboxyhemoglobin
COX	Cyclooxygenase
ECE	Endothelin Converting Enzyme
ET-1	Endothelin-1
ET_A	Endotelin Receptor type A
ET_B	Endotelin Receptor type B
FEV_1	Forced Expiratory Volume at the first second
GC	Guanylate Cyclase
GMPc	Cyclic Guanosine Monophosphate
GTP	Guanosine Triphosphate
HNE	Human Pancreatic Elastase
HAP	Hipertensió Arterial Pulmonar
HP	Hipertensió Pulmonar
ICAM-1	Intercellular Adhesion Molecule-1
IFN-γ	Interferon gamma
IL-6	Interleukin-6
HIV	Human Immunodeficiency Virus
LDL	Low-Density Lipoprotein
Lm	Mean Linear Intercept
L-NAME	N^G-monomethyl-L-arginine

LPS	Lipopolysaccharide
MPOC	Malaltia Pulmonar Obstructiva Crònica
MMP	Matrix Metalloproteinases
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Nor epinephrine
NF-KB	Nuclear Factor-Kappa B
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
eNOS	endothelial Nitric Oxide Synthase
iNOS	inducible Nitric Oxide Synthase
nNOS	neuronal Nitric Oxide Synthase
OMS	Organització Mundial de la Salut
PAP	Pulmonary Artery Pressure
PAPm	Mean Pulmonary Artery Pressure
PCNA	Proliferating Cell Nuclear Antigen
PDE	Phosphodiesterase
PE	l-phenylephrine
PeNOS	Phosphorilated endothelial Nitric Oxide Synthase
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin 2
PPE	Porcine Pancreatic Elastase
PS	Prostacyclin Synthase
ROS	Reactive Oxygen Species
TNFα	Tumor Necrosis Factor alpha
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VPH	Vasoconstricció Pulmonar Hipòxic

AGRAÏMENTS

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