Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2008;22:525-530

Accepted: November 03, 2008

Nitric Oxide and the Release of Lipoprotein Lipase from White Adipose Tissue

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Key Words

Immobilization • Acute stress • Nitric oxide synthase • WAT perfusion • NO donor

Abstract

Background/Aim: Lipoprotein lipase (LPL) is the main enzyme responsible for the distribution of circulating triacylglycerides in tissues. Its regulation *via* release from active sites in the vascular endothelium is poorly understood. In a previous study we reported that in response to acute immobilization (IMMO), LPL activity rapidly increases in plasma and decreases in white adipose tissue (WAT) in rats. In other stress situations IMMO triggers a generalized increase in nitric oxide (NO) production. Methods/Results: Here we demonstrate that in rats: 1) *in vivo* acute IMMO rapidly increases NO concentrations in plasma 2) during acute IMMO the WAT probably produces NO via the endothelial isoform of nitric oxide synthase (eNOS) from vessels, and 3) epididymal WAT perfused *in situ* with an NO donor rapidly releases LPL from the endothelium. Conclusion: We propose the following chain of events: stress stimulus / rapid increase of NO production in WAT (by eNOS) / release of LPL from the endothelium in WAT vessels. This chain of events could be a new mechanism that promotes the rapid decrease of WAT LPL activity in response to a physiological stimulus.

Introduction

Lipoprotein lipase (LPL, EC 3.1.1.34) is a homodimeric glycoprotein enzyme that hydrolyzes triacylglycerols (TAG) from plasma lipoproteins, thereby releasing non-esterified fatty acids for tissue uptake and metabolization. LPL is synthesized by parenchymal cells, from which it is secreted and then transported, by a poorly understood process, to the lumen surface of endothelial cells. The physiologically-active form of LPL is localized on the surface of the vascular endothelium of the tissues in which it is synthesized, where it is anchored by a membrane-bound glycosaminoglycan chain (heparan and dermatan sulphate) [1]. After a short half-life (about one hour) [2, 3], the enzyme is released from its anchorage in a monomeric, inactive form and travels through the blood to the liver, where it is captured [4] and degraded.

LPL regulation is limited and determined by its extracellular localization. It is regulated in the medium-

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term depending on the rate of its synthesis [5, 6]. In addition, LPL activity can also be regulated by its release from the endothelium, which transiently increases plasma enzyme activity. Although little is known about this, increased LPL activity levels in plasma have been described in several physiological conditions including fasting [7] and some types of exercise [8]. Moreover, the repeated injection of epinephrine and the β-adrenergic agonist isoproterenol in rats causes a decrease in LPL activity in WAT but an increase in plasma [9]. Similarly, in previous research we showed that after acute immobilization (IMMO) in rats, LPL activity decreases in retroperitoneal WAT depots and rises in plasma [10]. In recent studies these changes were observed within only 5 minutes of IMMO [11].

In rats, IMMO stress triggers a generalized increase in nitric oxide (NO) production [12], and induces iNOS expression and NO overproduction in the brain cortex [13]. NO is synthesized by a family of three isoenzymes: the constitutive neuronal (nNOS or NOS I), the endothelial (eNOS or NOS III), and the inducible (iNOS or NOS II) nitric oxide synthases (NOS). These isotypes have distinct tissue localization, eNOS being the main isoenzyme in endothelial cells, where it is involved in vasodilatation. NO is the main local modulator of blood flow [14]. Furthermore, NO, through its derivatives, called reactive nitrogen species, reacts with the thiol group of cysteine and the phenol ring of tyrosine (tyrosine nitration) of proteins, thereby modifying their biological activities [15-18].

Several authors have proposed a link between NO production and LPL down-regulation. In brown adipocytes, TNFα induces NO production by iNOS, whereas LPL activity decreases [19]. Lipopolysaccharide administration in rats *in vivo* induces iNOS expression in muscle and WAT, and increases nitrate/nitrite levels in plasma while decreasing LPL activity in both tissues [20].

To examine the possible role of NO in the short-term regulation of WAT LPL activity, here we used a) an *in vivo* acute stress model by IMMO and b) *in situ* perfusion of eWAT with an NO donor.

Materials and Methods

Stress by IMMO

A) Animals. Male Wistar rats (Harlan Interfauna Ibèrica, Barcelona, Spain) weighing 190-240 g were housed in a controlled noise-free environment (lights on from 8 am to 8 pm, temperature 23 ± 2°C, humidity 45-55%). The rats had access to a standard laboratory diet (Panlab, Barcelona, Spain) and water *ad libitum*. All procedures involving animals were approved by the Committee on Animal Bioethics and Care at the University of Barcelona and the Government of Catalonia (Spain).

B) Immobilization stress (IMMO). The body immobilization procedure was based on that described by Kvetnansky and Mikulaj [21] and used elsewhere [11, 22]. Rats were attached to wooden boards in prone position by taping their forelimbs and hind limbs to metal mounts. To restrict head movement, the head was placed inside a plastic tube and fixed over the neck area.

C) Experimental groups. The experimental groups used were as described in a previous study [11]. Briefly, rats were randomly assigned to one of four groups: control or non-stress (0 minutes of IMMO, time 0) and 5, 15, or 25 min of IMMO (times 5, 15 and 25). Experiments of stress were always done between 2:00 and 4:00 PM [10, 11, 22] to minimize the effects of circadian rhythms and the diet on the parameters studied. Rats were subjected to IMMO for 0, 5, 15 and 25 minutes and decapitated immediately thereafter. Blood was collected in EDTA and white adipose tissues (epididymal and retroperitoneal, eWAT and rWAT respectively) were removed and immediately frozen in liquid nitrogen. Plasmas and WAT were used for LPL activity quantification (published before, see reference 11). Plasma was obtained in EDTA from blood to measure nitrates to avoid interferences that other anticoagulants might cause with plasma nitrate determination [23]. In another set of animals, decapitated after 30 minutes of IMMO, epididymal and retroperitoneal WAT (eWAT and rWAT) were removed and used for histological NO detection and Western blot analysis of NOS synthases (eNOS and iNOS).

D) Plasma nitrate determination. We used the Griess assay with modifications adapted to plasma samples [23]. Briefly, samples were incubated with nitrate reductase (Cayman-Chemical, Ann Arbor, MI, USA) to reduce nitrate to nitrite. The Griess reagent (sulfanilamide 2% (w/v) and N-(1-naphthyl) ethylenediamine 0.2% (w/v) (1:1) (v/v)) was then added and absorbance was measured at 540 nm (Titertek Multiskan PLUS MKII).

E) eNOS and iNOS detection by Western blot in WAT. Tissues were homogenized (200 mg/mL) in a 50 mM Tris buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA, 1% (v/v) Triton X-100, 1 mM β-mercaptoethanol and 2 mM Pefabloc RSC and Complet Mini as protease inhibitors (both from Roche Diagnostics). Homogenates (170 µg of protein) and positive control were loaded onto an 8% (w/v) polyacrylamide gel and transferred to a PVDF membrane, followed by blocking in Tris-buffered saline (TBS) containing 5% (w/v) non-fat dry milk (blocking buffer). For iNOS detection, incubation with a rabbit anti-rat iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:500 (v/v) was carried out in blocking buffer overnight at 4°C. The same buffer was used in a Western blot for eNOS detection with a mouse anti-human eNOS monoclonal antibody (Transduction Laboratories, Lexington, KY, USA) 1:10,000 (v/v). The extent of the expression of eNOS activated (via the residue Ser1177) in WAT by specific antibodies was not measured due to the large amount of protein that is needed to detect it by Western blot.

A goat horseradish peroxidase-labeled anti-mouse IgG (Chemicon, Temecula, CA, USA) and a swine horseradish peroxidase-labeled anti-rabbit IgG (Dako, Glostrup, Denmark) were used 1:10,000 (v/v) for eNOS and iNOS immunoblotting detection respectively, using the SuperSignal West Pico Chemiluminescent Substrate system (PIERCE, Rockford, IL. USA).

F) NO production in histological sections from eWAT. Fresh tissue fragments were fixed for 2 h at 4ºC in 0.1 M PBS, pH 7.4, containing 4 % (w/v) paraformaldehyde. Tissue sections (18 µm-thick) obtained by cryostat were incubated with a mixture of L-arginine 100 µM and 10 µM 4,5-diaminofluorescein diacetate (DAF-2 DA, Calbiochem, Darmstadt, Germany) diluted in PBS for 1.5 h at 37°C as previously described [24]. Incubations were performed in the absence or presence of EDTA (a chelator of Ca^{2+}) to test the active eNOS (specific calcium required). Tissue sections maintained the vessel structure but not the adipocyte integrity. NO production by eNOS of vessels in the tissue sections was examined under a fluorescence microscope (Nikon Eclipse E600), using λ excitation at 495 nm and λ emission at 515 nm. Images were obtained using Ultraview Imaging Suite software (Perkin Elmer Life Sciences). Data were obtained only in eWAT because this methodology could not be applied to rWAT due to the low number of vessels and their small diameter.

Perfusion of eWAT

The technique used is described in our previous study [25]. Briefly, the method entails perfusion of the right epididymal fat pad, from the aorta and spermatic artery to the spermatic vein and cava. LPL activity measured in the perfused right fat pad is similar to that measured in the non-perfused left fat pad, and the perfused tissue is viable and metabolically active [25].

eWAT was perfused for 10 minutes with a medium (Minimum Essential Medium (MEM, Gibco, Carlsbad, CA, USA) containing 3% (w/v) BSA, 20 mM citrate and 1 nM insulin). After that, spermine-NONOate (Calbiochem, Darmstadt, Germany) or spermine (Sigma, St. Louis, MO, USA), the NO-carrying molecule (control group), was added to the medium (100 µM) at time 0. Spermine-NONOate is an NO donor selected on the basis of its stability in the same buffer used for perfusion [26]. The perfusate was collected at 5 minute intervals (until 30 minutes) in Eppendorf tubes kept in iced water, and immediately stored at -80ºC. In other sets of animals, perfusion was stopped at 5 and 30 minutes after the addition of spermine or spermine NONOate and the tissue was collected and frozen in liquid nitrogen.

LPL activity assay

Tissues were homogenized (≈ 200 mg) in 1 mL of buffer (10 mM HEPES, 1 mM EDTA, 1 mM DTT, 5 U/mL heparin, pH 7.5). LPL activity was determined in homogenates, plasmas and perfusates as previously described [9, 27]. Production of 1µmoL oleate per minute (from tri[9,10(*n*)-^{3}H]oleate) is equivalent to 1 U LPL.

Statistic analysis

Results are given as the means ± SEM. Data obtained in experiment of IMMO were statistically analyzed by one-way ANOVA (time of stress), and data obtained in experiment of perfusion, by two-way ANOVA (time and treatment, repeated-measures). A post hoc Tukey test was used. Differences were considered significant when $P < 0.05$.

Results and Discussion

Stress by IMMO

Acute stress by IMMO significantly increased (four-fold) nitrate levels (an index of NO production, [28]) at 15 minutes (Figure 1). After 25 minutes, levels had decreased to near baseline. This increase in NO production in response to IMMO is consistent with previous reports using the same stress model in rats [12]. Moreover in response to acute IMMO we found that LPL activity rose in plasma and decreased in rWAT, all after 5 minutes of IMMO [Table 1, summary of results obtained in the same animals and previously published, see reference 11]. The fact that NO_3^- increased in plasma later (at 15 minutes) than LPL (at 5 minutes) does not contradict our hypothesis that NO causes LPL release because the rate of basal production of NO *in vivo* [29] could explain the delay in nitrate accumulation in plasma.

Given that NO is a major local flow regulator [14] and we found that blood flow increases specifically in WAT after acute IMMO stress [10], NO synthesis by NOS in WAT could be partly responsible for the *in vivo* NO production observed in this study, although other tissues could be involved in its production. The NOS activity that caused this production must correspond to eNOS because: 1) in non-stressed rats it was the main isoenzyme in WAT (Figure 2); 2) although lipopolysaccharide administration in rats induces iNOS expression in WAT [18, 30] and chronic IMMO induces iNOS in brain cortex [13], iNOS was not expressed in WAT after 30 minutes of acute IMMO (Figure 2); and 3) NOS activity detected using a histochemical procedure (DAF) was blocked by EDTA, a chelator of calcium required for eNOS but not for iNOS activity ([31], Figure 3), or by the NOS inhibitor L-NAME, (D. Ricart, unpublished data). Furthermore: shear stress (enhanced in WAT by IMMO because of increased blood flow, [10]) stimulates eNOS activity, thereby increasing NO production [31-34].

Perfusion of eWAT

The effect of an NO donor on LPL bound to the capillary endothelium was studied in eWAT perfused *in situ* (Figure 4).

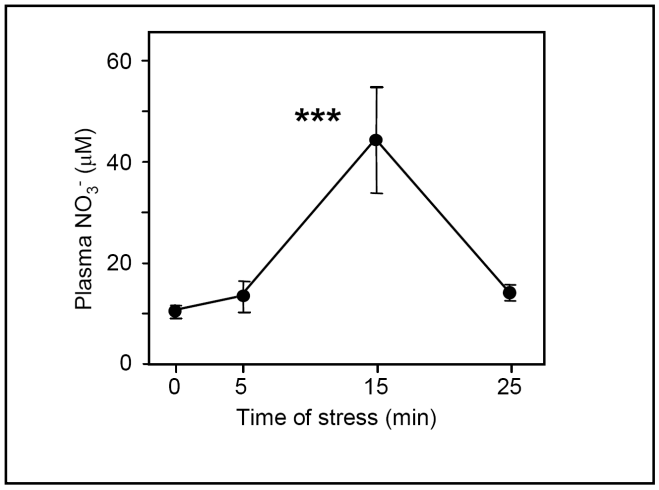

Fig. 1. The effect of acute stress by IMMO on nitrate concentration in plasma. Data are means ± SEM of 7 rats each time. Statistical comparisons by one-way ANOVA (time of IMMO) followed by Tukey tests. *** = $P < 0.001$ compared with control or basal situation (0 minutes).

Time of IMMO(min)	0	5	15	25
Plasma (mU/mL)	0.7±0.2	1.9±0.1*	1.6±0.3	1.8±0.4*
Whole tissues				
eWAT (mU)	666±111	470±75	640±63	491±51
rWAT (mU)	567±48	332±46*	324±16*	410±34

Table 1. LPL activity in plasma, epididymal (eWAT) and retroperitoneal (rWAT) white adipose tissues during acute stress by IMMO (summary of results published in Casanovas et al.[11]). Data are means ± SEM of 6 rats each time. Statistical comparisons by one-way ANOVA (time of IMMO) followed by Tukey tests. * = $P < 0.05$ compared with control or basal situation (0 minutes).

Since NONOate cross-reacts with the Griess reagent (N. Jané, unpuplished data), we were unable to measure the nitrite concentration in the perfusates. We thus chose a spermine NONOate dose of 100μM that, when prepared in a culture buffer, produces NO at a rate of 2-4 μM/minute on the basis of data taken from the literature [26]. Moreover, 100 μM lies within the low range of the concentrations used for assays *in vitro* [35].

The addition of spermine-NONOate (NO donor) resulted in a rapid (5 minutes) and significant increase in LPL activity in the perfusate (Figure 4, panel A), thereby demonstrating the release of LPL from its endothelial location. In accordance with this result, tissue LPL activity tended to decrease (although non-significantly) 5 minutes after the addition of this NO donor (Figure 4, panel B)

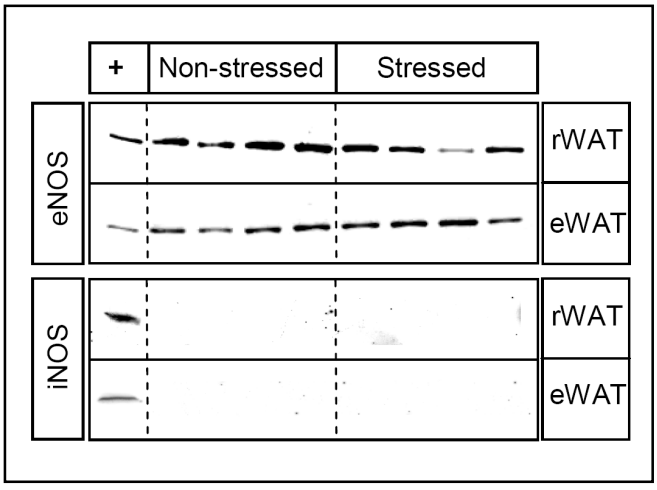

Fig. 2. eNOS and iNOS expression in WATs. Representative Western blots of eNOS (140kDa) and iNOS (130kDa) in epididymal (eWAT) and retroperitoneal (rWAT) white adipose tissue (170 μg of protein /well) from non-stressed (control, n= 4) and stressed (30 minutes of IMMO, n=4) rats. Each lane corresponds to a different animal. Positive controls (+) were performed at each gel and NOS type. Homogenates and positive controls were loaded in the same gel.

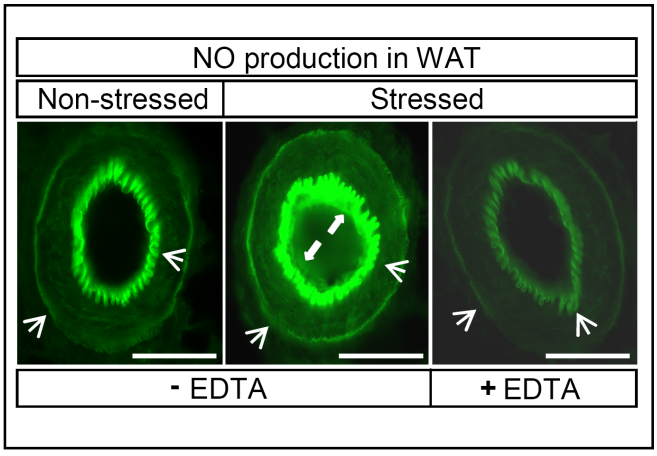

Fig. 3. Vascular NO production in histological sections from eWAT. Endothelial NO production in representative 18 μM-thick slices of rat eWAT (30 minutes stressed by IMMO and non-stressed). Tissue sections were incubated with a mixture of L-arginine and DAF-2 DA for NO detection, in the absence (-) or presence (+) of EDTA (a chelator of Ca^{2+}). eNOS activity was abolished with EDTA incubation. The DAF-NO complex was observed in a fluorescence microscope (λ: excitation-emission of 495-515 nm). Thick arrows indicate diffuse fluorescence corresponding to NO production. The spiral ring and the external ring (thin arrows) are caused by autofluorescence of the sample, and correspond to internal and external elastic layers of the vessels respectively. Bar = 100 μm.

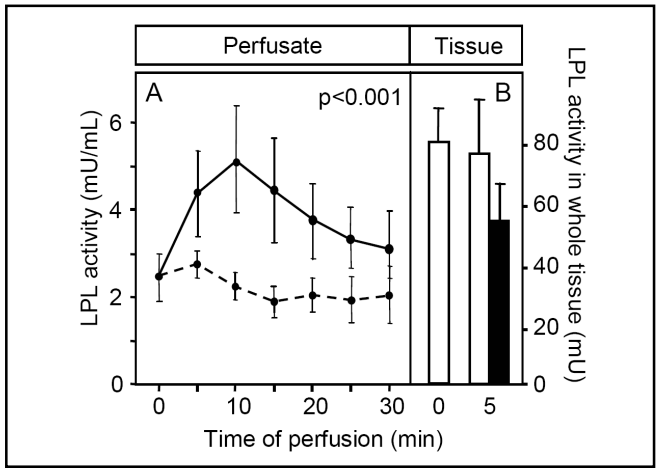

Fig. 4. The effect of NO donor on LPL activity in an *in situ* perfused eWAT. Rat eWAT was perfused *in situ* with 100 μM spermine-NONOate (NO donor at physiological concentrations, continuous line-panel A and black bar-panel B) or with 100 μM spermine (the carrier molecule, broken line-panel A and white bar-panel B). In panel A, LPL activity was measured in the perfusate (mU/mL) at several time points during perfusion. In panel B the experimental procedure was interrupted at 5 minutes when perfusion began and total LPL activity (mU) was measured in perfused tissue. Data are mean ± SEM of 4-5 animals per group. In panel A (perfusate), effect of treatment = $P < 0.001$, by ANOVA (repeated-measures). In panel B (tissue) no significant differences were found between groups, by Student *t*-test.

remaining to similar levels 30 minutes after (N. Jané, unpublished data). These effects must be specific to the NO produced because they were not observed after the addition of the NO-carrying molecule, spermine. The dose of the NO donor used in the perfusion does not interfere with the assay of LPL activity in WAT homogenates (N. Jané, unpublished data). Furthermore, the activity found in the perfusate after NO donor perfusion was lower than that found after heparin perfusion [25], probably because heparin has a greater capacity to displace LPL from its anchorage and it also stabilizes the dimer.

Physiological implications

During physiological stress, such as IMMO, catecholamines increase blood flow in WAT [10] and nitrates in plasma (Figure 1). Circulating catecholamines induce vasodilatation in WAT [36], perhaps increasing NO production by stimulating one of the protein kinases that activates eNOS [33]. Mechanical forces related to blood flow may strengthen and maintain NO production i.e., by continuous shear stress [31, 33, 34]. In this context, WAT switches from being an importer to an exporter of fatty acids. This switch results in increased blood flow, which may enable this tissue to rapidly export free fatty acids for energy requirements in other tissues (i.e. heart and muscle) [10, 11]. LPL activity then decreases rapidly in WAT (Table 1, [11]) and reduces the uptake of circulating lipids [10, 22]. The speed of this reduction is inexplicable by the known mechanisms of LPL regulation. Thus, the LPL release from the endothelium to the bloodstream was proposed as a fast mechanism for the down-regulation of tissue LPL activity [11].

Although several studies in adipose tissue suggest the mediation of NO production and NOS activity in LPL regulation [19, 20], our results using an *in situ* perfusion WAT model provide the first evidence of a cause-effect relationship between NO production and LPL release. Given the luminal co-localization of functional LPL and eNOS in the endothelium, the local NO and/or some NO derivatives could immediately act on LPL, thereby favoring its release into plasma and decreasing its activity in WAT. However, further studies are required to gain insight into LPL and NO interaction.

The proposed effect of NO releasing functional LPL from adipose tissue could be included in a more general role of NO modulation of energy metabolism in physiological and pathological conditions [30].

Abbreviations

LPL (lipoprotein lipase); NO (nitric oxide); NOS (nitric oxide synthase); eNOS (endothelial NOS (NOS III)); iNOS (inducible NOS (NOS II)); WAT (white adipose tissue); eWAT (epididymal WAT); rWAT (retroperitoneal WAT).

Acknowledgements

The authors thank the Language Advisory Service of the Universitat de Barcelona for revising the manuscript, and Rafael Castaño and José Terencio for their technical advice. This study was supported by grants SAF2005-02391 and BFU2007-6527/BMC from the Dirección General de Investigación Científica y Técnica, and the Spanish Ministry of Education and Culture. David Ricart-Jané received a fellowship from the Spanish Ministry of Education and Culture and Albert Casanovas received a fellowship from the Ministry of Universities, Research and Information Society of the Generalitat de Catalunya and European Social Funds.

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