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Cooperation of Adenosine with Macrophage Toll-4 Receptor Agonists Leads to Increased Glycolytic Flux through the Enhanced Expression of *PFKFB3* Gene*[S]

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Macrophages activated through Toll receptor triggering increase the expression of the A_{2A} and A_{2B} adenosine receptors. In this study, we show that adenosine receptor activation enhances LPS-induced *pfkfb3* expression, resulting in an increase of the key glycolytic allosteric regulator fructose 2,6-bisphosphate and the glycolytic flux. Using shRNA and differential expression of A_{2A} and A_{2B} receptors, we demonstrate that the A_{2A} receptor mediates, in part, the induction of *pfkfb3* by LPS, whereas the A_{2B} receptor, with lower adenosine affinity, cooperates when high adenosine levels are present. *pfkfb3* promoter sequence deletion analysis, site-directed mutagenesis, and inhibition by shRNAs demonstrated that HIF1α is a key transcription factor driving *pfkfb3* expression following macrophage activation by LPS, whereas synergic induction of *pfkfb3* expression observed with the A_2 receptor agonists seems to depend on Sp1 activity. Furthermore, levels of phospho-AMP kinase also increase, arguing for increased PFKFB3 activity by phosphorylation in long term LPS-activated macrophages. Taken together, our results show that, in macrophages, endogenously generated adenosine cooperates with bacterial components to increase PFKFB3 isozyme activity, resulting in greater fructose 2,6-bisphosphate accumulation. This process enhances the glycolytic flux and favors ATP generation helping to develop and maintain the long term defensive and reparative functions of the macrophages.

Macrophages are key cellular components of the innate immune system. They are present in all body tissues, where they normally assist in guarding against invading pathogens, and regulate normal cell turnover and tissue remodeling. Macrophages discriminate between pathogens and self through signals triggered by Toll-like receptors, which recognize different pathogen components, such as LPS, lipoproteins, or dsRNA, among others (1). Activation of most Toll receptors triggers a complex signaling pathway involving the transcription factor NFκB (2), which, upon activation, induces the expression of multiple genes implicated in the inflammatory response, including cytokines, effector enzymes such as iNOS and COX-2, and adhesion molecules that enable macrophages to mount an immediate attack against pathogens (1).

The activation of macrophages is dependent on various energy-requiring pathways, mainly glycolysis (3). To reach the inflammation sites, macrophages recruited from the blood need to move against oxygen gradients (4). Macrophages and other cells of the innate immune system need to adapt to this special condition to maintain viability and activity. Therefore, a high glycolytic flux is essential for macrophage activity in these conditions (5, 6).

Unlike almost all other cells, macrophages and myeloid cells do not typically shift to mitochondrial respiration, even in highly oxygenated environments. Indeed, glycolytic inhibitors have been shown to greatly reduce both the cellular ATP concentration and the functional activity of macrophages, whereas inhibitors of mitochondrial respiration have shown no effect on the inflammatory response (5, 7, 8). Macrophages activated by LPS increase the levels of lactate production (5), a marker of enhanced glycolytic function. Glycolytic flux is mainly controlled by 6-phosphofructo-1-kinase (9), with fructose 2,6-bisphosphate (Fru-2,6-P_2)[2] being its most powerful allosteric activator. This property confers to this metabolite a key role in the control of the glycolytic pathway (10). 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2) is the bifunctional enzyme that catalyzes the synthesis and degradation of Fru-2,6-P_2 and, hence, critically regulates the glycolytic rate under multiple pathophysiological conditions (10). Different PFK-2 isozymes generated from four genes (*pfkfb1–4*) have been identified in mammalian tissues (11). Proliferative and transformed cells and macrophages express the PFKFB3 isozyme, which is a product of the *pfkfb3* gene (12–16). This isoform presents the highest kinase-bisphosphatase activity ratio, thus generating

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[2] The abbreviations used are: Fru-2,6-P_2, fructose 2,6-bisphosphate; C/EBPβ, CCAAT/enhancer-binding protein β; NECA, 5′-(*N*-ethylcarboxamido)adenosine; A_2R, A_2 receptor; HRE, hypoxia-response element; CREB, cAMP-response element-binding protein.

the highest Fru-2,6-P_2 levels. PFKFB3 isozyme expression is induced by proinflammatory stimuli, hypoxia, and growth factors in different cells (12, 17), and the protein is degraded through the ubiquitin-proteasome proteolytic pathway (18). *pfkfb3* mRNA contains multiple copies of the AUUUA instability motif in its 3′-nontranslated region (14). This sequence motif confers both enhanced translation and instability to the mRNA molecule, and thus it plays an important role in regulating the half-life of the gene product in different physiological conditions. Analysis of the 5′ *pfkfb3* promoter sequence has revealed the presence of putative consensus binding sites for different transcription factors, which could play important roles in the regulation of *pfkfb3* gene expression. Binding of the transcription factor HIF1 seems critical for the hypoxia-dependent induction of this gene (17).

Although macrophage function is essential for the successful destruction of pathogens, failure to control macrophage activation or prolonged or inappropriate inflammatory processes will lead to unacceptable levels of collateral damage to surrounding cells. Multiple mechanisms controlling the extension of macrophage activation have been described (19). In the last years, adenosine has been shown to modulate the inflammatory response by limiting macrophage activation (20, 21). Utilization of ATP during periods of high metabolic activity leads to an increased concentration of intracellular adenosine that can be secreted through nucleoside transporters. Another major pathway contributing to high extracellular adenosine concentration during metabolic stress is the release from the cells of its precursor adenine nucleotides (ATP, ADP, and AMP), followed by extracellular degradation to adenosine. Adenosine accumulation is limited by its catabolic degradation to inosine and uric acid (20). Neutrophils and endothelial cells release large amounts of adenosine at sites of inflammation and infection. Activated macrophages can also serve as a major source of extracellular adenosine via ATP production (22). Adenosine acts at the cell surface through four G protein-coupled adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3). Lower concentrations of adenosine activate the high affinity A_1 and A_{2A} receptors, whereas a high adenosine concentration also stimulates the low affinity A_{2B} and A_3 receptors (20). Adenosine receptors expressed on monocytes and macrophages allow these cells to detect stressful conditions and modulate their cellular functions to adapt to their microenvironment. Activation of A_{2A} receptors in macrophages has been related to the anti-inflammatory effects of adenosine, such as the down-regulation of TNFα production (20, 23), and to a macrophage-regenerative phenotype, as suggested by the production of VEGF (24, 25). The use of A_{2A} receptor-deficient mice as a model of acute inflammation has demonstrated clearly the role of these receptors in immunosuppression (26–28).

In this study, we show that Toll receptor agonists and adenosine, through its A_{2A} and A_{2B} receptors, cooperate to increase glycolytic flux in macrophages by favoring the expression of the PFKFB3 isozyme. We have found that the Toll-4 receptor agonist LPS increases the expression of A_{2A} and A_{2B} receptors in macrophages, augmenting its sensibility to adenosine. We also show here that although LPS-dependent induction of *HIF1α* is essential for *pfkfb3* expression, the synergic induction of *pfkfb3*

expression observed with A_2R agonists critically depends on the transcription factor Sp1.

EXPERIMENTAL PROCEDURES

Chemicals—Adenosine, A_{2A} receptor agonist 2-p-(2-carboxyethyl) phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS-21680), A_2 receptor agonist 5′-(N-ethylcarboxamido)adenosine (NECA), A_{2B} receptor antagonist 8-[4-[4-cyanophenyl)carbamoylmethyl)oxy]phenyl]-1,3-di(n-propyl)xanthine (MRS 1754), deferoxamine, and $CoCl_2$ were purchased from Sigma. A_{2A} receptor antagonist 4-(2-triazolo(2,3-α)triazin-ylamino)ethyl)phenol (ZM 241385) was purchased from Tocris Cookson. Toll receptors agonists, LPS from *Salmonella typhimurium,* poly(I:C), LTA-SA, and CpG were from Sigma. Serum and culture medium were acquired from BioWhittaker (Walkersville, MD). Electrophoresis equipment and reagents were purchased from Bio-Rad.

Cell Culture—Elicited peritoneal macrophages were obtained from male mice 4 days after intraperitoneal inoculation of 1 ml of sterile 10% (w/v) thioglycollate. Cells were seeded at a density of $1 \times 10^5/cm^2$ in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 50 μg/ml each gentamicin, penicillin, and streptomycin. Raw 264.7 cells were subcultured at a density of $6 - 8 \times 10^4/cm^2$ in RPMI 1640 medium supplemented with 2 mM glutamine, 10% FCS, and the aforementioned antibiotics. After 2 days in culture, the medium was replaced by RPMI 1640 containing 5% FCS, and the cells were used in the following 24 h. Bone marrow-derived macrophages were obtained by flushing the femurs from IRF-3$^{+/+}$ or IRF-3$^{-/-}$ mice with DMEM containing 10% inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (all from Sigma) and subsequent culture in the same medium supplemented with 15% of L929 supernatant as a source of macrophage colony-stimulating factor.

Stable cell lines expressing dominant active forms of IκBα (Raw-IκB-DA) have been described elsewhere (29). Human monocytes were isolated from the blood of healthy donors by centrifugation on Ficoll-Paque^{TM} PLUS (Amersham Biosciences) following previously reported protocols (30). Hypoxia simulation conditions were achieved by growing cells in RPMI 1640 medium supplemented with 200 μM deferoxamine or 200 μM $CoCl_2$.

Metabolite Determinations—Fru-2,6-P_2 was determined as described previously (31). The concentration of lactate was measured enzymatically in neutralized $HClO_4$ extracts, as described previously (32). Biochemicals used for these measurements were from Roche Applied Science. ATP levels were determined by following the Sigma ATP bioluminescent somatic cells assay kit.

Generation of Plasmids and Mutant Constructs—Plasmids pCMV-SPORT6-$A_{2A}R$ and pCMV-SPORT6-$A_{2B}R$ were purchased from ATCC. shRNA plasmid for mouse $A_{2A}R$ and $A_{2B}R$ and shRNA plasmid for mouse HIF1α and C/EBPβ and -δ were purchased from SABiosciences; Sp1 siRNAs were from Ambion. p-GL2-basic luciferase expression vectors containing various lengths of 5′-flanking regions from the human *pfkfb3* gene promoter and an HIF1-luciferase reporter gene have been described previously (17, 33).

Mutated *pfkfb3* gene promoter constructs were prepared by gene synthesis following instructions included in the QuikChange (Stratagene) site-directed mutagenesis kit. Forward oligonucleotides used in binding site mutations (represented in boldface type) were as follows: SP1 (-110 CCC ACG TGG AAG GG**T AT**T GGG ACC **C**AA GGA ATG CG-75 and -83 ATG CGG CCC G**TA T**CG AGG CTG ACG TAG CGT C-48); HIF (-121 CCA CCC TCC TCC CC**T G**GT GGA AGG GGG CTG G-90); cEBP (-872 CCC TCG ACT TAG G**CT G**TG AAT TGC AGA TCG AAA TGG G-835). Mutated sites were confirmed by DNA sequencing. All plasmid DNA was purified using EndoFree columns (Qiagen).

Cell Transfections—Subconfluent Raw 264.7 cells were seeded in 12-well plates and transfected on the following day with 600 ng of plasmid DNA by using the FuGENE 6 reagent (Roche Applied Science), according to the manufacturer's recommendations. A β-galactosidase expression vector was used as an internal control for transfection efficiency. Luciferase and β-galactosidase activities were measured by using a commercial kit (Promega). To evaluate shRNA efficiency, Raw cells were cotransfected with GFP, and GFP$^+$ cells were analyzed by Western blot.

RNA Extraction and Analysis of Gene Expression by Quantitative RT-PCR—Total RNA was extracted (from 2 to 4×10^6 cells) by following the Qiagen RNA purification method. For reverse transcription (RT)-PCR, cDNA was generated from 1 μg of total RNA by using the RevertAid^{TM} HMinus First Strand (Fermentas) cDNA synthesis kit. Quantitative RT-PCR was performed following the SYBR Green protocol from Applied Biosystems. Amplification of the housekeeping gene GADPH was used as a quality and loading control. The forward and reverse primers, respectively, used for quantitative PCR amplification were as follows: GADPH, 5′-AAC TTT GGC ATT GTG GAA GG-3′ and 5′-GGA TGC AGG GAT GAT GTT CT-3′; A$_1$R, 5′-TTC TGC GTC CTG GTA TGT GAC CAA-3′ and 5′-GCA GAG CCC AGC TTT GGA AAT TGA-3′; A$_2$AR, 5′-ACA CGC CTC AGC AAG ACA CAA T-3′ and 5′-AAA GCC AGC GTT GTG CAT CT-3′; A$_2$BR, 5′-ATT CGC GTC CCG CTC AGG TAT AAA-3′ and 5′-AAT CCA ATG CCA AAG GCA GGG ACC-3′; A$_3$R, 5′-ACG CGA ACT CCA TGA TGA ACC CTA-3′ and 5′-ACT GAT ACC ACA TGC GAA GGC AGA-3′; HIF1α, 5′-CTA CTG CAG GGT GAA GAA TTA CTC AGA GC-3′ and 5′-GTG CAA TTG TGG CTA CCA TGT ACT GCT G-3′; PFKFB3, 5′-TCT AGA GGA GGT GAG ATC AG-3′ and 5′-CCT GCC ACT CTT ATC TTC TG-3′; iNOS, 5′-GCA AGT CCA AGT CTT GCT TGG-3′ and 5′-GGT TGA TGA ACT CAA TGG CAT CAT G-3′; TNFα, 5′-GAC GTG GAA CTG GCA GAA GAG-3′ and 5′-AAG CAG GAA TGA GAA GAG GCT G-3′; GLUT1, 5′-GGG CAT GTG CTT CCA GTA TGT-3′ and 5′-ACG AGG AGC ACC GTG AAG AT3′; phosphoglycerate kinase, 5′-CTG TGG TAC TGA GAG CAG CAA GA-3′ and 5′-CAG GAC CAT TCC AAA CAA TCT G-3′; and VEGF, 5′-AGT CCC ATG AAG TGA TCA AGT TCA-3′ and 5′-ATC CGC ATG ATC TGC ATG G-3′.

Preparation of Cell Extracts and Western Blot Analysis—Adherent macrophages ($1-3 \times 10^6$ cells) were washed twice with ice-cold PBS, scraped off the dishes, and collected by centrifu-

gation. Cell pellets were homogenized with 200 μl of lysis buffer (20 mM Tris-HCl, pH 7.8, 0.4 M NaCl, 5 mM MgCl$_2$, 10 mM KCl, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 10 μM leupeptin, 10 mM sodium fluoride, 1 mM sodium vanadate, and 0.5% Nonidet P-40). Cells were gently vortexed for 20 min at 4 °C, and following centrifugation at $8,000 \times g$ for 15 min, the supernatants were stored at -80 °C (total extracts). Sample protein concentrations were determined by using the detergent-compatible protein reagent from Bio-Rad. Samples containing equal amounts of protein were boiled in denaturing buffer (250 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol) and size-separated by loading $80-120$ μg of total cell extracts per well in a polyacrylamide gel in the presence of 10% SDS. The gels were transferred to Hybond^{TM}-C extra nitrocellulose membranes (Amersham Biosciences), which were then processed according to the recommendations provided by the antibody suppliers.

An antibody against PFKFB3 was obtained as described previously (16). Anti-HIF1α antibody was purchased from R&D Systems. Anti-phospho-AMPK-α-Thr-172 or anti-AMPK-α antibodies were purchased from Cell Signaling. α-Tubulin was detected using anti-α-tubulin antibody purchased from Sigma. Anti-C/EBPβ, anti-C/EBPδ, anti-Sp1, anti-Ser(P), anti-A$_2$$_A$R, and anti-A$_2$$_B$R antibodies were purchased from Santa Cruz Biotechnology. Proteins were detected by ECL (Amersham Biosciences). In each assay, several film exposure times were used to avoid film saturation.

Data Analysis—The number of experiments performed and analyzed is indicated in the corresponding figure legends. Statistical differences ($p < 0.05$) between mean values were determined by one-way analysis of the variance, followed by Student's *t* test.

RESULTS

Toll Receptor Agonists Increase pfkfb3 Gene Expression in Macrophages—Macrophage activation with LPS increased the expression of *pfkfb3* in both murine peritoneal macrophages and human monocytes/macrophages (Fig. 1*A*). Maximal mRNA levels were reached about 6 h after LPS triggering and decreased to near control levels 24 h after stimulation. These results agree with previous observations in human monocytes (14). Increased PFKFB3 isozyme levels could be detected for at least 24 h (Fig. 1*B*), and in accordance with that, elevated levels of the metabolite Fru-2,6-P$_2$ were detected 8 h after macrophage activation by LPS and could be observed for 24 h (Fig. 1*C*). We have extended these initial studies and confirmed that, besides LPS, other Toll receptor agonists, such as viral double-stranded RNA and bacterial lipopeptides, also increase *pfkfb3* gene expression (Fig. 1*D*). These data indicate that up-regulation of *pfkfb3* is a common process in the course of macrophage activation.

Adenosine Cooperates with LPS to Increase pfkfb3 Gene Expression—As macrophage activation by pathogens increases ATP consumption (34), and high metabolic activity leads to the liberation of adenosine (35), we explored whether adenosine could affect *pfkfb3* expression and thus modulate the glycolytic flux in activated macrophages. Adenosine, the A$_2$$_A$R-specific agonist CGS1680, or the A$_2$R-nonspecific agonist NECA ex-
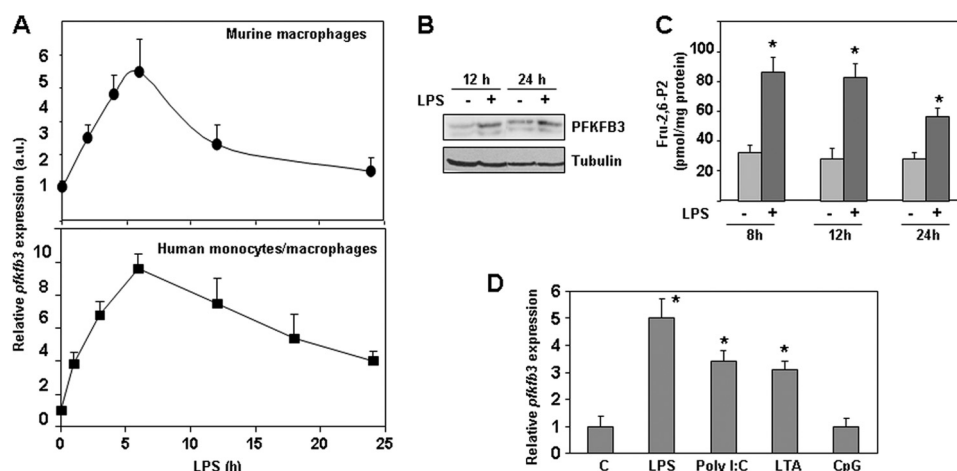
FIGURE 1. **Toll receptor agonists increase *pfkfb3* gene expression in macrophages.** *A,* quantitative RT-PCR analysis of *pfkfb3* mRNA levels in peritoneal murine macrophages and human monocytes/macrophages activated for different times with the TLR4 agonist LPS (100 ng/ml). *B,* Western blot analysis of PFKFB3 protein. *C,* Fru-2,6-P_2 levels in peritoneal macrophages activated for different times with LPS. *D,* expression of *pfkfb3* mRNA analyzed by RT-PCR after triggering macrophages with TLR3 (poly(I:C) 200 ng/ml), TLR2 (LTA-SA 2 μg/ml), or TLR9 (CpG 500 ng/ml) agonists for 6 h. Results represent the mean $\pm$ S.D. of at least three independent experiments. *, $p < 0.05$ *versus* control cells (*A, C,* and *D*) or correspond to a representative experiment out of three (*B*). *a.u.,* arbitrary units.

erted little effect by themselves in resting macrophages, but they all cooperated with LPS to increase *pfkfb3* expression (Fig. 2, *A* and *B*). Increased levels of the metabolite Fru-2,6-P_2 were found also in macrophages treated with LPS and adenosine or A_2 receptor agonists (Fig. 2*C*).

In the few last years, adenosine has been shown to modulate the inflammatory response by limiting macrophage activation through the A_{2A} receptors and to favor the development of an angiogenic phenotype (20, 21). To assess whether adenosine and the other A_2R agonists worked correctly in our cells, we evaluated their effects on *TNFα*, *VEGF,* and *iNOS* expression after induction with LPS. In agreement with previous reports (25, 36), *TNFα* expression was repressed in macrophages treated with adenosine or CGS1680, whereas LPS-induced *iNOS* and *VEGF* expression was increased ([supplemental Fig. 1](#)). Therefore, taken together, these results identify, for the first time, *pfkfb3* as a gene whose expression is synergistically up-regulated by LPS and adenosine, leading also to a synergistic effect on Fru-2,6-P_2 production.

Toll-4 Signaling Increases the Expression of A_{2A} and A_{2B} Receptors—A previous report has shown that adenosine receptors are modulated by LPS (37). We also report here a potent increase in the expression of A_{2A} and A_{2B} receptors in murine macrophages activated with LPS (Fig. 3*A*). The mRNA levels of A_1 and A_3 adenosine receptors decreased in this condition (Fig. 3*A*), as described previously (37). Although the induction relative to basal levels of $A_{2B}R$ by LPS was lower than that observed for $A_{2A}R$ (Fig. 3, *A* and *B*), basal expression levels of $A_{2B}R$ in control macrophages were higher than the levels of $A_{2A}R$ (data not shown). Both A_{2A} and A_{2B} receptors were induced transiently and rapidly by LPS, reaching maximal mRNA levels 4 h after treatment and diminishing to basal levels in a short period of time (Fig. 3*B*).

We have also observed that A_{2A} receptor induction by LPS is highly dependent on NFκB activation, as Raw-IκBα-SR cells, which overexpress the super-repressor IκBα (unable to undergo phosphorylation and degradation and therefore maintaining low levels of NFκB activity), showed a drastic reduction in the expression levels of A_{2A} induced by LPS (Fig. 3*C*). However, A_{2B} receptor expression was only slightly diminished in this cell line (Fig. 3*C*). We also evaluated the contribution of IRF-3, another transcription factor directly activated by LPS, to A_2 receptor expression, in IRF3$^{+/+}$ and IRF3$^{-/-}$ bone marrow-derived macrophages activated by LPS. Expression of the A_{2A} receptor was not affected by the lack of IRF3; however, A_{2B} receptor expression was significantly diminished ([supplemental Fig. 2](#)). Taken together, these results show that different signaling events control the induction by LPS of A_{2A} and A_{2B} receptors in murine macrophages.

Adenosine Cooperates with LPS to Increase pfkfb3 Promoter Activity—To investigate the mechanism responsible for the increased expression of *pfkfb3* in macrophages activated by LPS, we studied both in the absence or in the presence of adenosine or adenosine receptor agonists, the activity of the human *pfkfb3* promoter, previously characterized (17). In our studies, we used Raw 264.7 cells, which conserve the *pfkfb3* expression pattern observed in bone marrow-derived macrophages, peritoneal macrophages, and human monocyte/macrophages when treated with LPS (data not shown). We transfected this cell line with a construct in which luciferase expression was driven by a 3,566-bp fragment from the human *pfkfb3* promoter. As shown in Fig. 4*A*, adenosine, CGS1680, or NECA by themselves exerted little effect on *pfkfb3* promoter activity, but they all cooperated with LPS to increase its effect. The activity of the *pfkfb3* promoter increased about 15-fold by LPS alone, an induction level similar to that observed with the endogenous *pfkfb3* gene in Raw 264.7 cells (data not shown) or human monocytes/macrophages stimulated by LPS (Fig. 1*A*) but higher than that observed in LPS-activated murine peritoneal macrophages (about 4-fold, Figs. 1 and 2). Activation of A_2 receptors enhanced the promoter activity induced by LPS by ~2-fold, thus reaching total induction levels close to 30-fold. This promoter behavior was coherent with the increased expression levels of the endogenous *pfkfb* gene observed in
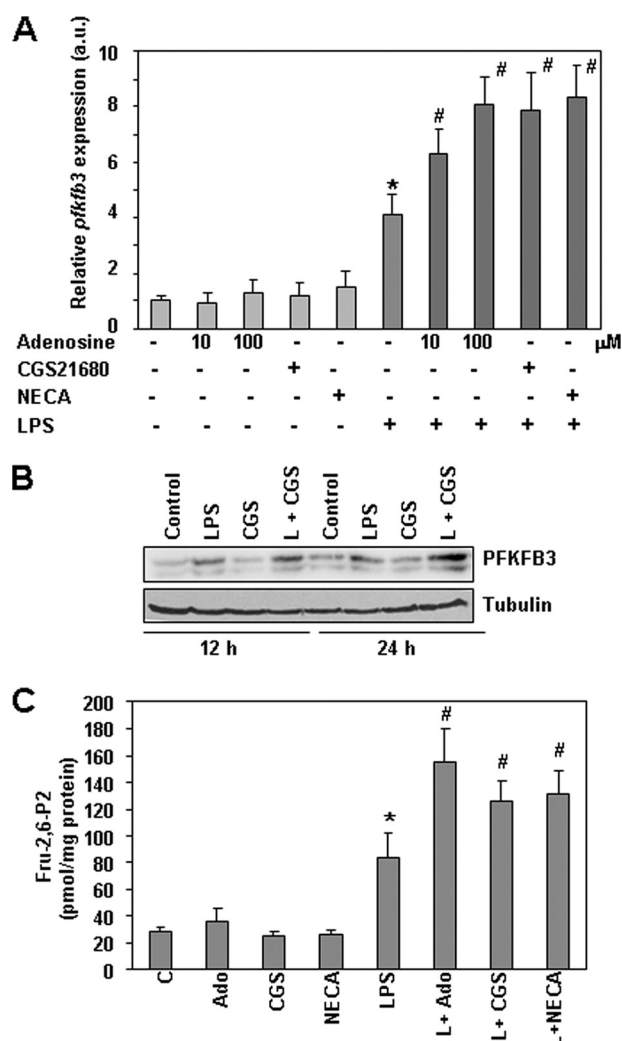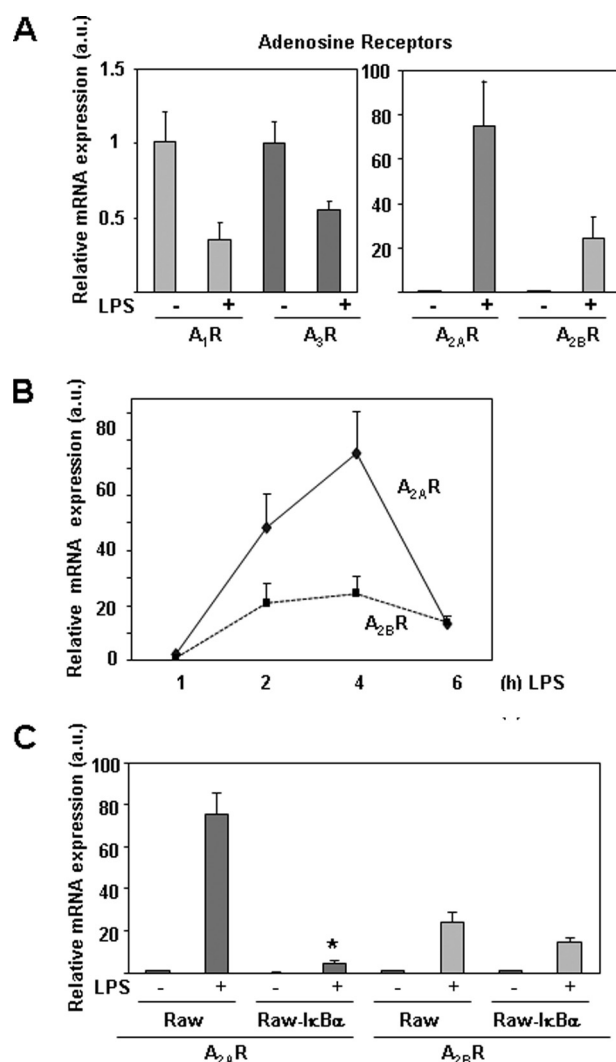
FIGURE 2. **Adenosine receptor agonists modulate LPS-induced *pfkfb3* gene expression in macrophages.** *A*, quantitative RT-PCR analysis of *pfkfb3* mRNA expression in peritoneal macrophages treated for 6 h with LPS (100 ng/ml), adenosine (10 or 100 μM), the specific A_2 receptor agonist CGS21680 (2 μM), or the nonselective A_2 receptor agonist NECA (10 μM). *B*, analysis of PFKFB3 expression by Western blot performed with extracts from peritoneal macrophages treated for 12 or 24 h with the same concentrations as above of the indicated agents. A representative experiment is shown. *C*, Fru-2,6-P$_2$ levels in macrophages treated for 12 h as in *A*. Results represent the mean $\pm$ S.D. of at least three independent experiments. *, $p < 0.05$ *versus* control cells. #, $p < 0.05$ *versus* LPS-treated cells. *a.u.*, arbitrary units.

FIGURE 3. **LPS increases the expression of A_2 receptors in macrophages.** *A*, RT-PCR analysis of the four adenosine receptors in peritoneal macrophages treated with LPS (100 ng/ml) for 4 h. *B*, time course of A_{2A} and A_{2B} receptor expression in macrophages treated with LPS for the indicated times. *C*, analysis by quantitative RT-PCR of A_{2A} and A_{2B} receptor expression in control or Raw 264.7 cells expressing the super-repressor protein IκBα (Raw-IκBα-SR), 4 h after treatment with LPS. Results represent the mean $\pm$ S.D. of at least three independent experiments. *a.u.*, arbitrary units.

macrophages (Fig. 2*A*) and Raw 264.7 cells (data not shown) treated with LPS or/and A_2 receptor agonists.

pfkfb3 expression is induced in different cell types by hypoxia (17). We evaluated the effect of two hypoxia mimics, cobalt chloride (CoCl$_2$) and the iron chelator deferoxamine, in the activity of the human *pfkfb3* promoter in macrophages. Promoter activity increased in both cases by about 5-fold, an induction level lower than that observed with LPS alone or LPS with A_2 receptor agonists. No cooperation between hypoxia mimics and adenosine receptor agonists was observed (data not shown).

We studied the DNA regions required for LPS and adenosine to increase *pfkfb3* promoter activity by using a series of promoter constructs (see promoter diagram shown in Fig. 4*B*) containing successive deletions from the 5′ end, as described pre-

viously (17). For each construct, the fold increase in luciferase activity, elicited by either LPS alone or together with NECA, was determined over basal luciferase activity. As shown in Fig. 4*C*, 5′ deletion mutants revealed that sequences between −3,566 and −148 from the transcription start site did not substantially affect induction of the *pfkfb3* gene promoter by LPS and NECA, although some variations in inducibility were observed. In contrast, both effects were completely abolished by deleting the region between positions −148 and −50. These results show that sequences between −148 and −50 are sufficient for LPS and A_2R agonist induction of the *pfkfb3* gene promoter.

Previous studies have identified two proximal hypoxia-response element sequences (HRE) between nucleotides 1,269 and 1,297 of the *pfkfb3* gene promoter (17). This region is the major hypoxia-responsive region of this gene, and it is essential
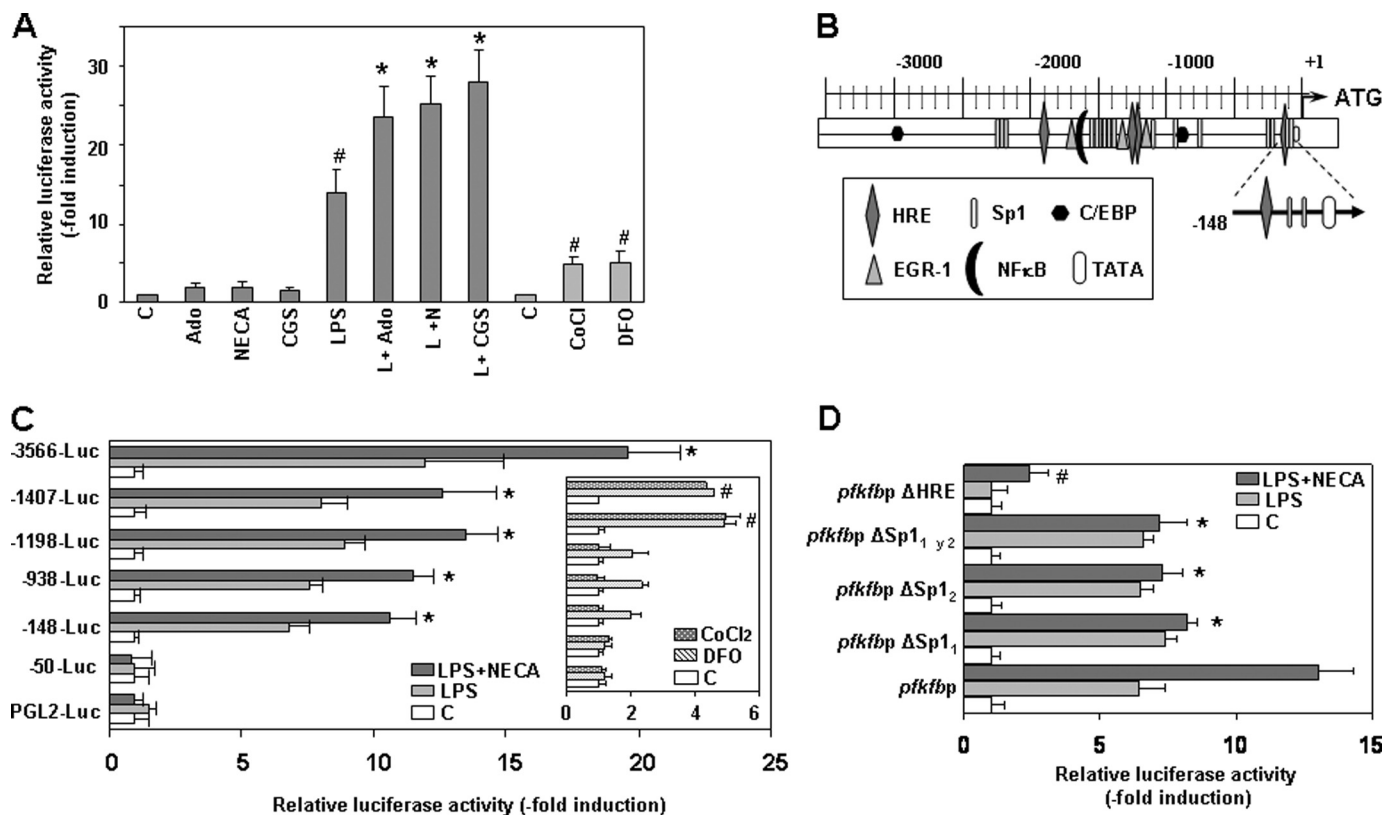
FIGURE 4. **Specific regions of the *pfkfb3* promoter mediate its transcriptional induction by LPS and further up-regulation by adenosine and A₂ receptor agonists.** *A,* Raw 264.7 cells were transiently cotransfected with a pGL2-basic luciferase expression vector, containing 3,566 bp of the human *pfkfb3* gene promoter, and a β-galactosidase expression vector. Cells were activated for 18 h with LPS (100 ng/ml) and/or adenosine (*Ado*) (100 μM), CGS21680 (*CGS*) (2 μM), or NECA (10 μM), or treated with deferoxamine (*DFO*) (200 μM) or CoCl₂ (200 μM). Transfections were performed in triplicate. Results represent the mean ± S.D. of the luciferase activity determined in at least three independent transfection experiments. *, $p < 0.05$ *versus* LPS treatment; #, $p < 0.05$ *versus* control. *B,* schematic representation of the *pfkfb3* promoter with its putative regulatory elements. *C,* Raw 264.7 cells were transiently transfected with pGL2-basic luciferase expression vectors containing various lengths of the human *pfkfb3* gene promoter 5'-flanking region and a β-galactosidase expression vector as a transfection control. Cells were activated for 18 h with LPS or LPS plus NECA or treated with deferoxamine (*DFO*) or CoCl₂. *, $p < 0.05$ *versus* LPS treatment; #, $p < 0.05$ *versus* control. *D,* activity of *pfkfb3* minimal promoter following site-directed mutations of the HRE and the two Sp1-binding sites. In all cases, transfections were performed in triplicate. Results represent the mean ± S.D. of at least three independent transfection experiments. *, $p < 0.05$ *versus* LPS plus NECA-treated control promoter; #, $p < 0.05$ *versus* its own control.

for the hypoxia-induced transcription of the *pfkfb3* gene promoter in macrophages, as showed at the *right panel* of Fig. 4*C*.

Accordingly to these data, some differences could be appreciated between *pfkfb3* gene promoter induction by LPS/A₂R agonists and by hypoxia mimics. First, the level of induction was higher with LPS than with hypoxia mimics in macrophages. The second difference resided on the sequences responsible for the induction. Stimulation of the *pfkfb3* promoter by LPS essentially depended on proximal promoter sequences from position −148 to −50, whereas induction by hypoxia mimics was mainly observed in constructs containing the region between nucleotides 1,269 and 1,297.

Sequence analysis of the proximal *pfkfb3* promoter (−148 nucleotides) revealed two binding sites for the transcription factor Sp1 (starting at −72 and −97 nucleotides, respectively) and one HRE sequence (107 nucleotides) (Fig. 4*B*). As shown in Fig. 4*D*, site-directed mutagenesis of the Sp1 transcription factor-binding sites revealed that Sp1 is a key protein for adenosine cooperation with LPS in the induction of *pfkfb3* promoter activity, for each one of the Sp1-binding site mutations are individually sufficient to eliminate the adenosine-dependent induction of the *pfkfb3* promoter without affecting induction by LPS. However, mutation of the HRE-binding site completely abol-

ishes *pfkfb3* promoter induction by LPS alone, although LPS in combination with the A₂ receptor agonist NECA still results in a little induction. These results point at HIF proteins as being responsible for LPS-dependent induction of the *pfkfb3* promoter (Fig. 4*D*).

Analysis of the Transcription Factors Involved in pfkfb3 Induction by LPS/A₂R Agonists—In addition to Sp1 and HRE, multiple binding sites for relevant transcription factors have been previously identified in the *pfkfb3* gene promoter, including NFκB, C/EBP, EGR-1, and estrogen receptor-binding elements, among others (Fig. 4*B*) (17). To identify additional transcription factors that could modulate *pfkfb3* induction by LPS/A₂R agonists, we analyzed the effect of LPS and adenosine/NECA in the activity of proteins potentially capable of binding to specific *pfkfb3* promoter sequences and related to the LPS or the adenosine response. Reporter constructs carrying binding sites for HIF1, NFκB, C/EBP, Sp1, and EGR-1 were transfected into Raw 264.7 cells, which were stimulated afterward with LPS, NECA, or both. The results showed that the activity of HIF1, C/EBP, and Sp1 reporter constructs increased upon activation with LPS, and this activity was enhanced by the addition of NECA (Fig. 5*A*). On the contrary, NFκB activity increased with LPS, but it was
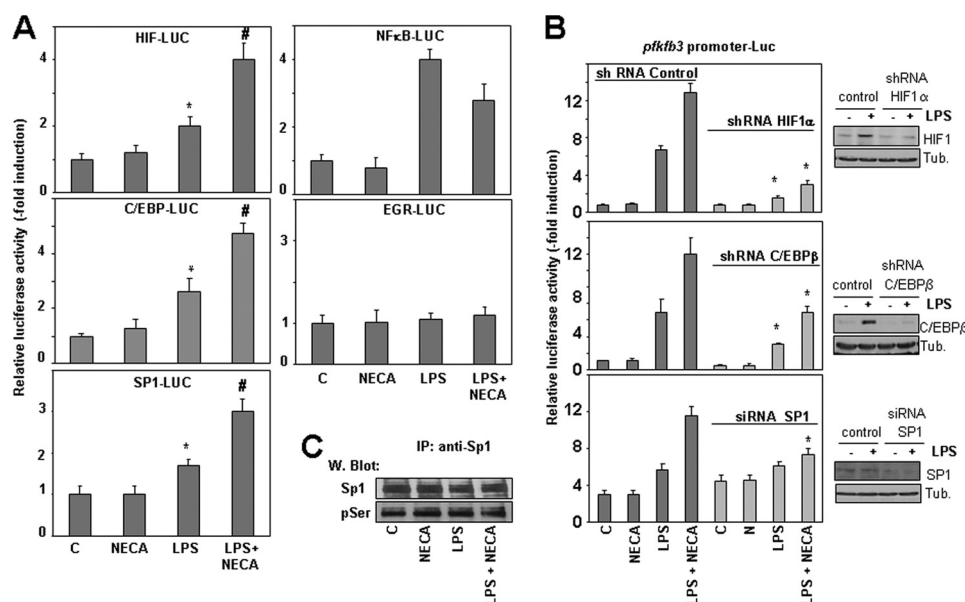
FIGURE 5. **Analysis of the transcription factors involved in *pfkfb3* induction by LPS and A₂ receptor agonists.** *A,* transcriptional activity of reporter constructs carrying binding sites for HIF1, NFκB, C/EBP, SP1, or EGR-1 transfected in Raw 264.7 cells stimulated with LPS, NECA, or both. Results represent the mean ± S.D. of at least three independent experiments. *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* LPS treatment. *B,* analysis of *pfkfb3* promoter activity in Raw 264.7 cells transfected with control, HIF1α, cEBPβ shRNAs, or Sp1 siRNA and stimulated for 18 h with LPS and/or NECA. All transfection experiments were performed in triplicate. Results represent the mean ± S.D. of at least three independent transfection experiments. *, $p < 0.05$ *versus* control shRNA or siRNA. In each case, efficiency of shRNAs knockdown is shown by Western blot (*W blot*). *C,* analysis by Western blot of Sp1 expression and phosphorylation in Raw 264.7 cells activated by LPS, NECA, or both for 6 h. Total cell proteins (1 mg) were immunoprecipitated (*IP*) with anti-Sp1 antibody and revealed afterward with anti-Ser(P) or anti Sp1 antibodies. A representative experiment out of three is shown.

inhibited by NECA, whereas EGR-1 reporter activity was not affected by either treatment.

To further analyze the role of HIF1 and Sp1, previously identified as key transcription factors by site-directed mutations of proximal binding sites, and the role of C/EBP, which possesses several putative binding sites on the *pfkfb3* promoter (Fig. 4*B*), we used an shRNA or siRNA approach to diminish their expression and explore the effects of this decrease on *pfkfb3* promoter activity. We first confirmed that specific shRNAs or siRNAs diminished protein expression at least by 80% of control levels. Then we observed (Fig. 5*B*) that reduced expression of HIF1α greatly decreased *pfkfb3* induction by LPS and LPS/A₂R agonists, corroborating its key role in LPS-induced *pfkfb3* expression. However, enhanced expression of *pfkfb3* by A₂R agonists was still observed under these circumstances. In line with the results of the site-directed mutagenesis experiments, reduced Sp1 expression clearly blocked the A₂R agonist-dependent enhancement of the *pfkfb3* promoter activity, whereas basal and LPS-induced *pfkfb3* promoter activity levels were slightly up-regulated. However, overexpression of Sp1 increased *pfkfb3* promoter activity (supplemental Fig. 3). Reduced expression of C/EBPβ (Fig. 5*B*) or C/EBPδ (data not shown) also diminishes *pfkfb3* induction by LPS and LPS/A₂R agonists. Nevertheless, we have not observed changes in the *pfkfb3* promoter activity after mutation of a C/EBP-binding site (−855 nucleotides) (data not shown). However, other putative C/EBP-binding sites could be responsible for its effect; moreover, an indirect effect could be involved as well.

We analyzed *HIF1α*, C/EBPβ, and Sp1 expression in macrophages activated with LPS in the presence or in the absence of A₂ receptor agonists. As shown in supplemental Fig. 4, *HIF1α* mRNA and protein levels increased after LPS treatment. Aden-

osine or NECA exerted little effect by themselves on *HIF1α* expression, but both A₂ agonists cooperated with LPS to increase it. Similarly, LPS induced the expression of C/EBPβ and C/EBPδ proteins, and this induction was further enhanced by NECA 3 h after LPS treatment. At this time, using macrophage nuclear extracts, we also observed a cooperative effect between LPS and NECA to trigger protein binding to a consensus C/EBP sequence. Expression of C/EBPβ and C/EBPδ proteins also increased *pfkfb3* promoter activity (supplemental Fig. 5).

However, in agreement with previously reported results (38, 39), we did not observe changes in Sp1 expression after macrophage activation with LPS in the presence or in the absence of A₂ agonists (Fig. 5*C*). As the activity of Sp1 can be modulated by post-translational modifications, we evaluated whether its phosphorylation state changed in the course of macrophage activation with LPS or A₂ receptor agonists. As shown in Fig. 5*C*, similar Sp1 Ser(P) content was detected in all conditions. Thus, other post-translational modifications, different from phosphorylation, are probably involved in Sp1 activation (40).

A₂A Receptors Are Essential for LPS and Adenosine Induction of pfkfb3 Expression—To assess the role of both A₂A and A₂B adenosine receptors in *pfkfb3* induction, we evaluated the expression of this gene in the presence of ZM 241385 or MRS1754, which are A₂A and A₂B receptor antagonists, respectively. Both inhibitors diminished the induction of *pfkfb3* by a combination of LPS and A₂R agonists by around 40%. Surprisingly, both inhibitors also affected to a similar extent the induction of *pfkfb3* by LPS alone, arguing for a role of A₂ receptors in the induction of this gene by LPS (supplemental Fig. 6). To further evaluate the role of these receptors, we overexpressed each of them in Raw 264.7 cells along with the largest *pfkfb3*
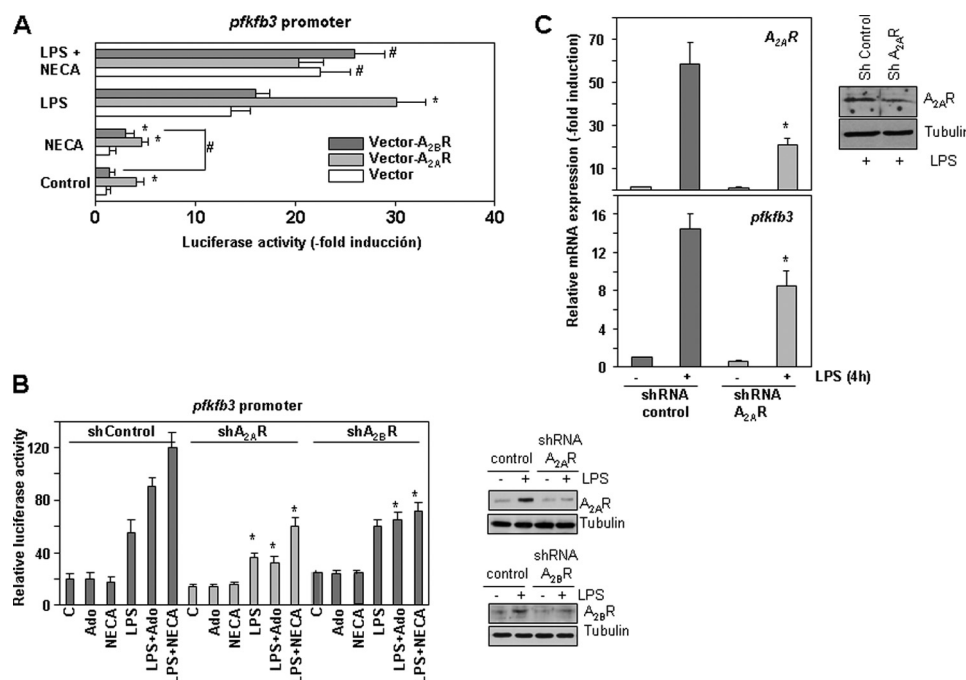
FIGURE 6. **A$_{2A}$ and A$_{2B}$ receptors collaborate to increase LPS-induced *pfkfb3* expression.** *A,* overexpression of A$_{2A}$ or A$_{2B}$ receptors increases *pfkfb3* promoter activity in macrophages. Raw 264.7 cells were cotransfected with −3,566-*pfkfb3*-Luc and A$_{2A}$ or A$_{2B}$ receptor expression vectors and treated with LPS, NECA, or both for 18 h. A β-galactosidase expression vector was used as a transfection control. All experiments were performed in triplicate. Results represent the mean ± S.D. of the luciferase activity determined in at least three independent transfection experiments. *, $p < 0.05$ *versus* control transfection; #, $p < 0.05$ *versus* NECA treatment. *B,* effect of A$_{2A}$ or A$_{2B}$ receptor shRNAs in the activity of the −3,566-*pfkfb3* promoter construct. Raw 264.7 cells were transiently cotransfected with −3,566-*pfkfb3*-Luc and control or A$_{2A}$ or A$_{2B}$ receptor shRNAs and treated with LPS, NECA, or both for 18 h. A β-galactosidase expression vector was used as a transfection control. All experiments were performed in triplicate. Results represent the mean ± S.D. of the luciferase activity determined in at least three independent transfection experiments. *, $p < 0.05$ *versus* control transfection. *C,* expression of A$_{2A}$ receptors and *pfkfb3* mRNAs in stably transfected Raw 264.7 cells expressing control or A$_{2A}$ receptor shRNAs and activated or not with LPS or LPS/NECA. *, $p < 0.05$ *versus* control shRNA. In each case efficiency of shRNAs knockdown is shown by Western blot.

promoter construct. As shown in Fig. 6*A*, expression of A$_{2A}$, but not of A$_{2B}$ receptor, in the absence of any exogenous stimulation, increased the promoter activity by about 5-fold. Treatment with NECA did not increase further the activity of the *pfkfb3* promoter in cells transfected with the A$_{2A}$ receptor, but increased its activity in cells overexpressing the A$_{2B}$ receptor. These differences are probably related to the different affinities of each receptor for adenosine (0.7 μM for A$_{2A}$ and 24 μM for A$_{2B}$ (35)) and suggest that endogenous adenosine production by macrophages is sufficient to activate A$_{2A}$ but not A$_{2B}$ receptors. Induction of the *pfkfb3* promoter by LPS is also stronger when A$_{2A}$ rather than A$_{2B}$ is overexpressed, but as observed previously in the absence of LPS, treatment with NECA was unable to further increase luciferase activity by A$_{2A}$, although it increased *pfkfb3* promoter activity in cells transfected with the A$_{2B}$ receptor.

In an additional approach to clarify the role of A$_{2A}$ and A$_{2B}$ receptors in *pfkfb3* induction by LPS and adenosine, we transiently transfected Raw 264.7 cells with the −3,566 *pfkfb3* promoter construct and A$_{2A}$ or A$_{2B}$ receptor shRNAs. As shown in Fig. 6*B*, the LPS-induced increase in *pfkfb3* promoter activity diminished in the presence of A$_{2A}$ shRNA, but it was not affected by A$_{2B}$ shRNA. However, again, both A$_{2A}$ and A$_{2B}$ shRNAs diminished the effects caused by adenosine or NECA. To confirm the role of A$_{2A}$ receptor in the LPS-dependent induction of *pfkfb3* gene expression, we generated stably transfected Raw 264.7 cells with a control or with an A$_{2A}$ receptor shRNA to decrease its expression. As shown in Fig. 6*C*, diminished A$_{2A}$ receptor expression levels resulted in reduced *pfkfb3* expression after macrophage activation by LPS. Taken together, these results suggest that, although both A$_{2A}$ and A$_{2B}$ receptors are implicated, the A$_{2A}$ receptor is essential for the induction of *pfkfb3* expression by LPS when low concentrations of adenosine are present in the medium. A$_{2B}$ receptors are less relevant in this role, but they can further increase *pfkfb3* expression when adenosine accumulates in stressful situations.

LPS Increases AMP Kinase Activity in Macrophages—Different reports (41, 42) have shown that PFKFB3 activity can be modulated by phosphorylation at Ser-461 by the AMP-activated protein kinase (AMPK). AMPK is activated by phosphorylation when the AMP/ATP ratio is elevated and can switch on catabolic pathways by different mechanisms, including phosphorylation of metabolic enzymes (43), as described for the PFKFB3 isozyme (41). To evaluate the possible contribution of LPS and adenosine toward the activation of AMPK to modulate PFKFB3 isozyme activity, we analyzed AMPK phosphorylation and expression in macrophages activated by LPS and/or adenosine. As shown in Fig. 7*A*, AMPK phosphorylation increases over the control levels in long term LPS-activated macrophages (8–24 h). When AMPK expression was analyzed, higher levels of protein could be observed after LPS treatment. No differences between LPS and LPS/adenosine conditions were observed. These data lead us to conclude that increased AMPK activity, due to increased AMPK expression and phosphorylation, is present in long term activated macrophages, which
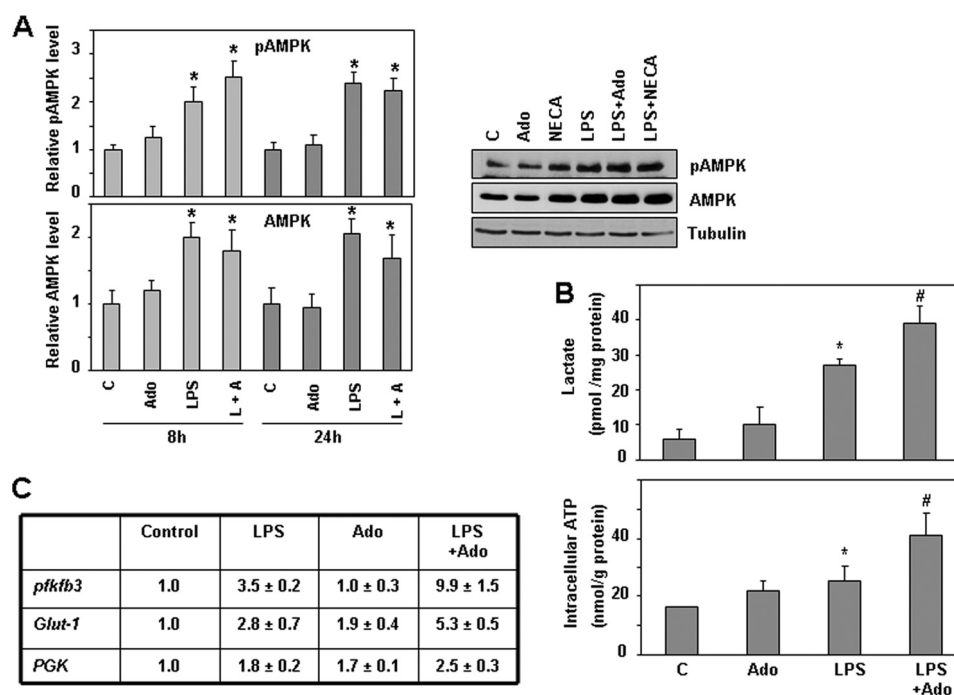
FIGURE 7. **Adenosine and LPS cooperate to increase the glycolytic flux and ATP production in macrophages.** *A,* analysis by Western blot of pAMPK and AMPK levels in macrophages activated with LPS (100 ng/ml) and/or adenosine (100 μM) or NECA (10 μM) for different times. Results represent the mean $\pm$ S.D. of at least three independent experiments. Tubulin expression is used as a reference. *, $p < 0.05$ *versus* control condition. A representative Western blot is also shown. *B,* determination of lactate production (*upper panel*) and intracellular ATP levels (*lower panel*) in macrophages activated as described previously for 12 or 24 h, respectively. Results represent the mean $\pm$ S.D. of at least three independent experiments. *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* LPS treatment. *C,* expression analysis by RT-PCR of different genes implicated in glucose metabolism in macrophages stimulated for 4 h with LPS and/or NECA. Results represent the mean $\pm$ S.D. of at least three independent experiments. *PGK,* phosphoglycerate kinase.

could contribute also to increased PFKFB3 activity and Fru-2,6-P$_2$ accumulation in these cells.

Increased Lactate Production and ATP Supply in Macrophages Activated by LPS and Adenosine—As the glycolytic flux is mainly controlled by 6-phosphofructo-1-kinase (9), and the main consequence of enhanced glycolytic function is an increase in lactate production, we analyzed this metabolite in macrophages activated by LPS and adenosine. As reported previously (5), increased levels of lactate are observed in LPS-activated macrophages (Fig. 7B). Statistically significant higher lactate levels, over those reached with LPS alone, were observed after treatment with adenosine.

We also analyzed the ATP levels in activated macrophages. In agreement with previous data (34), decreased ATP levels were observed during the first hours after LPS treatment (data not shown). However, long term macrophage activation with LPS (from 12 to 36 h) resulted in increased ATP levels (Fig. 7B). This increase was enhanced by adenosine. Thus, it appears that adenosine cooperates with macrophage-activating signals to increase energy supply, permitting macrophage long term activity. Along this line, besides PFKFB3, expression of other glycolytic enzymes, such as PGK and the glucose transporter Glut1, is also increased by LPS and A$_2$ receptor agonists, as shown in Fig. 7C.

DISCUSSION

Macrophages and phagocytic cells accomplish their defensive roles in hostile environments, poor in oxygen and rich in free radicals able to block electron transport in mitochondria.

In these conditions, ATP supply by anaerobic glycolysis is an essential source of energy for these cells. In this work, we show that molecules present in the macrophage microenvironment, particularly adenosine, cooperate with some pathogen-induced activation signals to increase the glycolytic flux through the induction of *pfkfb3* expression and PFKFB3 isozyme activity. Our results identify, for the first time, *pfkfb3* as a gene synergistically up-regulated by LPS and adenosine, which produces a synergistic effect on the expression and activity of PFKFB3. We have analyzed the molecular mechanisms underlying the cooperative induction of *pfkfb3* expression by LPS and adenosine, and we concluded that different processes are implicated. First, LPS induces the expression of A$_{2A}$ and A$_{2B}$ receptors, increasing the sensitivity of the macrophage to adenosine. Second, both LPS and adenosine increase the expression of *HIF1α*, C/EBPβ, and C/EBPδ, which are key transcription factors for *pfkfb3* expression. Third, adenosine increases Sp1 activation, which cooperatively up-regulates *pfkfb3* expression. Moreover, long term LPS treatment induces AMPK activation, favoring phosphorylation of PFKFB3 at Ser-461, and thus increasing its kinase activity. Therefore, increased expression and phosphorylation of the PFKFB3 isozyme must be responsible for the increased levels of Fru-2,6-P$_2$ and glycolytic rate observed in activated macrophages in the presence of adenosine.

Bacterial LPS and inflammatory cytokines have been reported to modify the expression of adenosine receptors, although somewhat different results are reported in the literature. A small ($<$2-fold) increase in the expression of the A$_{2A}$

receptor mRNA has been reported in the case of human monocytic cell lines (44). However, Murphree *et al.* (37) reported greater than 100-fold increase in A_{2A} receptor expression in human or mice macrophages, in addition to a concomitant up-regulation of the A_{2B} receptor. Our results agree with this last work in that macrophage activation by LPS significantly increases A_{2A} and A_{2B} adenosine receptors. On the contrary, A_1 and A_3 receptors are down-regulated by proinflammatory signals. A_{2A} and A_{2B} receptors increase cAMP levels, whereas A_1 and A_3 exert the opposite effect (35); thus macrophage activation by LPS causes an adenosine receptor isoform expression pattern switch that can selectively modify the macrophage phenotype. We have also analyzed the molecular mechanism responsible for this effect and unveiled, by using genetic approaches, that NFκB is a key transcription factor in LPS-induced A_{2A} receptor expression, whereas IRF3 is not implicated. On the contrary, A_{2B} receptor expression depends on IRF3 and is less affected by NFκB. These results suggest that different Toll receptor agonists would induce different adenosine receptor expression patterns depending on the main transcription factors activated by the similar, but not identical, Toll receptor signaling pathways, thus modifying in this way macrophage sensitivity to adenosine.

One of the more surprising findings of this work is that the endogenous production of adenosine by activated macrophages functions as a second messenger in the LPS signaling pathway leading to increased *pfkfb3* expression. A role for adenosine in the induction of IL-10 synthesis by *Escherichia coli* has also been described (45). Therefore, increasing evidence shows that endogenous adenosine production by macrophages mediates part of the effects triggered by LPS on macrophage gene expression.

In the last decade, multiple evidence has shown that adenosine, A_{2A}, and, to a lesser extent, A_{2B} receptors exert a crucial anti-inflammatory and immunosuppressive effect (reviewed in Refs. 21, 35), essential in limiting macrophage activity and protecting surrounding tissues of collateral damage during infection. *In vivo* genetic experiments have confirmed the anti-inflammatory role of A_2 receptors (26). Our results are consistent with these observations, as they show that adenosine diminishes TNFα expression and increases iNOS synthesis, which also agrees with previous reports (46). In this context, we have observed increased *pfkfb3*, *PGK*, and *Glut1* gene expression (Fig. 7). Thus adenosine, besides diminishing macrophage activation, seems to cooperate with LPS to increase the expression of proteins implicated in glucose transport and catabolism. Among them, PFKFB3 is especially relevant due to the essential role that the metabolite it produces, Fru-2,6-P_2, plays in the control of the glycolytic flux (11).

Our analysis of the *pfkfb3* promoter activity and studies performed with specific shRNAs or siRNAs identified HIF1α as a key transcription factor implicated in LPS induction (Fig. 6). HIF1 is an essential mediator allowing cell adaptation to low oxygen levels. Cramer *et al.* (5), using conditional gene targeting deletion of HIF1α in the myeloid cell lineage of mice, have clearly shown that this transcription factor is essential for macrophage energy supply and function and for the orchestration of the inflammatory response *in vivo*. Although hypoxia is the main cause of HIF1α activation, an increasing body of evidence indicates that this transcription factor may also be up-regulated by nonhypoxic stimuli. Indeed, cytokines, such as TNFα and IL-1β, and inflammatory mediators, such as NO, have been shown to increase HIF1α levels during normoxia (47–49). This induction involves transcriptional (47) and post-transcriptional mechanisms for HIF1 stabilization (50, 51). In macrophages, adenosine, through its A_2 receptor, increases HIF1α levels in a process depending on protein kinase C and PI3K pathways, which involve transcriptional and, to a large extent, translational mechanisms (52). HIF1α has been previously related to adenosine and LPS cooperation in the expression of VEGF (25). Our results extend these observations and show the essential role played by HIF1α in LPS and adenosine-induced expression of *pfkfb3* in macrophages.

C/EBP transcription factors play a fundamental role in the process of macrophage differentiation and in the regulation of different functions in activated macrophages (53–55). Toll-4 receptors increase C/EBPβ expression, favoring the transcription of multiple proinflammatory genes, such as *IL-6*, *TNFα*, and *iNOS* (56). C/EBPδ is also induced after LPS treatment by a mechanism depending on NFκB (57). Indeed, it has been recently described that C/EBPδ cooperates with NFκB to maintain long term gene expression in persistent Toll receptor signaling (57). Our results show that LPS and adenosine cooperate to increase C/EBPβ and C/EBPδ expression and that all these proteins are able to increase *pfkfb3* promoter activity. Expression of anti-inflammatory cytokines, such as IL-10, induced by LPS, is mediated by C/EBPβ (39, 45), and this process depends on the expression of A_2 receptors (45). Transduction through these receptors implicates the activation of cAMP-dependent protein kinase A, which phosphorylates the transcription factor CREB (58). Recently, it has been described that CREB increases the expression of C/EBPβ (45). Activation of CREB has been also related to the strong anti-inflammatory properties of A_2 receptors by competing with NFκB and other transcription factors for the important cofactor CREB-binding protein.

Induction of HIF1α and C/EBPβ and C/EBPδ activity by adenosine in activated macrophages has been previously reported to explain the cooperative effect of adenosine and bacterial components in VEGF and IL-10 expression, respectively (25, 45). However, in this work we show, for the first time, that the transcription factor Sp1 is also implicated in the regulation of gene expression by adenosine (Fig. 6). Sp1 was thought to serve mainly as a constitutive activator for housekeeping genes, but growing evidence shows that post-translational modifications can modulate its transcriptional activity (reviewed in Ref. 40). Sp1 can be phosphorylated by various kinases at different sites. Decreased Sp1 phosphorylation has been related to the expression of IL-21 receptor in activated T cells (59). On the contrary, increased expression of apolipoprotein A-I by the action of Sp1 seems to be modulated in its phosphorylation by protein kinase A and C, among others (60, 61). The influence of phosphorylation on Sp1 activity is not completely understood. Although it was tempting to speculate that activation of protein kinase A after adenosine receptor triggering is implicated in the increased Sp1 activity observed in macrophages activated with LPS and A_2 receptor agonists, our results discard this hypoth-

esis, because we observed that the increased Sp1 activity does not appear to depend on Ser phosphorylation. Further work is needed to clarify the post-translational modifications that explain these molecular events.

Expression of *pfkfb3* occurs mainly in proliferative and transformed cells (13, 14). Previous reports have also described its expression in human and mouse macrophages, astrocytes, and monocytes (14, 15, 62). Besides, there is evidence of *pfkfb3* up-regulation by hypoxia, serum, insulin, and proinflammatory molecules (14, 17, 62, 63). In addition to transcription, PFKFB3 isozyme activity is regulated by phosphorylation by AMPK at Ser-461, which increases its V_{max} without affecting its K_m values (11). Our results show that expression of the *pfkfb3* gene is highly up-regulated in macrophages activated by LPS and adenosine agonists by increasing the transcriptional activity of its promoter. In addition, our results show increased levels of activated and phosphorylated AMPK in long term LPS-activated macrophages, which further increase PFKFB3 activity through AMPK-dependent phosphorylation in those cells. This effect could be related to NO accumulation, as it has been described in astrocytes, in which increased NO levels results in mitochondrial inhibition and AMPK activation (15). Increased glycolytic flux originated by the NO/AMPK induction of PFK2.3 activity increases, in turn, the resistance of astrocytes to apoptosis (15). Elevated glycolysis also seems to be essential for the activation of other immune cells, such as T lymphocytes. Although the energetic yield of glycolysis is much smaller than that of glucose oxidation in the mitochondria, in conditions of unlimited glucose supply, glycolysis produces ATP substantially faster, so the stimulation of glucose uptake and glycolysis is also essential for proper T cell activation, and a defective energy supply results in the death of T cells by apoptosis (64). Therefore, we can hypothesize that the up-regulation of PFKFB3 isozyme expression observed in activated macrophages could also favor their viability in the inflammatory microenvironment. Along this line, a recent work reports that diminishing PFKFB3 expression increases macrophage apoptosis and limits macrophage activation (62).

In summary, our results demonstrate an important role for adenosine signaling in inflammation. Besides its crucial anti-inflammatory and immunosuppressive effects, essential in limiting macrophage activity and protecting surrounding tissues of collateral damage, adenosine, through A_2 receptor activation, cooperates with signaling pathways triggered by some pathogen components to increase glycolytic flux and energy supply in macrophages, favoring the expression and activation of the key glycolytic isozyme PFKFB3. This process could be essential in protecting the viability of macrophages to develop their long term defense and reparative functions.

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