Adenosine Modulates HIF-1α, VEGF, IL-8, and Foam Cell Formation in a Human Model of Hypoxic Foam Cells

Stefania Gessi, Eleonora Fogli, Valeria Sacchetto, Stefania Merighi, Katia Varani, Delia Preti, Edward Leung, Stephen MacLennan, Pier Andrea Borea

Objective—Foam cell (FC) formation by oxidized low-density lipoprotein (oxLDL) accumulation in macrophages is crucial for development of atherosclerosis. Hypoxia has been demonstrated in atherosclerosis and hypoxia-inducible factor-1 (HIF-1) has been shown to promote intraplaque angiogenesis and FC development. As hypoxia induces HIF-1α stabilization and adenosine (ado) accumulation, we investigated whether this nucleoside regulates HIF-1α in FCs.

Methods and Results—Ado, under hypoxia, stimulates HIF-1α accumulation by activating all adenosine receptors (ARs). HIF-1α modulation involved extracellular signal-regulated kinase 1/2 (ERK 1/2), p38 mitogen-activated protein kinase (p38 MAPK), and protein kinase B (Akt) phosphorylation in the case of A1, A2A, A2B, and ERK 1/2 phosphorylation in the case of A3 receptors. Ado, through the activation of A3 and A2B receptors, stimulates vascular endothelial growth factor (VEGF) secretion in a HIF-1α–dependent way. Furthermore, ado, through the A2B subtype, induces an increase of Interleukin-8 (IL-8) secretion in a ERK 1/2, p38, and Akt kinase–dependent but not HIF-1α–mediated way. Finally, ado stimulates FC formation, and this effect is strongly reduced by A3 and A2B blockers and by HIF-1α silencing.

Conclusions—This study provides the first evidence that A3, A2B, or mixed A3/A2B antagonists may be useful to block important steps in the atherosclerotic plaque development ado-induced. (Arterioscler Thromb Vasc Biol. 2010; 30:90-97.)

Key Words: adenosine receptors ■ atherosclerosis ■ HIF-1α ■ foam cells ■ A3/A2B antagonists

Macrophage foam cell formation is an important process in atherosclerotic plaque development.1 Atherosclerosis is initiated by dysfunction of endothelial cells at lesion-prone sites in the walls of arteries, which results in monocyte infiltration into the arterial intima. These cells differentiated into macrophages, which then internalize large amounts of oxidized low-density lipoprotein forming cholesterol-laden macrophages called “foam cells” (FCs), which in turn give rise to fatty streaks in the arterial wall.2 As the atherosclerotic lesion develops, the arterial wall thickness increases and oxygen diffusion into the intima is markedly reduced. These hypoxic regions contain large number of FCs revealing that these cells experience hypoxia during the development of atherosclerotic lesions.3–4 Hypoxia-inducible factor-1 (HIF-1), the most important factor involved in the cellular response to hypoxia, is a heterodimeric transcription factor composed of an inducibly expressed HIF-1α subunit and a constitutively-expressed HIF-1β subunit.5 It is well established that HIF plays a major role in vascular endothelial growth factor (VEGF) expression and angiogenesis, mediating important alterations associated with atherosclerosis and angiogenic activity of macrophages.6–7 Moreover, under atherogenic conditions, the high expression of HIF-1 in macrophages promotes FC formation and atherosclerosis.8

Recently, it has been shown that another angiogenic chemokine, interleukin-8 (IL-8), is upregulated by FCs located in hypoxic areas in rabbit and human atherosclerotic plaques.4 Hypoxia-induced secretion of IL-8 from FCs may lead to the recruitment of smooth muscle, vascular endothelial, and T cells into the atherosclerotic plaques and thus to plaque progression.9 It has been also demonstrated that in vascular endothelium, under hypoxia, IL-8 expression is increased by HIF.10,11 However, the relationships between HIF and IL-8 has been questioned by other authors.12

Adenosine (Ado) is a proangiogenic purine nucleoside released from ischemic and hypoxic tissues. Under these conditions, it is released into the extracellular space and signals through the stimulation of 4 extracellular G protein–coupled receptors named A1, A2A, A2B, and A3 (ARs).13 All 4 adenosine subtypes have been recently associated to the modulation of angiogenesis. Therefore, because of the link between ado, inflammation, and angiogenesis and the increasing evidence that these factors play a role in atherosclerosis, we thought to investigate HIF-1α, VEGF, IL-8, and FC...
formation by ado receptors in human macrophages and in an in vitro model of human FCs.

Methods
Please see the supplemental materials (available online at http://atvb.ahajournals.org) for more detailed methods.

Cell Culture
The human myelomonocytic cell line U937 was obtained from ATCC and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine (2 mmol/L), 100 U/mL penicillin, 100 μg/mL streptomycin, at 37°C in 5% CO2/95% air.

Preparation of Human Macrophages From Peripheral Blood
Peripheral blood mononuclear cells were isolated fromuffy coats by the Ficoll-Hypaque gradient. Monocytes were selected by adherence in RPMI 1640 medium containing 2 mmol/L glutamine, 5% human AB serum, 100 U/mL penicillin, and 100 μg/mL streptomycin and differentiated into human macrophages (HMs) by adherence over 7 days.

Hypoxic Treatment
Hypoxic exposures were done in a modular incubator chamber and flushed with a gas mixture containing 1% O2, 5% CO2 and balance N2 (MiniGalaxy, RSBiotech).

FC Formation
U937 cells were induced to differentiate into HMs by treatment with 40 nmol/L PMA for 72 hours and then incubated with oxLDL to form FCs (Intracel). Then all treatments to the cells with ado were carried out in the presence of the adenosine deaminase (ADA) inhibitor, EHNA 5 μmol/L, and those with ado agonists were performed in the presence of ADA.

Real-Time RT-PCR
Total cytoplasmic RNA was extracted and quantitative real-time RT-PCR assay were performed as previously reported.

Binding Experiments
Binding assays were carried out as we previously reported.

Western Blot Analysis
Detection of adenosine receptors, HIF-1α, HIF-2α, and phosphorylated proteins was carried out as we previously described. Immunoreactivity was assayed and quantified by using a VersaDoc Imaging System (Bio-Rad).

HIF-1 DNA Binding Activity
Nuclear extracts from U937, FCs, and HMs were prepared by using the Nuclear Extract Kit (Active Motif), and HIF-1α–binding activities in the nuclear extracts were detected by using an ELISA-based HIF binding kit (TransAM HIF-1, Active Motif) according to the manufacturer’s recommendations.

ELISA
The levels of VEGF and IL-8 protein secreted by the cells in the medium were determined by VEGF and IL-8 ELISA kits (R&D Systems) according to the manufacturer’s instructions.

Knockdown of ARs and HIF-1α by siRNA
Transfection of siRNA was performed at a concentration of 100 nmol/L using Lipofection 2000 in Opti-MEM (Invitrogen). A non-specific random control ribonucleotide sense strand (5’-ACU UCU GCA CGC UGA CdTdT-3’) and antisense strand (5’-dTdT UGA GAU AGA CGU GCG ACU G-3’) were used under identical conditions as we already reported.

Oil Red O Stain Analysis
Oil red O (in 60% isopropanol) staining was done for 15 minutes. Cells were viewed under a bright-field microscope in 100X fields using a Nikon Eclipse E800 microscope. The number of foam cells formed under each condition were calculated manually and presented as percentage foam cell formation.

Statistical Analysis
All values in the figures and text are expressed as mean±SE of n observation (with n≥3). Data sets were examined by analysis of variance (ANOVA) and Dunnett test (when required). A probability value less than 0.05 was considered statistically significant.

Results
Expression of ARs mRNA in U937, HMs, and FCs
mRNA expression of ARs was evaluated in U937, HMs, and FCs in normoxia and hypoxia. Hypoxia induced a significant increase of A2BRs in all the 3 cellular models investigated, while it did not change the level of the other ARs (supplemental Figure IA through ID).

Expression of ARs Protein in U937 Cells, HMs, and FCs
The protein evaluation of all ARs was examined, through immunoblots, in U937, HMs, and FCs in normoxia and hypoxia. We observed the presence of all ARs in the cells investigated according to mRNA data, as reported in supplemental Figure II through IH. These results were also confirmed by [3H]DPCPX, [3H]ZM 241385, [3H]MRE 2029F20, and [3H]MRE 3008F20 radioligands, used in receptor binding studies to evaluate affinity and density values of A1, A2A, A2B, and A3 ARs, respectively (supplemental Table I).

Ado Induces HIF-1α Protein Accumulation
To evaluate the effect of ado on HIF-1α protein accumulation, FCs, HMs, (Figure 1A and 1B, respectively) and U937 cells (supplemental Figure IIA) were incubated with ado 100 μmol/L in normoxia and hypoxia. In hypoxia ado stimulated HIF-1α accumulation, time-dependently, in all cells investigated. As for normoxia, ado effect slightly appears after 24 hours in FCs, whereas HIF-1α protein was undetectable in HMs and in U937 cells. In FCs the effect was similar with 50 or 100 μg/mL of oxLDL (data not shown), therefore the concentration of 50 μg/mL was used in all experiments. No changes in cell viability were observed after treatment of cells with ado 100 μmol/L for 24 hour of hypoxia (data not shown). Furthermore, treatment of ado stimulated, in a time-dependent way, HIF-1α DNA binding activity in hypoxia and also induced a minor but statistically
significant effect in normoxia in FCs, HMs (Figure 1C–D) and U937 cells (supplemental Figure IIB). Ado did not affect HIF-1α mRNA levels in normoxia and after 2 hours hypoxia, whereas it induced a slight increase of 1.6±0.1-, 1.9±0.1- and 1.5±0.1-fold after 4, 8, 24 hours of hypoxia, respectively (P<0.05 versus control); after addition of actinomycin D (actD), ado did not increase HIF-1α mRNA excluding a role in mRNA stability (supplemental Figure IIIA). The lack of mRNA modulation after 2 hours, time at which ado start to affect protein increase, suggests that ado does not affect transcription. Furthermore, we evaluated the ado-induced regulation of HIF-2α in hypoxia. Supplemental Figure IIIIB shows that ado slightly increased HIF-2α, and this effect was blocked by actD suggesting that at variance with HIF-1α, HIF-2α was transcriptionally regulated by ado.

Then we investigated the ado modulation of HIF-1α protein stability in hypoxia and normoxia. Ado in normoxia, at variance with hypoxia, increases HIF-1α stability; furthermore rapamycin, inhibitor of mTOR pathway, reduced ado effect suggesting also an increase in translation (supplemental Figure IVA through IVC). However, as the ado effect on HIF-1 was most evident in hypoxia, all the other experiments were carried out in this condition for 4 hours.

**Involvement of ARs in Ado-Induced HIF-1α Expression**

To evaluate which AR was involved in the ado-induced HIF-1α expression we treated FCs with antagonists of ARs before addition of ado in hypoxia. As shown in Figure 2A the ado effect was partially antagonized by 100 nmol/L DPCPX, SCH 58261, MRE 2029F20, and MRE 3008F20 suggesting the involvement of A1, A2A, A2B, and A3 ARs, respectively. Therefore we evaluated the effect of high affinity agonists, CHA, CGS 21680, Compound 24,21 and Cl-IB-MECA on HIF-1α accumulation. Probes selectivity is provided in supplemental Table II. All the agonists were able to induce HIF-1α in FCs (Figure 2B). Analogous results were obtained...
in U937 cells and in HMs (data not shown). Therefore in the second part of the work we focused our attention on FCs.

To further ascertain the involvement of the different ARs in the ado-induced HIF-1α accumulation we knocked-down ARs. After 48 and 72 hours posttransfection with siRNA targeting each AR, mRNA and protein levels were significantly reduced; the specificity of a given siRNA to the other AR subtypes is also shown in supplemental Figure V. Treatment of cells with siRNAs for A1, A2A, A2B, and A3 subtypes reduced the effect of ado on HIF-1α modulation supporting again a role for all ado subtypes in this effect; silencing of all ARs together abrogated the ado-mediated increase of HIF-1α protein (Figure 2C).

**Involvement of MAPK and Akt Pathways in ARs-Induced Modulation of HIF-1α**

To investigate the role of MAPK and Akt kinases in ARs-induced HIF-1α accumulation, we performed experiments with U0126, SB202190, and SH-5, inhibitors of MEK1/2, p38 MAPK, and Akt, respectively, in FCs. All the blockers were able to abrogate the effect induced by A1, A2A, and A2B agonists, whereas the A1-mediated HIF-1α accumulation was antagonized only by U0126 (Figure 3A). Addition of CHA, CGS 21680, and Compound 24 induced a concentration-dependent increase of pERK1/2, pp38, and pAkt, whereas CI-IB-MECA was involved only in ERK1/2 phosphorylation (Figure 3B).

**ARs Induce VEGF Increase in Hypoxia**

We tested VEGF production by FCs after ado treatment for 24 hours of hypoxia. Ado 100 μmol/L increased VEGF levels of 165±10%, and the effect was strongly reduced by MRE 2029F20 and MRE 3008F20 100 nmol/L suggesting the involvement of A2B and A3 ARs and inhibited to a lesser extent by the A2A antagonist (Figure 4A). DPCPX 100 nmol/L produce a moderate blunting of ado-induced VEGF release, but at this
concentration it can have antagonistic actions against A2B receptors (see supplemental Table II). Indeed a lower dose of DPCPX 10 nmol/L did not reduce ado effect (161 ±10%). U0126 and SB202190 followed by SH-5 were able to block the ado increase on VEGF levels. Treatment of the cells with siRNA of HIF-1α abrogated the VEGF increase induced by ado suggesting that the nucleoside was acting through HIF-1α modulation (Figure 4B). The increase induced by ado 100 μmol/L on VEGF was also observed at mRNA level (2.4 ±0.2-fold of increase, P<0.05 versus control). Other HIF-1α-responsive genes, aldolase and PGK were increased at mRNA level after ado treatment for 24 hours of 4.5 ±0.2- and 1.8 ±0.2-fold, respectively, and the effect was abrogated in the presence of HIF-1α siRNA, 1.1 ±0.1- and 1.0 ±0.1-fold, respectively (P<0.05 versus control).

A2BAR Induces IL-8 Increase in Hypoxia

We tested IL-8 production by FCs after ado treatment for 24 hours in hypoxia. Ado 100 μmol/L increased IL-8 levels of 158 ±10%, and the effect was blocked by MRE 2029F20 or A2B silencing, but not by DPCPX, SCH 58261 and MRE 3008F20 (Figure 4C and 4D). A dose–response curve of Compound 24 revealed an EC50 value of 58 ±6 nmol/L for stimulation of IL-8 secretion. The effect of Compound 24 1 μmol/L (142 ±8% of IL-8 secretion) was completely blocked by MRE 2029F20 (102 ±6% of IL-8 secretion). All these data suggest the involvement of A2B subtype in this response. U0126, SB202190, and SH5 were able to revert the ado increase on IL-8 levels suggesting a role for ERK 1/2, p38 and Akt pathways (Figure 4D). Finally, treatment of cells with siRNA of HIF-1α for 72 hours before stimulation
with ado shows that IL-8 modulation was not affected by HIF-1α silencing (Figure 4D). IL-8 was not altered by ado at mRNA level (1.14 ± 0.0-fold of increase versus control).

Oil Red O Staining in FCs
U937 cells without oxLDL did not contain high levels of neutral lipids and were not stained with Oil red O, a dye specific for neutral lipids (Figure 5). After treatment of U937 cells with 50 μg/mL ox-LDL for 24 hours, we observed FC formation characterized by large cytoplasmic lipid droplets. This effect was increased after incubation with ado 100 μmol/L, not significantly affected by DPCPX and SCH 58261, and strongly blocked by MRE 2029F20 and MRE 3008F20 antagonists and HIF-1α silencing, suggesting the involvement of HIF-1α and A2B and A3 ARs in the ado-induced FC formation. Also the high affinity A2B and A3 agonists were able to increase FC formation (Figure 5).

The normoxic modulation of HIF-1α by ado was only barely appreciated by means of western blotting experiments. However, by evaluating the HIF-1 DNA binding activity through an ELISA assay, ado was able to induce a significant increase of this response in hypoxia and a lower but significant effect in normoxia, according to the elegant study by De Ponti et al.27 This result also suggests that, in the case of low signals, ELISA approach on nuclear extract is more sensitive than Western blot on whole cell extracts.

Discussion
Hypoxia, HIF-1, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis.7,22–23 Furthermore, hypoxia stabilizes HIFs and leads to the accumulation of ado.5–24

This study reports, for the first time, that ado increases HIF-1α protein levels in U937, HM, and FCs in hypoxia as already observed in cancer cells,19,25–26 A2B and A3 subtypes play a major role in the VEGF increase and FC formation and only the A2B is responsible for IL-8 stimulation induced by adenosine.
MRE 2029F20, and MRE 3008F20, used at 100 nmol/L, a dose that may be considered selective for A1, A2A, A2B, and A3 ARs, respectively, were able to reduce HIF-1α protein accumulation induced by ado. The involvement of all ARs was also confirmed by the increase of HIF-1α protein levels induced by high-affinity AR agonists like CHA, CGS 21680, Compound 24, and Cl-IB-MECA for A1, A2A, A2B, and A3 ARs, respectively. Furthermore, we found that silencing of A1 or A2A, or A2B, or A3 ARs was able to reduce HIF-1α modulation induced by ado and that the simultaneous knocking down of all 4 ARs abrogated the ado effect. Addition of oxLDL did not modify the responses of FCs versus macrophages and U937 cells, but we concentrated on FCs because the effects of ado modulation of HIF-1α in this cellular type, crucial in atherosclerosis, have not been addressed before. However, ox-LDL are recognized by different receptors than minimally-oxidized LDL (mm-LDL), and it is likely that alternative LDL ligands such as mm-LDL might have different effects, with greater relevance to atherosclerosis.29 Different receptor subtypes have been reported to play a role in the ado-induced HIF-1α accumulation depending on the cellular model investigated.9,25–27,30–32 The results of this study suggest that HIF-1α accumulation may be triggered by all ARs in FCs analogously to their effect in activating other intracellular signaling factors like ERK1/2.33 For example, the ado-induced activation of myocardial ERK1/2 by statins has been found to involve A1, A2A, and A2B ARs in mice.34 It is well known that HIF-1 expression and activity, in addition to O2 concentration, are also regulated by important signal transduction pathways including those involving ERK/MAPK and Akt.55 As these pathways are also modulated by ado, our aim was to investigate the intracellular signaling triggered by this nucleoside in HIF-1α modulation. Our results show the involvement of ERK1/2, p38 MAPK, and Akt phosphorylation, whereas the Cl-IB-MECA effect was abrogated only by U0126. Indeed A1, A2A, and A2B Receptors activate ERK1/2, p38 MAPK, and Akt, whereas the A3 subtype was involved in the modulation of ERK1/2.

Several studies demonstrated a link between ado and HIF-1α at first in human cancer cell lines and then also in murine macrophages and in liver cells.19,25–26,30–31 In most of these cases its accumulation was related to an increase of VEGF, which regulates important functions associated with angiogenesis. According to these results we found that ado increased VEGF levels through A1 and A2B receptors and to a lesser extent by the A2A subtype and was dependent by HIF-1α, pERK1/2, pp38 MAPK, and pAkt. Recently, it has been reported that HIF-1α is also linked to IL-8 expression in human endothelial cells,11 whereas other authors point to different mechanisms of IL-8 regulation.12 IL-8 is another crucial angiogenic factor found to be expressed by FCs in human atheroma4,9 and is also modulated by ado in different cellular models by activation of A2A ARs.36 In this study we found that ado increased IL-8 secretion in hypoxic FCs through activation of the only A2B subtype. However, in agreement with other authors, its modulation was not dependent on HIF-1α accumulation, suggesting that other transcription factors, possibly AP-1, may be involved.12 Finally, as HIF-1α has been demonstrated to promote FC formation,8 we evaluated the involvement of ado in FC development. Our results clearly demonstrate that ado increases FC formation and that this effect is strongly reduced by A1 and A2B antagonists and by silencing HIF-1α; this suggests that under hypoxic conditions, ado, by increasing HIF-1α through activation of A1 and A2B ARs, promotes FC formation. The marginal role of the A2A agonist in the modulation of this effect may be in line with recent relevant studies carried out by Reiss and Cronstein. These authors demonstrated that A2A agonists in normoxic conditions inhibited FC formation in stimulated THP-1 macrophages by increasing expression of cholesterol 27-hydroxylase and adenosine 5’-triphosphate-binding cassette transporter A1, which are proteins involved in reverse cholesterol transport.37 The same authors demonstrated that A2A receptors were responsible for the atheroprotective effects induced by methotrexate.38 Therefore, it seems that adenosine by regulating FCs may play both anti- or proatherogenic effects, depending on the receptors activated and the oxygen conditions present.

Altogether, these data suggest that in hypoxic conditions ado, through A1 and A2B AR activation, induces HIF-1α protein accumulation thus leading to an increase of VEGF secretion and of FC formation; in addition, the A2B subtype is responsible for IL-8 accumulation. Therefore AR antagonists and in particular A1 and A2B or mixed A1/A2B blockers may be useful to block important steps in the atherosclerotic plaque development mediated by ado.

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Disclosures
None.

References


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Supplemental Material

Materials and Methods

5-N-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2(2furyl)-pyrazolo-[4,3e]-1,2,4-triazolo [1,5-c] pyrimidine ($[^{3}H]$MRE 3008F20, specific activity 67 Ci mmol$^{-1}$), N-benzo[1,3]dioxol-5-yl-2-[5-(1,3-dipropyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yl-oxy]-acetamide ($[^{3}H]$MRE 2029F20, specific activity 123 Ci mmol$^{-1}$) were obtained from Amersham International (Buckinghamshire, UK), (4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo-[2,3-2][1,3,6]triazinyl-amino]ethyl)-phenol) ($[^{3}H]$ZM 241385, specific activity 20 Ci mmol$^{-1}$) was purchased from Tocris (Boston, Mass; USA) and 1,3-dipropyl-8-cyclopentyl-xanthine ($[^{3}H]$DPCPX, specific activity 120 Ci mmol$^{-1}$) was derived from NEN Research Products (Boston, Mass; USA). 7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]-pyrimidine (SCH 58261), 1-Deoxy-1-[6-{4-[(phenylcarbamoyl)-methoxy]phenylamino}-9H-purin-9-yl]-N-ethyl-β-D-ribofuranuronamide (Compound 24) were synthesized by Prof. P.G. Baraldi (Department of Pharmaceutical Sciences, University of Ferrara, Italy). Phorbol myristate acetate (PMA), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), cyclohexyl-adenosine (CHA), 2-[p-(carboxyethyl)-phenethylamino]-NECA (CGS 21680), N$^{6}$-(3iodobenzyl)-2-chloroadenosine-5′N-methyluronamide (Cl-IB-MECA), rapamycin were purchased by Sigma Aldrich (Milano, Italy). The antibodies for A$^{1}$, A$^{2A}$, A$^{2B}$ adenosine receptor subtypes were purchased from Alpha Diagnostic (S. Antonio, Texas, USA) and the antibody for A$^{3}$ was from AVIVA System Biology (Milano, Italy). Human anti-HIF-1α and HIF-1β antibodies were obtained from BD Transduction Laboratories (Milano, Italy). The Anti-ACTIVE® mitogen-activated protein kinase anti-ERK1/2 (pAb) was from Promega (Milano, Italy). Phospho-Akt (Ser473) and phospho-p38 (Thr180/Tyr182) were purchased from Cell Signaling Technology (Milano, Italy). Ficoll-Hypaque was obtained by Amersham Pharmacia Biotech AB. The assays-on-demandTM. Gene expression Products NM 000674, NM 000675, NM 000676 and NM 000677 for A$^{1}$, A$^{2A}$, A$^{2B}$ and A$^{3}$ adenosine subtypes and the assays-on-demandTM Gene expression Products Hs00936368_m1, Hs00173626_m1, Hs00174103_m1, Hs00765620_m1,
Hs99999906_m1, for HIF-1α, VEGF, IL-8, Aldolase A and Phosphoglycerate kinase (PGK) were purchased from Applera (Milano, Italy). A1, A2A, A2B, A3AR, HIF-1α small interfering RNAs (siRNAs) and HIF-2α antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Nuclear Extract Kit and HIF-1α binding activities were from Active Motif (Belgium). All other reagents were of analytical grade and obtained from commercial sources.

Cell culture- The human myelomonocytic cell line U937 was obtained from ATCC and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), 100 U/ml penicillin, 100 µg/ml streptomycin, at 37°C in 5% CO2/95% air.

Preparation of human macrophages (HM) from peripheral blood- Peripheral blood mononuclear cells were isolated from buffy coats by the Ficoll-Hypaque gradient (Amersham Pharmacia Biotech AB) as described previously.1 Monocytes were selected by adhesion in RPMI 1640 medium containing 2 mM glutamine, 5% human AB serum (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin and differentiated into HM by adhesion over 7 days.

Hypoxic treatment- Hypoxic exposures were done in a modular incubator chamber and flushed with a gas mixture containing 1% O2, 5% CO2 and balance N2 (MiniGalaxy, RSBiotech, Irvine, Scotland).

FC formation- U937 cells were induced to differentiate into HM by treatment with 40 nM phorbol myristate acetate (PMA) for 72 hours. Before use oxLDL (Intracel, Frederick, MD) were dialyzed against 1 liter of 0.15 M sodium chloride and 0.3 mM EDTA (pH 7.4) for 12 h at 4 °C, then against RPMI 1640 medium (two changes, 1 liter/each change) for 24 h. All dialysis was carried out with Pierce Slide-A-Lyzer cassettes (10,000 molecular weight cut-off). After dialysis, lipoproteins were sterilized by passing them through a 0.45 µm (pore-size) filter and added (50 or 100 µg/ml) to PMA-treated U937 cells for 48 h in serum-free RPMI.2 Then all treatments to the cells with ado were carried out in the presence of the adenosine deaminase (ADA) inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) 5 µM and those with ado agonists were performed in the presence of ADA.
**Real-time RT-PCR**—Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method. Quantitative real-time RT-PCR assay was performed as previously described.\(^3\) The assays-on-demand\(^\text{TM}\) Gene expression Products NM 000674, NM 000675, NM 000676 and NM 000677 for A1, A2A, A2B and A3 ARs were used, respectively. Quantification of AR messages was made by interpolation from standard curve of Ct values generated from the plasmid dilution series.\(^4\) Analogue results were obtained when the expression level of ARs was normalized to that of β-actin. For the real-time RT-PCR of HIF-1α, VEGF, IL-8, Aldolase A and PGK the assays-on-demand\(^\text{TM}\) Gene expression Products Hs00936368_m1, Hs00173626_m1, Hs00174103_m1, Hs00765620_m1, Hs99999906_m1 were used, respectively. For the real-time RT-PCR of the reference gene the endogenous control human β-actin kit was used, and the probe was fluorescent-labeled with VIC\(^\text{TM}\) (Applera).

**Binding experiments**—Binding assays were carried out as reported previously.\(^4\) In saturation experiments, membranes (70 µg of protein/assay) were incubated with 50 mM Tris HCl buffer (10 mM MgCl\(_2\) for A2A; 10 mM MgCl\(_2\), 1 mM EDTA, 0.1 mM benzamidine for A2B and 10 mM MgCl\(_2\), 1 mM EDTA for A3) pH 7.4 and increasing concentrations of 1,3-dipropyl-8-cyclopentyl-xanthine ([\(^3\)H]DPCPX) (0.4-40 nM); (4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo-[2,3-d]-[1,3,6]-triazinyl-amino] ethyl)-phenol) ([\(^3\)H]ZM 241385) (0.3-30 nM); N-benzo[1,3]dioxol-5-yl-2-[5-(1,3-dipropyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yl-oxy]-acetamide ([\(^3\)H]MRE 2029F20) (0.4-40 nM); 5-N-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2furyl)-pyrazolo-[4,3c]-1,2,4-triazolo [1,5-c] pyrimidine ([\(^3\)H]MRE 3008F20) (0.4-40 nM) to label A1, A2A, A2B and A3 ARs, respectively. The filter bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20.

**Western Blot Analysis**—Whole cell lysates were prepared as described previously.\(^5\) ARs were evaluated by using specific antibodies towards human A1, A2A, A2B (Alpha Diagnostic) and A3 ARs (Aviva) (1:1000 dilution). In experiments aimed to detect HIF, western blot analyses were
performed using antibody against HIF-1α (1:250 dilution) (BD Biosciences), HIF-2α (1:1000) (Novus Biologicals) and HIF-1β (1:1000 dilution) (BD Biosciences) in 5% non-fat dry milk in PBS/0.1% Tween-20 overnight at 4 °C. For detection of phosphorylated proteins antibodies specific for phosphorylated (Thr183/Tyr185) p44/p42 MAPK (1:5000 dilution), phosphorylated (Thr180/Tyr182) p38 MAPK (1:1000 dilution) and phosphorylated Akt (Ser473) (1:1000 dilution) were used. The protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL). Tubulin (1:250) was used to ensure equal protein loading. Immunoreactivity was assessed and quantified by using a VersaDoc Imaging System (Bio-Rad).

**HIF-1 DNA binding activity**

Nuclear extracts from U937, FC and HM were prepared by using the Nuclear Extract Kit (Active Motif) and HIF-1α binding activities in the nuclear extracts were detected by using an ELISA-based HIF binding kit (TransAM™ HIF-1, Active Motif) according to the manufacturer’s recommendations.

**Enzyme-Linked Immunosorbent Assay (ELISA)** - The levels of VEGF and IL-8 protein secreted by the cells in the medium were determined by VEGF and IL-8 ELISA kits (R&D Systems) according to the manufacturer’s instructions. The data were presented as mean ± SD from four independent experiments.

**Knockdown of ARs and HIF-1α by small interfering RNA (siRNA)** - FC were plated in six-well plates and grown to 50–70% confluence before transfection. Transfection of siRNA was performed at a concentration of 100 nM using Lipofection 2000 in Opti-MEM (Invitrogen). A non-specific random control ribonucleotide sense strand (5’-ACU CUA UCU GCA CGC UGA CdTdT-3’) and antisense strand (5’-dTdT UGA GAU AGA CGU GCG ACU G-3’) were used under identical conditions as already reported.\(^5\) A\(_1\), A\(_2\)A, A\(_2\)B, A\(_3\)AR and HIF-1α siRNAs were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Oil red O-stain analysis** - Treatment of PMA-differentiated U937 cells with ado and ado ligands was performed before addition of oxLDL. After exposition to oxLDL under hypoxia for 24 h cells
were fixed in saline-buffered 4% paraformaldehyde solution for 15 min and then air dried. Oil red O (in 60% isopropanol) staining was done for 15 min essentially as described before.\(^6\) Cells were viewed under a bright-field microscope in 100 X fields using a Nikon’s Eclipse E800 microscope. FC were defined as macrophages in which cytoplasm was filled with Oil Red O-stainable lipid droplets.

**MTS Assay.** The MTS assay was performed to determine foam cells viability according to the manufacturer's protocol from the CellTiter 96 AQueous One Solution (Promega) cell proliferation assay. Cells \((10^5)\) were plated in 24-multiwell plates; 500 µl of complete medium was added to each well with ado. The cells were then incubated for 24 h. At the end of the incubation period, MTS solution was added to each well. The optical density of each well was read on a spectrophotometer at 570 nm. Experiment was repeated three times.

**Statistical analysis**- All values in the figures and text are expressed as mean ± standard error (S.E.) of N observation (with N ≥ 3). Data sets were examined by analysis of variance (ANOVA) and Dunnett’s test (when required). A P-value less than 0.05 was considered statistically significant.
Supplemental Figure Legends

Figure 1 - mRNA and protein expression of ARs in U937 cells, HM and FC in normoxia (N) and hypoxia (H). Bargraph showing µg mRNA/µg total RNA of human A₁, A₂ₐ, A₂ₚ and A₃ ARs (A-D, respectively). ARs detection by western blot analysis (E-H). Cellular extracts were prepared and subjected to immunoblot assay using anti-A₁, A₂ₐ, A₂ₚ and A₃ antibodies. Tubulin shows equal loading protein. Values are the means and vertical lines S.E. of the mean of four separate experiments performed in triplicate. *P<0.05 compared with normoxia.

Figure 2 - Time course of HIF-1α modulation induced by ado. Effect of 100 µmol/L Ado on HIF-1α protein expression (panel A) and DNA binding activity (panel B) in U937 cells, in normoxia (N) and hypoxia (H). HIF-1β shows equal loading protein. Densitometric quantification of HIF-1α western blots is the mean ± S.E. values (N=3); *P<0.05 compared with 2 h hypoxia in the absence of Ado; #P<0.05 compared with cells in the absence of Ado at each time. DNA binding activity data are means ± S.E (N=3); *P<0.05 compared with 24 h normoxia in the absence of Ado; #P<0.05 compared with cells in the absence of Ado at each time.

Figure 3- Effect of actinomycin D (ActD) on HIF-1α and HIF-2α in foam cells. Real-time RT-PCR analysis of HIF-1α mRNA level. Total RNA was extracted from cells treated in the absence or in the presence of Ado for 2,4,8,24 h hypoxia before and after addition of ActD (10 µg/ml). Data were expressed as fold of increase vs control arbitrarily fixed as 1 (cells in the absence of Ado). *P<0.05 compared with control (A). Western blot analysis of HIF-1α and HIF-2α protein level after treatment with 100 µmol/L Ado in the absence and in the presence of ActD. Densitometric quantification of western blots is the mean ± S.E. values (N=3); *P<0.05 compared with control (cells in the absence of Ado) (B).

Figure 4- Effect of cycloheximide (CHX) on induction of HIF-1α in foam cells. Western blot analysis of HIF-1α protein level. Cells were treated in hypoxia without or with 100 µmol/L ado for 4 hours, 1 µmol/L CHX was added to inhibit new HIF-1α protein synthesis, and incubation was continued for 0.25, 0.5, 1, 2 and 4 h (A). Densitometric quantification of HIF-1α western blots is the
mean ± S.E. values (N=3); *P<0.05 compared with control (cells in the absence of Ado and CHX treated in hypoxia for 4 h), # P<0.05 compared with control (cells in the absence of CHX treated in hypoxia plus Ado for 4 h) (B). Effect of CHX and 100 nmol/L rapamycin on HIF-1α DNA binding activity induced by ado in normoxia (C). Cells were treated in normoxia without or with 100 µmol/L ado for 8 hours, 1 µmol/L CHX was added to inhibit new HIF-1α protein synthesis, and incubation was continued for 0.25, 0.5 and 1 h. Cells were treated in normoxia without or with 100 µmol/L ado for 8 hours in the presence of rapamycin. Nuclear extracts were isolated and subjected to a colorimetric assay. Means ± S.E (N=3); *P<0.05 versus control (cells in the absence of CHX treated in normoxia plus Ado for 8 h).

Figure 5 - ARs silencing by siRNA transfection in FC. Relative ARs mRNA quantification, related to β-actin mRNA, by real-time RT-PCR; FC were transfected with siRNA of A1, A2A, A2B and A3 ARs (A-D, respectively) and cultured for 24, 48 and 72 h. Plots are mean ± S.E. values (N = 3); *P < 0.05 compared with the control (time = 0). Western blot analysis using anti A1, A2A, A2B and A3 receptor polyclonal antibodies (E-H, respectively), of protein extracts from FC treated with siRNA of each AR subtype and cultured for 24, 48 and 72 h. Tubulin shows equal loading protein. Specificity of adenosine receptors siRNAs (I). Western blot analysis using anti A1, A2A, A2B and A3 receptor polyclonal antibodies of protein extracts from FC transfected with control ribonucleotides (ctr.) or with siRNA of each AR subtype and cultured for 72 h. Tubulin shows equal loading protein.
Figure 1E-H

E

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A1 50kDa

Tubulin

F

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A2A 50kDa

Tubulin

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A2B 37 kDa

Tubulin

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A3 50kDa

Tubulin
Figure 2

A

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Ado 100 μM

HIF-1α

110 kDa

HIF-1β

B

DNA binding activity (Absorbance)

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* * *

H

Ado 100 μM

hours

2 4 8 24

* #
Figure 3

A

B

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HIF-2α

HIF-1β

HIF-1α

HIF-1β
**Figure 4**

### A

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**HIF-1α**

110kDa

**HIF-1β**

### B

### C
Figure 5I

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<th>A2B</th>
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Arrows indicate:
- A1
- A2A
- A2B
- A3
- Tubulin
Table 1- Affinity (K_D, nM) and density (Bmax, fmol/mg of protein) values of A_1, A_{2A}, A_{2B} and A_3 ARs evaluated through [^3H]DPCPX, [^3H]ZM 241385, [^3H]MRE 2029F20 and [^3H]MRE 3008F20 radioligands, respectively, in PMA-treated U937, HM and FC cells in normoxia (N) and hypoxia (H).

<table>
<thead>
<tr>
<th></th>
<th>A_1</th>
<th></th>
<th>A_{2A}</th>
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<th>A_{2B}</th>
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<td>4.0±0.3</td>
<td>52±6</td>
<td>2.8±0.3</td>
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<td>33±3</td>
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<td>235±26</td>
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<td>4.4±0.4</td>
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<td>73±6</td>
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<td>HM N</td>
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<tr>
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Table 2-Affinity (Ki, nM) of selected adenosine receptor agonists and antagonists to $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ adenosine receptors.

<table>
<thead>
<tr>
<th></th>
<th>$A_1$</th>
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References


