Mitochondria as signaling organelles in the vascular endothelium

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Contribution of Salvador Moncada, February 8, 2006

**Results**

**Contribution of Mitochondrial Respiration and Glycolysis to the Generation of ATP in Human Umbilical Vein Endothelial Cells (HUVECs).** The role of mitochondrial O2 consumption on ATP production in HUVECs was investigated by measuring ATP concentrations ([ATP]) after exposing the cells to different O2 concentrations (21, 3, 1.5, and 0.5%) for up to 2 h. Table 1 shows a small (≈25%) but significant decrease in [ATP] when the O2 concentration was 1.5% or below. The use of pharmacological electron transport chain inhibitors such as rotenone or antimycin at ambient O2 concentration had similar effects, causing an ATP reduction of ≈30%. No further reduction in [ATP] was seen when electron transport chain-inhibited cells were incubated at a lower O2 concentration (i.e., <0.5%). Pretreatment of the cells with an inhibitor of the glycolytic pathway [20 mM 2-deoxy-D-glucose (2DG)] led to a greater decrease in the intracellular [ATP] (≈50%) at any O2 concentration studied. The AMP:ATP ratio from cells exposed to 3% O2 was not significantly different from those at 21% O2 (Table 2). However, a significant increase (≈2-fold) in the AMP:ATP ratio was observed after incubation of HUVECs with 2DG; this increase was even greater (6- to 7-fold) when 2DG was combined with rotenone.

**NO Prevents the Accumulation of HIF-1α at Low Oxygen Concentrations.** To investigate the effect of constitutively generated NO on the stabilization of HIF-1α by low O2 concentration, we exposed HUVECs to a variety of O2 concentrations (21%, 6%, 3%, 1.5%, and 0.5%) in the presence or absence of the NO synthase inhibitor Nω-monomethyl-L-arginine (L-NMMA). Unlike other human cells [smooth muscle cells and human epithelial kidney cells (HEK293)], no significant accumulation of HIF-1α was observed in HUVECs until the O2 concentration was reduced to 0.5% O2 (Fig. 1A). Treatment of HUVECs with L-NMMA led to HIF-1α stabilization at a higher O2 concentration compared with control cells (Fig. 1A) but did not affect the O2 concentration at which nonendothelial cells stabilize HIF-1α (data not shown). Human microvascular endothelial cells, which also generate NO from endothelial NO synthase (eNOS) (16), responded in a similar manner to HUVECs when they were exposed to low O2 concentrations in the absence or presence of the NOS inhibitor (Fig. 1B). In cells that were pretreated with the inhibitor of mitochondrial respiration myxothiazol, however, L-NMMA did not affect HIF-1α stabilization at 1.5% O2, indicating that inhibition of the electron transport chain (and subsequent redistribution of O2) was responsible for the observed destabilization of HIF-1α (Fig. 1C). Studies on DNA binding in nuclear extracts confirmed the activation of HIF-1α.

Conflict of interest statement: No conflicts declared.

Abbreviations: AMPK, AMP-activated protein kinase; HIF-1, hypoxia-inducible factor 1; HUVEC, human umbilical vein endothelial cell; DHE, dihydroethidine; eNOS, endothelial NO synthase; ROS, reactive oxygen species; L-NMMA, Nω-monomethyl-L-arginine; 2DG, 2-deoxy-D-glucose.

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**Endogenous nitric oxide (NO) is a highly diffusible gas that has a variety of physiological functions, some of which are mediated by activation of the soluble guanylate cyclase enzyme (1). In the last decade cytochrome c oxidase, the terminal enzyme in the mitochondrial electron transport chain, has also been identified as a target of the action of NO (2–4). Acting on the latter enzyme, NO can regulate cellular oxygen (O2) consumption (5) and the mitochondrial redox state, facilitating the release of free radicals, which act as a signaling mechanism (6). Furthermore, inhibition of mitochondrial O2 consumption by NO leads to a situation in which, though O2 might be available, cells and tissues are unable to use it. This phenomenon has been termed “metabolic hypoxia,” a condition that differs from true hypoxia in which O2 availability is insufficient (5). In metabolic hypoxia there is also a redistribution of O2 away from mitochondria toward nonrespiratory O2-dependent targets (7). Inhibition of cell respiration by NO is also known to activate glycolysis in some cells through a mechanism involving activation of 6-phosphofructo-2-kinase (8).

Although we have previously demonstrated that endogenous NO regulates O2 consumption in vascular endothelial cells and other cell types (RAW264.7) (6, 9), the consequences of this effect have yet to be studied in detail. Of particular interest are the consequences in vascular endothelial cells, which have been known for some time to be glycolytic (10) and to possess high energetic functions.

Vascular endothelial cells are highly glycolytic and consume relatively low amounts of oxygen (O2) compared with other cells. We have confirmed that oxidative phosphorylation is not the main source of ATP generation in these cells. We also show that at a low O2 concentration (<1%) endogenous NO plays a key role in preventing the accumulation of the α-subunit of hypoxia-inducible factor 1. At higher O2 concentrations (1–3%) NO facilitates the production of mitochondrial reactive oxygen species. This production activates the AMP-activated protein kinase by a mechanism independent of nucleotide concentrations. Thus, the primary role of mitochondria in vascular endothelial cells may not be to generate ATP but, under the control of NO, to act as signaling organelles using either O2 or O2-derived species as signaling molecules. Diversion of O2 away from endothelial cell mitochondria by NO might also facilitate oxygenation of vascular smooth muscle cells.

AMP-activated protein kinase | hypoxia-inducible factor 1α | hypoxia | nitric oxide
Control (Fig. 1D). To investigate whether the observed effect of L-NMMA was due to inhibition of NO production, eNOS expression in endothelial cells was blocked by using RNA interference methodology. Western blot analysis of eNOS short-hairpin RNA-infected cells showed a reduction (80%) of eNOS expression (Fig. 1E) and HIF-1α stabilization at a higher O2 concentration (i.e., 1.5%) compared with mock-infected cells (0.5%) (Fig. 1F).

Reactive Oxygen Species (ROS) Generation Depends on Oxygen Concentrations. The production of superoxide ($O_2^-$) was studied in HUVECs incubated in the presence or absence of antioxidants (50 μM MnTBAP plus 1 mM ascorbic acid) at different O2 concentrations. Fig. 2A shows representative confocal images of the cells after incubation with dihydroethidine (DHE) at 21% and 3% O2. Fig. 2B summarizes the quantitative analysis of DHE oxidation product measurements. No significant difference was observed in the fluorescence of cells incubated at 21% in the presence or absence of antioxidants. When cells were incubated at 3% O2 however, there was a significant increase in the fluorescence of control cells, which was prevented by antioxidants (6.38 ± 0.73 vs. 3.41 ± 1.01 arbitrary units for control and antioxidant-treated cells, respectively). To address the role of NO in the generation of ROS observed at 3% O2, HUVECs were incubated with and without 1-NMMA at 21% and 3% O2. Inhibition of NO synthesis abolished the increase in the fluorescence observed at 3%, but it did not affect the fluorescence at 21% (Fig. 2B). Cells incubated at 0.5% O2 did not show an increase in fluorescence.

AMPK Activation Depends on O2 Concentration. When tested at different O2 concentrations, the activation of AMPK was found to be undetectable at 21% O2, to be maximal at 3% O2, and to decrease at lower O2 concentrations (Fig. 3A). Activation at 3% O2, however, did not appear to be mediated by AMP, because the AMP:ATP ratio was not different from that observed under normoxic conditions (Table 2). Moreover, AMP levels did not seem to increase even when O2 concentrations were reduced further, despite the fact that under these conditions a small decrease in ATP was evident (compare Table 2 with Table 1). In contrast, at 21% O2, treatment with the glycolytic inhibitor 2DG, alone or together with a mitochondrial inhibitor, increased the AMP:ATP ratio substantially (Table 2) and activated AMPK (Fig. 3B).

Table 1. Bioenergetic assessment in HUVECs

<table>
<thead>
<tr>
<th>[O2]</th>
<th>Control</th>
<th>2DG</th>
<th>Rotenone</th>
<th>Antimycin</th>
<th>2DG plus rotenone</th>
</tr>
</thead>
<tbody>
<tr>
<td>21%</td>
<td>11.3 ± 1.1</td>
<td>5.7 ± 0.9**</td>
<td>9.6 ± 1.0**</td>
<td>7.3 ± 0.5**</td>
<td>0.6 ± 0.1**</td>
</tr>
<tr>
<td>3%</td>
<td>9.3 ± 1.7</td>
<td>5.3 ± 1.0**</td>
<td>7.2 ± 1.4**</td>
<td>7.8 ± 1.5</td>
<td>0.5 ± 0.3**</td>
</tr>
<tr>
<td>1.5%</td>
<td>9.1 ± 0.6*</td>
<td>4.2 ± 0.2**</td>
<td>9.3 ± 1.1</td>
<td>7.2 ± 1.3**</td>
<td>0.6 ± 0.3**</td>
</tr>
<tr>
<td>&lt;0.5%</td>
<td>8.3 ± 1.2*</td>
<td>2.9 ± 0.4**</td>
<td>8.5 ± 1.1</td>
<td>7.7 ± 0.7</td>
<td>0.4 ± 0.1**</td>
</tr>
</tbody>
</table>

Effects of glycolytic and mitochondrial inhibitors on [ATP], at different O2 concentrations. Treatments were carried out for 2 h at the indicated O2 concentration before [ATP] was determined by chemiluminescence. For details of procedures, see Materials and Methods. Data represent the mean ± SEM of at least four independent experiments. 2DG, 20 mM; rotenone, 0.5 μM; antimycin, 0.5 μM. *, Significantly different from control (P < 0.05); **, significantly different from respective control (P < 0.05).

Table 2. AMP:ATP ratios in HUVECs

<table>
<thead>
<tr>
<th>[O2]</th>
<th>CTRL</th>
<th>2DG</th>
<th>2DG plus rotenone</th>
</tr>
</thead>
<tbody>
<tr>
<td>21%</td>
<td>0.095 ± 0.010</td>
<td>0.187 ± 0.009**</td>
<td>0.664 ± 0.012**</td>
</tr>
<tr>
<td>3%</td>
<td>0.091 ± 0.024</td>
<td>0.176 ± 0.017*</td>
<td>0.657 ± 0.055**</td>
</tr>
<tr>
<td>1.5%</td>
<td>0.127 ± 0.007</td>
<td>0.291 ± 0.013**</td>
<td>1.007 ± 0.148**</td>
</tr>
<tr>
<td>&lt;0.5%</td>
<td>0.116 ± 0.030</td>
<td>0.362 ± 0.029*</td>
<td>1.157 ± 0.172**</td>
</tr>
</tbody>
</table>

Effects of glycolytic and ETC inhibitors on AMP:ATP ratios at different O2 concentrations. Treatments were carried out for 2 h before samples were processed. Each individual sample was neutralized, centrifuged, and filtered before HPLC separation. For further details of procedures, see Materials and Methods. Data represent the mean ± SEM of at least three independent experiments. 2DG, 20 mM; rotenone, 0.5 μM. *, Significantly different from control (P < 0.05); **, significantly different from control (P < 0.01).

ROS-Dependent AMPK Activation at Low O2 Concentration in HUVECs. The contribution of ROS to AMPK activation was investigated by treatment of HUVECs with different prooxidant regimens. Fig. 4A shows that incubation of HUVECs with tert-butyldihydroperoxide led to the phosphorylation of AMPK at 21% O2. Similarly, exposure of HUVECs to angiotensin II, which is known to activate NADPH oxidase (17), resulted in AMPK activation in a dose-dependent manner (Fig. 4B). Based on these results, we investigated whether the observed activation of AMPK at 3% O2 was due to the ability of HUVECs to generate ROS under these conditions. As shown in Fig. 5A, the presence of antioxidants (MnTBAP plus ascorbic acid) led to a decrease in the amount of phosphorylated AMPK (pAMPK) in HUVECs exposed to 3% O2. Similar results were observed with β0 cells, which lack functional mitochondria (Fig. 5B).

Discussion

We have found that endothelial cells from various origins, including microvascular endothelium, stabilize HIF-1α at a lower O2 concentration than other cells. Indeed, whereas human epithelial kidney and smooth muscle cells stabilize this transcription factor at 3% O2, the endothelial cell does this only at <1% O2. Our experiments suggest that this observation, which has been described previously but not remarked on (18, 19), may be attributed, at least in part, to NO acting on the mitochondrion to divert O2 away from it toward the cytosol; this diversion maintains the activation of the prolyl hydroxylases as we have reported before (7). This hypothesis is supported by the observation that treating HUVECs with L-NMMA to inhibit NO generation or silencing eNOS both lead to an increase in the O2 concentration at which HIF-1α is stabilized, i.e., to 1.5%. Furthermore, this effect of L-NMMA is not observed in cells that have previously been treated with myxothiazol to inhibit their mitochondrial O2 consumption, thus adding support to the contention that HIF-1α destabilization by NO occurs as a consequence of the redistribution of O2 to the prolyl hydroxylases.

A ROS-dependent mechanism has been suggested to play a role in HIF-1α stabilization in hypoxia in different cell types (20). This does not seem to be the case in HUVECs, where our
results show that the highest release of ROS occurs at an O2 concentration around 3%. At this point we did not observe maximal HIF-1α stabilization; however, we did observe HIF-1α stabilization at a very low O2 concentration, at which the release of ROS is greatly decreased or disappears altogether (21). Nevertheless, evidence for a mitochondrial ROS involvement in HIF-1α stabilization is documented in other systems; therefore, the reasons for the absence of this mechanism in endothelial cells deserve further investigation.

HIF-1α stabilization at a very low O2 concentration, at which the release of ROS is greatly decreased or disappears altogether (21). Nevertheless, evidence for a mitochondrial ROS involvement in HIF-1α stabilization is documented in other systems; therefore, the reasons for the absence of this mechanism in endothelial cells deserve further investigation.

Fig. 1. Effect of endogenous NO on HIF-1α accumulation at different O2 concentrations. HIF-1α protein levels were detected by Western blotting of nuclear extracts from HUVECs, HEK293 cells, and smooth muscle cells (A) or human microvascular endothelial cells (B) exposed for 8 h to the indicated O2 concentration in the absence or presence of L-NMMA (1 mM). (C) HIF-1α protein levels from nuclear extract of HUVECs exposed to 1.5% O2 in the presence or absence of L-NMMA and myxothiazol. (D) HIF-1α DNA-binding activity in nuclear extracts from HUVECs exposed to O2 concentration as in A. The values represent the mean ± SEM from three independent experiments. (E) HUVECs infected with short-hairpin RNA eNOS exhibited markedly reduced eNOS expression levels after 2 weeks when compared with wild-type cells. GAPDH was used as protein loading control. (F) HIF-1α protein levels from nuclear extract of silenced eNOS cells exposed to different O2 concentrations. Values in brackets are the estimations from densitometry analysis.

Fig. 2. ROS generation in HUVECs at different O2 concentrations. (A) Representative confocal images of HUVECs exposed to two different O2 concentrations. (B) Fluorescence of DHE oxidation products, as measured by flow cytometry, from intact cells incubated with DHE indicating intracellular superoxide production in the presence or absence of L-NMMA and antioxidants (50 μM MnTBAP plus 1 mM ascorbic acid) at 21%, 3%, and 0.5% O2, MFI, mean fluorescence intensity. *, significantly different from control (C) 21% (P < 0.05); †, significantly different from control (C) 3% (P < 0.05).

Fig. 3. Effect of O2 concentration and metabolic inhibitors on AMPK activation in HUVECs. (A) HUVECs were incubated for 2 h at different O2 concentrations (21%, 3%, 1.5%, and <0.5%) in a controlled hypoxic chamber, and AMPK activation was assessed by Western blotting. (B) AMPK activation by bioenergetic crisis under normoxic conditions. Phosphorylated AMPK (pAMPK) in HUVECs treated with different glycolytic and mitochondrial inhibitors (20 mM 2DG and 0.5 μM rotenone) was assessed by Western blotting. These are representative data from at least three independent experiments. The values in brackets are the estimations from densitometry analysis. Rot, rotenone; AMPK, loading control.
whether mitochondria also play a key role in diverting O2 from the other substrates such as glutamine and palmitate, especially in situations in which glucose is low (27) or when fatty acids are increased (31). Therefore, our results suggest that mitochondria in HUVECs are not preferentially used for bioenergetic purposes; instead, under the control of NO, they seem to be acting as signaling organelles. Indeed, at a significantly higher O2 concentration than that at which HIF-1α is stabilized, AMPK is activated through a mechanism that is mitochondria-dependent but independent of changes in nucleotide levels.

Since its discovery, AMPK has been suggested to serve as an energy gauge in cells, detecting changes in the AMP:ATP ratio (32). The consequences of AMPK activation include inhibition of acetyl CoA carboxylase and fatty acid synthesis, activation of 6-phosphofructo-2-kinase and glycolysis, β-oxidation promotion, and, as recently claimed, modulation of gene expression (33, 34). Recently, however, activation of the enzyme by mechanisms independent of the AMP:ATP ratio has been described (35, 36), including a ROS-mediated mechanism (37–39).

Our experiments indicate that AMPK activation in HUVECs at low O2 concentrations depends on mitochondrial ROS generation because (i) it coincides with the O2 concentration at which maximum release of ROS is observed, (ii) the use of antioxidants prevents AMPK activation, and (iii) it does not occur in rat HUVECs devoid of functional mitochondria. A ROS-mediated mechanism for AMPK activation is further supported by our experiments using a prooxidant such as tert-butylhydroperoxide or angiotensin II, as others have previously demonstrated (40).

The activation of AMPK by a ROS-dependent mechanism is intriguing and might indicate a role in cellular defense. Indeed, a protective role for AMPK activation in endothelial cells has recently been suggested related to cardioprotection and the inactivation of caspase-3 (41, 42). It is not known whether other protective mechanisms are also activated. Release of ROS has been shown to contribute to the activation of other transduction mechanisms involved in cellular defense such as NF-κB (43), AP1 (44), and p53 (45). It has also been claimed to mediate pharmacological responses such as pulmonary vasoconstriction to hypoxia (46) and cold-induced constriction of cutaneous arteries (47). Our present and previous experiments (6) suggest that this is not a response to hypoxia per se because it takes place at O2 concentrations higher than those that would be considered hypoxic. Moreover, the release of ROS occurs in the absence of significant changes in O2 consumption and in endothelial cells which, because of their glycolytic nature, are able to preserve their bioenergetic status under hypoxic conditions. It is more likely that, as we have suggested, this release of ROS is an early stress response dependent primarily on the mitochondrial redox status, and it is modulated by NO (6).

Interestingly, the catalytic AMPK-α1 subunit is the only one present in endothelial cells (our unpublished observations). This distribution is unlike liver, cardiomyocytes, and skeletal muscle (48), where both catalytic subunits (α1 and α2) are expressed. Although there is clear evidence for the activation of the α2-subunit of AMPK being dependent on bioenergetic crisis (49), the α1-subunit is known to be far less responsive to changes in AMP. Our experiments are in agreement with this observation, because very substantial changes in the AMP:ATP ratio were required for us to observe activation of AMPK in HUVECs. Whether there are differences between the downstream mechanisms activated by each of the catalytic subunits is not known at present.

In conclusion, our results indicate that in vascular endothelial cells mitochondria prevent the stabilization of HIF-1α and generate ROS for activation of AMPK. We suggest that these two actions, which are NO-dependent, are important for the maintenance of the nonangiogenic and highly resistant phenotype of endothelial cells. Whether mitochondria also play a key role in diverting O2 from the...
glycolytic endothelium to the O$_2$-requiring vascular smooth muscle needs further investigation.

Materials and Methods

Cells and Reagents. HUVECs were purchased from PromoCell (Heidelberg, Germany). Cells were grown until passage 3–5 in endothelial cell growth medium (EGM-2, PromoCell) at 37°C in a 5% CO$_2$/humidified air incubator. All experimental procedures were carried out when the cells were 80% confluent. pB293 endothelial cells were prepared essentially as described by King and Attardi (50). Briefly, after 3 weeks of treatment with ethidium bromide, the lack of mitochondrial function was verified by the absence of expression of two intrinsic mitochondrial genes using RT-PCR [cytochrome oxidase subunit 1 (GenBank accession no. AF381998), 5'-ATTTAGCTGACTGCCA-CACCTCA-3' and 5'-TAGGCAGAAAGTTGGTGGA-3', and ATPas subunit 6 (GenBank accession no. AY963585), 5'-ACATTACGTGACCCACTCTCA-3' and 5'-ACGTAAGCTTTAATAGGCCACA-3']. Smooth muscle cells from corpus cavernosum were provided by S. Cellek (GlaxoSmithKline, Harlow, Essex, U.K.). Human epithelial kidney cells (HEK293) were purchased from Invitrogen. Hypoxia (GlaxoSmithKline, Harlow, Essex, U.K.). Human epithelial kidney cells from corpus cavernosum were provided by S. Cellek (GlaxoSmithKline, Harlow, Essex, U.K.). Human epithelial kidney cells (HEK293) were purchased from Invitrogen. Hypoxia was achieved by incubation of the cells at 37°C in an O$_2$-controlled hypoxic chamber (Coy Laboratory Products, Ann Arbor, MI) for 2–8 h. N-acetyl-l-cysteine, l-ascorbic acid, 2DG, antimycin A, myoxthiazol, tert-butylhydroperoxide, angiotensin II, and rotenone were purchased from Sigma; l-NMMA was purchased from Alexis (Nottingham, U.K.). Anti-eNOS antibodies were obtained from Santa Cruz Biotechnology, and anti-phospho-AMPK-α-Thr172 and anti-AMPK antibodies were from Cell Signaling Technology (Beverly, MA) and used as indicated by the supplier.

eNOS Silencing. HUVECs were infected with pSIN-sh eNOS-Puro, a self-inactivating lentiviral vector expressing a short-hairpin RNA molecule against eNOS (GenBank accession no. NM.000603) by using the following oligos: 5'-TTCATACGAGTACTAAGTCCgctgGTAGTGTGGATGATTGAG-gtttt-3' and 5'-tgatgAAAAAACCTTACCAGCAGTACATGTTGAAGT-GTTTT-3'. These oligos were annealed in Mg$^{2+}$-free PCR buffer (Promega) for 2 min at 95°C and allowed to cool slowly to room temperature using a water bath. The resulting mixture was inserted into pGEM-U6L (a gift from Sam Wilson, Windeyer Institute, University College London) cut with SalI (blunted with PhosphoSafe buffer (Novagen), and centrifuged for 10 min at 13,000 × g (4°C). From clear supernatants, protein concentration was determined by the DC Bio-Rad kit using BSA as control. Sample aliquots were boiled for 2 min, and equal amounts (usually 20 μg) of total protein were electrophoresed in precast SDS/PAGE 4–15% gradient gels (Bio-Rad). Proteins were transferred to nitrocellulose membranes (Amersham Pharmacia), assessed for equal loading/transference by Red Ponceau tinction, and immunoblotted overnight with the indicated primary antibodies (typically 1:1,000 dilution) followed by secondary antibody conjugated with horseradish peroxidase (1:5,000 dilution). The ECL Plus Western blotting detection kit (Amersham Pharmacia Biosciences) was used for detection.

Accumulation and Activation of HIF-1α. HUVECs were incubated for 8 h under different treatments. Nuclear extraction was carried out as described in ref. 6. Protein content in the nuclear extracts was determined to adjust the amount to 60 μg per well for each sample. Samples were analyzed by Western blotting using a mouse monoclonal antibody (BD Biosciences) against HIF-1α (1:2,500), followed by an anti-mouse horseradish peroxidase conjugate (1:2,500; DAKO) and enhanced chemiluminescence (Amersham Pharmacia). HIF-1α activation was quantified in 5–10 μg of nuclear extracts by specific binding of HIF-1α to the hypoxia response element, a 5'-RCGTG-3' consensus sequence, using the TransAM HIF-1 Kit (Active Motive, Reixensart, Belgium) according to the manufacturer’s instructions.

Intracellular Superoxide Production. HUVECs were incubated with 10 μM DHE in the absence or presence of different treatments. After 1-h exposure to different O$_2$ concentrations, cells were washed with PBS or with 3% O$_2$-equilibrated PBS, respectively, to remove excess DHE. Cells were fixed for 10 seconds with 0.5 ml of 70% ethanol on ice and were then resuspended in 1 ml of normal PBS and analyzed immediately by flow cytometry (FACScalibur, Becton Dickinson). Data were acquired and analyzed using CELLQUEST software. Results are expressed as the mean fluorescence intensity.

Determination of Adenine Nucleotides. For experiments involving ATP determination HUVECs were grown for 24 h in 96-well plates (PerkinElmer 3603 clear bottom, black walls, seeding density 5,000 cells per well) in a phenol red-free medium. On the day of the experiment, fresh medium was added and treatments were performed as indicated. ATP was measured by the luciferin/luciferase method with a chemiluminescence kit (PerkinElmer) following the manufacturer’s protocol. Chemiluminescence was determined in a TopCount (Packard Biosciences), and data were analyzed in EXCEL (Microsoft). Alternatively, for simultaneous determination of AMP and ATP, HUVECs were grown for 24 h on six-well plates (3 × 10$^5$ cells per well). After treatments, nucleotides were extracted, separated by HPLC, and quantified as described by Smolenski et al. (52).

Statistics. Values stated are means ± SEM. To compare data obtained under different conditions an ANOVA test was used. Results were considered to be significantly different when $P < 0.05$.

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