Evidence of synergistic/additive effects of sildenafil and erythropoietin in enhancing survival and migration of hypoxic endothelial cells

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1Department of Human Morphology and Biomedical Sciences, Città Studi, Università degli Studi di Milano, Milan, Italy; 2Institute for Veterinary Physiology, Vetsuisse Faculty, Zurich; 3Zürich Centre for Integrative Human Physiology (ZIHP), Zurich; 4Institute of Human Movement Sciences and Sport, ETH Zurich, Switzerland

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Gammella E, Leuenberger C, Gassmann M, Ostergaard L. Evidence of synergistic/additive effects of sildenafil and erythropoietin in enhancing survival and migration of hypoxic endothelial cells. Am J Physiol Lung Cell Mol Physiol 304: L230–L239, 2013. First published November 30, 2012; doi:10.1152/ajplung.00112.2012.—Endothelial cell dysfunction is a common event to several pathologies including pulmonary hypertension, which is often associated with hypoxia. As the endothelium plays an essential role in regulating the dynamic interaction between pulmonary vasodilatation and vasoconstriction, this cell type is fundamental in the development of vascular remodeling and increased vascular resistance. We investigated the protective effects of sildenafil, a phosphodiesterase type 5 inhibitor, given in combination with erythropoietin (Epo), as it has been demonstrated that both drugs have antiapoptotic effects on several cell types. Specifically, we examined the viability and angiogenic properties of rat pulmonary artery endothelial cells upon exposure to either 21% or 1% oxygen, in presence of sildenafil (1 and 100 nM) and Epo (5 and 20 U/ml) alone or in combination (1 nM and 20 U/ml). Cell proliferation and viability were analyzed by Trypan blue staining, MTT assay, and Annexin V/propidium iodide stainings. In all assays, the ability of the combination treatment in improving cell viability was superior to that of either drug alone. The angiogenic properties were studied using a migration and a 3D collagen assay, and the results revealed increases in the migration potential of endothelial cells as well as the ability to form tube-like structures in response to sildenafil and the combination treatment. We therefore conclude that both drugs exert protective effects on endothelial cells on hypoxia and that sildenafil enhances the migratory and angiogenic properties, especially in hypoxic conditions. Furthermore, we present evidence of possible additive or synergistic effects of both drugs.

hypoxia

MAINTENANCE OF AN ADEQUATE supply of oxygen is important for the function of all organs and tissues. Thus it is crucial for the body to detect and respond rapidly to hypoxia to avoid the development of pathophysiological conditions such as stroke, cancer, inflammation, sleep apnea, heart failure, and hypertension (40, 41). Oxygen sensing is not limited only to specialized cells, such as the glomus cells of the carotid body, which depolarize within milliseconds in response to hypoxemia (13, 34), but occurs in all cells in the body. In other words, all cells sense and respond to hypoxia to adapt to changes in the oxygen supply (32). Hypoxia-induced pulmonary hypertension (PH) is a common clinical problem in patients suffering from chronic hypoxic lung disease, sleep disorder breathing, or high-altitude exposure. Hypoxia has been demonstrated to be one of the triggering factors in the development of endothelial cell dysfunction (35), which can lead to, for instance, morphological changes of the vascular wall, thereby playing a crucial role in the pathogenesis of PH (5, 45).

Endothelial cells are a specialized type of epithelial cells that form the inner layer of blood vessels. Their structure and functional integrity are important for the preservation of the vessel wall and circulatory functions. Furthermore, they are important for several functions including immune and inflammatory reactions, angiogenesis, and vasculogenesis. This makes the endothelium indispensable for the general body homeostasis, and consequently endothelial dysfunction is a common event for several pathologies including arteriosclerosis, congestive heart failure, inflammatory syndromes, and PH. In particular, when exposed to hypoxia, endothelial cells undergo marked changes in their survival and inflammatory and protein synthetic capabilities, which will also affect their permeability and migration (44, 47).

Thus alterations in the normal functions and structure of endothelial cells play a key role in the development of PH, which is a disorder of unknown etiology characterized by progressive remodeling of the pulmonary vasculature, leading to a rise in pulmonary arterial pressures (exceeding 25 mmHg at rest or 30 mmHg during exercise) and pulmonary vascular resistance (39, 46). This often culminates in right ventricular failure and eventually death. It can occur as a primary or secondary disease following pulmonary or cardiac illnesses, many of which are associated with persistent or intermittent hypoxia (44).

The present treatment possibilities for PH are very limited and often associated with discomfort for the patients, whereby new therapeutic options are highly needed. Sildenafil, which was developed for the treatment of erectile dysfunction, inhibits cyclic guanosine monophosphate-specific phosphodiesterase-5, an enzyme that is abundantly present in the pulmonary vasculature, and thereby promotes nitric oxide-mediated vasodilatation, which in turn decreases pulmonary vascular resistance (14, 16, 37). Recently several studies have reported the beneficial effects of sildenafil for the treatment of PH (14, 17, 28, 37).

The blood hormone erythropoietin (Epo) is a glycoprotein that is mainly produced in the adult kidney whose production is increased in response to hypoxia. Its main function is to regulate the production of red blood cells (6), but, as it has also been found expressed in several nonhematopoietic tissues, other functions of Epo are now emerging (25, 48), such as the prevention of apoptosis due to hypoxia, toxicity, or injury and its antihypertensive effect (38, 49). These protective effects are
mainly known and studied in cardio- and neuroprotection (6, 19, 29), and recently the ability of Epo to reduce endothelial dysfunction in a disease model of Alzheimer’s has been demonstrated (24).

Accordingly, as both drugs demonstrate protective effects on hypoxia (14, 6, 27, 20) and are antipulmonary hypertensive agents, we hypothesized that the combination of the two drugs, which to our knowledge are used together for the first time, could lead to a beneficial effect by attenuating the hypoxia-induced endothelial cell dysfunction, thereby allowing the use of the two drugs in a lower concentration. Thus the aim of this study was first to analyze the effects of the combined treatment on the proliferation, viability, and migration of endothelial cells on exposure to hypoxia. Furthermore, we investigated the signaling pathways involved and whether the two drugs can modulate these pathways in different ways, thereby contributing to the synergistic/additive effects (Fig. 1).

**MATERIALS AND METHODS**

**Cell cultures.** The rat pulmonary artery endothelial cell (RPAEC) line was a kind gift from Dr. Roscoe Warner, PhD, Department of Pathology, University of Michigan, Ann Arbor, MI. Cells were cultured at 37°C in 5% CO₂-95% air in endotoxin-free Dulbecco’s modified Eagle’s Medium containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 0.1 ng/ml streptomycin, sodium pyruvate, and non-essential amino acids. Upon reaching 80% confluency, cells were trypsinized in trypsin-EDTA and prepared for experiments. Cells were cultured at 37°C in 5% CO₂-95% air in endotoxin-free Dulbecco’s modified Eagle’s Medium containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 0.1 ng/ml streptomycin, sodium pyruvate, and non-essential amino acids. Upon reaching 80% confluency, cells were trypsinized in trypsin-EDTA and prepared for experiments. Cells were seeded at day 0, and the following day treatment was applied for the indicated amount of time. All cells were used at passages 8–9.

Experiments were performed at different days with different cell batches to determine repeatability.

The cells were seeded at a confluence of 8% in complete medium and allowed to settle for 24 h before being exposed to 21% O₂ (normoxia) or 1% O₂ (hypoxia) and treated with either Epo (Eprex, Janssen-Cilag) (5 U/ml and 20 U/ml) or sildenafil (kindly provided by Pfizer) (1 nM and 100 nM) alone or in combination (5 U/ml Epo and 1 nM sildenafil) in complete medium for 48 h, unless otherwise stated. All the analyses were done after 48 h of treatment except for the cell proliferation analysis that was done after 8 h, 24 h, and 48 h and for the 3D gel analysis that required 72 h for optimal tube formation.

**Immunoblotting.** Total protein extracts were prepared from the cell culture with a lysis buffer containing 10 mM Tris pH 8, 1 mM EDTA pH 8, 400 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor cocktail. Protein content was assessed using Bradford kit (Bio-Rad), and aliquots containing equal amounts of proteins were separated in an 8% PAGE gel and electroblotted onto Hybond ECL membranes (Amersham). After transfer was assessed by means of Ponceau S staining, the membranes were first blocked in 5% milk and then incubated with primary antibodies against hypoxia-inducible factor (HIF)-1α (NB 100-479, 1:1,000, NOVUS), and VEGF receptor 2 (ab2349-500, 1:500, Abcam) overnight at 4°C. The next day the membrane was incubated with a secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature and was then developed using chemiluminescent detection (Amersham). All proteins were normalized against β-actin.

**Cell proliferation and viability.** Cell proliferation was analyzed after 8, 24, and 48 h of exposure to normoxia or hypoxia in the presence or absence of treatments. Cell viability was evaluated only after 48 h. The number of cells (proliferation) was evaluated by staining with Trypan blue and counting in a Neubauer chamber. Viability was estimated by the ratio of white vs. blue cells, i.e., the percentage of nonviable cells were calculated.

**Annexin V assay.** Externalization of phosphatidylserine to the outer leaflet of the plasma membrane of apoptotic cells was assessed with Annexin V-fluorescein isothiocyanate (FITC). After the various treatments, RPAEC grown on a coverslip were washed with PBS and incubated at room temperature for 5 min in the dark with Annexin V-FITC and propidium iodide (PI) and then observed under a fluorescence microscope according to the instructions of the kit (PromoCell). The number of Annexin V-positive cells was determined on at least four randomly selected areas from three coverslips for each experimental group.

**MTT assay.** RPAEC were seeded in quadruplicate in 24-well plates. At the end of the treatments, cell viability was measured as

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**Fig. 1.** The proposed pathways involved in the migratory/angiogenic response and how they can be modulated by Epo and sildenafil. Dark gray ovals represent the kinases investigated. PI3K, phosphatidylinositol 3-kinase; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; sGC, soluble guanylyl cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; PDE5, phosphodiesterase type 5.
previously described (3, 9) using thiazolyl blue (MTT, Sigma) as an indicator of mitochondrial function. Briefly, 50 μl of MTT solution (5 mg/ml) was added to each well with 450 μl of medium. After incubation at 37°C for 2 h, formazan crystals were dissolved by adding 500 μl of MTT solubilization solution and thorough up-and-down pipetting. Absorbance was read at 570 nm, and the background absorbance at 690 nm was subtracted.

*Ca*spase activation. Caspase 3 activation was evaluated by a colorimetric assay (R&D Systems). Cells exposed for 48 h to normoxia or hypoxia in the presence or absence of the treatments were collected and lysed in a lysis buffer provided in the assay kit. The enzymatic reaction was carried out in a 96-well flat-bottom microplate. In brief, each reaction requires 50 μl of cell lysate (derived from 2 × 10^6 cells or 100–200 μg of total protein), 50 μl of 2× reaction buffer, and 5 μl of caspase 3 colorimetric substrate. The reaction mixture was incubated for 1–2 h at 37°C, and absorbance was measured on a microplate reader at 405 nm.

*Migration*. Migration was assayed in a Transwell-like set up, using a 48-well chamber from Neuro Probe. Cells were seeded (200,000 cells per well, unless otherwise stated) onto a polycarbonate membrane with 8-μm pores and incubated over 48 h in normoxia and hypoxia, respectively, with and without treatments. After incubation, the membrane was stained with DAPI, and the migrating cells were quantified by counting under the microscope with a 20× magnification.

In a separate set of experiments, the following inhibitors were added before drug treatment: Rp-8-Br-PET-cGMPS (Kᵢ = 100 μM, B6684, Sigma Aldrich), a PKG inhibitor. PD98059, an ERK inhibitor (Kᵢ = 50 μM, P215, Sigma Aldrich), SP600125, a JNK inhibitor (Kᵢ = 10 μM, S5567, Sigma Aldrich), SB203580, a p38 inhibitor (Kᵢ = 10 μM, S8307, Sigma Aldrich), and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (Kᵢ = 50 μM, 70920, Cayman Chemical). All inhibitors were added at the beginning of the experiment, after which migration was evaluated.

*3D gel*. Collagen was isolated from rat tails by acid extraction. Briefly, tendons were isolated from rat tails, lacerated, and soaked overnight in 15 mM acetic acid; all animal procedures were approved by the Institutional Animal Care and Use of Committee at Institute for Veterinary Physiology, Vetsuisse Faculty, Zurich, Switzerland. The next day, the solution was centrifuged, and the isolated collagen was lyophilized. A solution of 2.5 mg/ml of collagen was prepared in 15 mM acetic acid and allowed to polymerize. After polymerization, cells were seeded on the top surface and allowed to attach for 24 h. The cells were subjected to the aforementioned treatments for 72 h, after which the collagen gel was permeabilized with 4% paraformaldehyde for 20 min and stained with 50 nM DAPI. The formation of tube-like structures was observed under the fluorescent microscope.

**VEGF ELISA.** VEGF content in the media was measured by ELISA (Peprotech) according to the manufacturer’s instructions. Briefly, the plate was coated with the primary antibody overnight. The following day, the standards and the samples were prepared and incubated in the plate for 2 h. After washing the samples, we incubated them with the detection antibody for 2 h, and then absorbance was measured at 405 nm.

*Statistical analysis.* Data are expressed as means ± SD of the indicated number of observations. Statistical comparisons between groups were performed using the one-way ANOVA with a Newman-Keul’s post hoc test except for the data in Table 1, where Dunnett’s post hoc test was applied. Differences were considered significant when P < 0.05.

### RESULTS

**HIF-1 activation.** To verify whether the cells were hypoxic and whether the treatments had an effect on HIF-1α stabilization, we analyzed the protein levels of this transcription factor by immunoblotting using a whole cell protein lysate. The exposure to 1% O₂ for 48 h induced a 10-fold increase in the expression of HIF-1α (Fig. 2, A and B), but no differences with the drug treatments were observed, except for the treatment with 100 nM sildenafil at hypoxia, which induced a small, but significant increase in HIF-1α expression. Table 1 shows the measured ratio of hypoxia to control for the different groups and treatments. Statistical analysis was performed using the Student t-test.

**Table 1. Migration with inhibitors**

<table>
<thead>
<tr>
<th>Kinase Inhibited</th>
<th>N, C</th>
<th>Hx, C</th>
<th>Hx, 5</th>
<th>Hx, 20</th>
<th>Hx, 1</th>
<th>Hx, 100</th>
<th>Hx, ES</th>
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<tr>
<td>No Inhibitor</td>
<td>6.4 ± 2.6</td>
<td>5.4 ± 2.7</td>
<td>17.8 ± 17.2</td>
<td>5.2 ± 2.5</td>
<td>31.7± 16.5</td>
<td>33.7± 3.5</td>
<td>42.2± 23.5</td>
</tr>
<tr>
<td>PKG</td>
<td>13.4± ± 12.1</td>
<td>50.8± ± 34.5</td>
<td>52.3± ± 6.7</td>
<td>48.8± ± 14.1</td>
<td>48.4± ± 17.1</td>
<td>36.0± ± 14.5</td>
<td>39.8± ± 7.4</td>
</tr>
<tr>
<td>ERK</td>
<td>3.7 ± 2.5</td>
<td>16.6 ± 11.8</td>
<td>13.0 ± 4.9</td>
<td>17.6 ± 9.3</td>
<td>17.2± ± 12.3</td>
<td>11.0± ± 7.7</td>
<td>21.3± ± 16.6</td>
</tr>
<tr>
<td>JNK</td>
<td>26.8 ± 17.6</td>
<td>33.4± ± 14.5</td>
<td>39.3± ± 18.6</td>
<td>36.9± ± 5.9</td>
<td>31.6± ± 22.0</td>
<td>46.7± ± 14.1</td>
<td>44.1± ± 13.6</td>
</tr>
<tr>
<td>p38</td>
<td>6.4 ± 2.9</td>
<td>13.2 ± 8.7</td>
<td>7.3 ± 4.3</td>
<td>3.9± ± 1.9</td>
<td>8.0± ± 3.2</td>
<td>5.6± ± 2.0</td>
<td>9.0± ± 2.7</td>
</tr>
<tr>
<td>PI3K</td>
<td>56.0± ± 21.3</td>
<td>8.9 ± 5.4</td>
<td>3.8 ± 1.1</td>
<td>9.1± ± 4.3</td>
<td>4.6± ± 0.5</td>
<td>8.2± ± 1.1</td>
<td>15.6± ± 10.0</td>
</tr>
</tbody>
</table>

Values are shown as means ± SD. *P < 0.05 comparing each value in a column to the corresponding value in the “No Inhibitor” row. †P = 0.06 ± SD comparing each value in a row to the respective “Hx, C”. Boldface values represent those of statistical significance. N, normoxia; Hx, hypoxia; C, control; PI3K, phosphatidylinositol 3-kinase.
significant, increase in HIF-1α levels compared with the hypoxic control.

**Cell viability and proliferation.** Proliferation was estimated by cell counting, and no significant changes between normoxic and hypoxic cells were observed at the two first time points (8 and 24 h), whereas the 48-h time point revealed a decrease in the proliferation of hypoxic cells compared with the control cells that were cultured in normoxia (Fig. 3A). Furthermore, sildenafil at high concentration attenuated this hypoxic decrease in proliferation, whereas the cotreatment with both drugs reverted the number of cells to that of the normoxic control (Fig. 3B). No differences in cell numbers with treatment under normoxia were observed (data not shown).

Under hypoxic conditions at 48 h, there was an approximately threefold increase in the number of dead cells compared with normoxia (Fig. 4A). Epo and sildenafil treatments did not have any effects under normoxia, but in hypoxia they showed a dose-dependent effect in reducing the number of dead cells. In addition, the cotreatment with low doses of both drugs returned the ratio of dead vs. total cells to similar values as the normoxic control (Fig. 4A). Comparable results were observed with the MTT assay (Fig. 4B).

**Apoptosis.** Stainings with Annexin V and PI showed that hypoxia induces apoptosis and necrosis of the cells (Fig. 5, C and D). Treatment with the single drugs reduced apoptosis and abolished necrosis of the cells as shown by the lower staining of green (Annexin V) and absence of red (PI) cells. Furthermore, the effect of the combination of the two drugs in the lower concentrations is even more pronounced compared to the single treatment, as there are no necrotic cells and only very few apoptotic cells (Fig. 5, I and J).

Exposure to hypoxia for 48 h induced a twofold increase of caspase 3 activity. There was a tendency to a decrease following the treatments with Epo and sildenafil, and this was significant with the combined treatment (Fig. 6).

**Migration and angiogenesis.** The combination treatment and sildenafil in the highest dose significantly increased the number of migrating cells under hypoxic conditions after 48 h (Fig. 7, A and B). For 1 mM sildenafil, a tendency of an increase was observed (P < 0.1). Similar results were observed after 4 h (data not shown). Epo did not seem to influence migration significantly.

In parallel to the migration analysis, we also investigated the effects of the treatment on the capabilities of the endothelial cells to undertake an angiogenic phenotype under hypoxic conditions. The 3D collagen assay that we utilized revealed that the drug treatments increased the potential of
Fig. 5. Apoptosis and necrosis of pulmonary endothelial cells. A: hypoxia-induced apoptosis visualized by Annexin V staining (left) and necrosis visualized by propidium iodide (PI) staining (right). Normoxia (Nx) (top), hypoxia (Hx) (top middle), hypoxia and 20 U/ml Epo (middle), hypoxia and 100 nM Sildenafil (bottom middle), hypoxia and 5 U/ml Epo and 1 nM sildenafil (bottom). B: quantification of Annexin V (solid bars) and PI staining (shaded bars); n = 4, *P < 0.05 vs. hypoxia, control, #P < 0.05 vs. hypoxia, Epo and sildenafil.
the endothelial cells to interconnect with each other and to form tube-like structures in the presence of the two drugs (Fig. 8, A–E). With normoxia the cells exhibited the usual cobblestone pattern at the surface of the gel matrix, whereas hypoxia could facilitate the invasion of single cells and small clusters. When applying the single drugs, cells were observed to sprout from the surface and form small tube-like structures. These structures were larger and more pronounced with the combination treatment and could also be observed deeper in the gel. Similar results were observed with normoxia (data not shown).

Activated kinases. The phosphorylation of ERK and p38 was decreased upon hypoxia. This reduction was reversed by either using sildenafil in the high concentration or by the combination treatment of both drugs in the low concentrations (Fig. 9, A and B). Activation of JNK and Akt was unchanged after hypoxic exposures, but both kinases exhibited increased phosphorylation again with either sildenafil in the high concentration or the combination treatment (Fig. 9, C and D).

Migration in the presence of kinase inhibitors. As mentioned, treatment with sildenafil and the combination of the two drugs could increase the number of migrating cells. This increase was attenuated by inhibition of ERK (Hypoxia, ES vs. Hypoxia, ES + ERK inhibitor: P = 0.06), p38, and PI3K (Table 1), whereas no change was observed in the groups treated with Epo or in the hypoxic control. On the contrary, the presence of PKG or JNK inhibitors induced increases in the number of migrating cells with control conditions and Epo treatments, matching those observed with sildenafil and combination treatments. Furthermore, PI3K inhibition could enhance migration of normoxic control cells.

VEGF and VEGF receptor 2. The levels of VEGF released from the endothelial cells were highly increased upon hypoxia, and this could be further potentiated by the combination treatment. Under normoxia, there was a statistically nonsignificant tendency for an increased VEGF release with all sildenafil treatments. The expression of VEGF receptor 2 was not altered under any conditions (Table 2).

DISCUSSION

The present study provides convincing evidence of a synergistic/additive effect of a cotreatment with Epo and sildenafil for restoring endothelial function after hypoxic exposure. Interestingly, in most assays, the lower concentration of both drugs had the optimal effect, also over either drug given in a higher concentration. The protective effect of both drugs upon hypoxia is in agreement with previous reports, where these drugs were used alone: sildenafil has been shown to alleviate the endothelial cell dysfunction that can occur under conditions of ischemia reperfusion injury (8, 18), chronic heart failure (22), and coronary artery disease (20, 23). Also, Epo has been

**Fig. 6.** Activation and modulation of Caspase 3. Caspase 3 activity was assayed by use of a fluorescent substrate. Values are expressed as a ratio of the normoxic control. Open bar represents normoxia, and shaded bar represents hypoxia; n = 4. *P < 0.05 vs. hypoxia, control.

**Fig. 7.** Effect of hypoxia and drug treatments on endothelial migration. A: number of cells that migrated through a porous membrane. Open bar represents normoxia, and shaded bar represents hypoxia, both for 48 h. *P < 0.05 vs. hypoxia, control, #P < 0.05 vs. hypoxia, Epo and sildenafil. B: migrating cells were visualized by DAPI stainings. i: Hypoxic control. ii: Hypoxia and sildenafil (1 nM) and Epo (5 U/ml).

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*SILDENAFIL AND ERYTHROPOIETIN IN HYPOXIC ENDOTHELIAL CELLS*
demonstrated to promote proliferation and survival of endo-
thelial cells in vitro and to stimulate angiogenesis in vivo (26,
35). To our best knowledge, this is the first report of the
simultaneous use of both drugs.

In this study, we have investigated the signaling pathways at
the basis of the survival and angiogenic mechanisms. Survival
of endothelial cells is important for normal function/homeo-
stasis, and this will allow for a “nonfavorization” of the
apoptosis-resistant phenotype forming plexiform lesions. They
are generally thought to originate from a misled neoangiogen-

Fig. 8. Visualization of angiogenesis in a 3D collagen gel. Tube-like formation
was observed in 3D collagen gels after 72 h of hypoxia. DAPI stainings of the
top layer of the gel (A), in a control gel (no treatment) (B), with 20 U/ml Epo
(C), with 100 mM sildenafil (D), and with 5 U/ml Epo and 1 mM sildenafil (E).

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esis with an imbalance between apoptosis/necrosis and subse-
quent proliferation of the endothelium (1, 21, 27). We observed
that prolonged hypoxia (48 h) decreased the proliferation of
endothelial cells and that this can be reverted only after the
treatment with sildenafil in the high concentration or after
cotreatment with the two drugs in the low concentrations,
underlining their synergistic/additive effects (Fig. 3).

The hypoxic exposure also induced a threefold increase in
endothelial cell death that was dose dependently counteracted
by treatments with Epo and sildenafil alone or by the cotreat-
ment, revealing an additive effect by resembling the effect of
the high doses of the single treatments. The same tendency was
observed with the MTT assay, indicating reduced mitochondrial
activity under hypoxia and the ability of the two drugs to
prevent this (Fig. 4).

Apoptosis vs. necrosis was investigated by Annexin V and
PI stainings (Fig. 5). During hypoxia, several necrotic, e.g.,
PI-positive, cells could be observed, which were then greatly
reduced upon treatments and significantly more when combi-
ning the two drugs. In addition, all treatments reduced the
hypoxia-dependent increase of early apoptotic, annexin V-pos-
itive cells and significantly more by sildenafil and the combi-
nation treatment compared with Epo. The additional analysis
of the apoptotic pathway revealed a nonsignificant tendency for
a dose-dependent effect of the two drugs alone and a significant
effect of the cotreatment in reducing the hypoxia-induced
caspase-3 activation (Fig. 6).

The influence of Epo and sildenafil on endothelial cells to
migrate and interconnect and form tube-like structures was also
investigated. Migration and angiogenesis is important for new
vessel formation, leading to better perfusion and thereby oxy-
gen uptake. Migration of the cells through a porous membrane
was examined after 4 (data not shown) and 48 h, at normoxia
and hypoxia and with drug treatments (Fig. 7). Overall, no
effect of Epo was observed on the migratory capacity of the
cells in this setting, under normoxia or hypoxia. Some groups
have reported on this phenomenon, suggesting that the data
depend on the exact type of endothelial cells. For instance, Epo
could promote migration of human umbilical vein endothelial
cells (HUVEC) and bovine adrenal capillary endothelial cells
(2) as well as mouse embryoid body endothelial cells and
human endothelial progenitor cells (30), whereas migration of
human dermal microvascular endothelial cells was reduced by
Epo under normoxia and unaffected under hypoxia (4).

Sildenafil on the other hand had a positive effect on migra-
tion under hypoxia and at both time points. This effect of
sildenafil has previously been reported in HUVEC and endo-
thelial progenitor cells (12).

We found that several kinases were regulated in response to
hypoxia. This includes ERK, p38, JNK, and Akt, in all of
which the activity was increased with sildenafil and the com-
bination treatment (Fig. 9). As this fits very well with what we
observed with the migration experiments, we employed spe-
cific inhibitors of the individual kinases to elucidate which of
the kinases might be responsible for the migratory response. In
addition, we tested an inhibitor of PKG, as this is a central
factor in the nitric oxide pathway.

The observed increase in migration with sildenafil and the
combined treatment under hypoxia could be attenuated with
inhibitors of ERK, p38, and PI3K, indicating that these kinases
are important for the migratory response (Table 1). PKG and
JNK inhibitors, on the other hand, increased the number of migrating cells under control conditions and with Epo treatment, whereas no effect was observed in the presence of sildenafil or of the combination treatment. The involvement of ERK and p38 in the sildenafil-mediated induction of migration, but surprisingly not of PKG, suggests that ERK and p38 are PKG independently activated, which could imply the involvement of the ras/raf pathway activated by cGMP (7, 31), but this needs further investigation.

As mentioned, the PKG inhibitor increased the number of cells that had migrated under control conditions and with Epo treatments, indicating a negative influence of PKG in this setting. This is in agreement with other published studies showing that PKG can in fact inhibit the migratory pathway of human microvascular endothelial cells (43), colon cancer cells (11), and hepatic stellate cells (36), whereas no effect was observed in retinal endothelial cells (42). The same was the case when using the JNK inhibitor, and this is also in agreement with previous reports (50).

The switch to an angiogenic phenotype of the endothelial cells in response to the drugs was verified by an enhanced ability of the cells to migrate into the gel and to form tube-like structures in a 3D collagen matrix (Fig. 8). It appeared that the treatments with sildenafil as well as the combination treatment had the best effect, i.e., the structures formed were bigger, there were more branching points, and they were observed deeper in the gel, but this was not quantified.

VEGF expression was, as expected, highly elevated under hypoxic conditions. As we did not detect any general increase in migration under hypoxia compared with normoxia, we conclude that this growth factor does not play a major role in the migratory response of the RPAEC, at least under hypoxic conditions. We also assessed the expression of the VEGF receptor 2 (Flk-1), but this was unchanged under all experimental conditions (Table 2).

Endothelial dysfunction is a key event in the initiation and progression of PH (33) as well as a range of other diseases, and for this reason the identification of drugs that could improve the endothelial function is of great interest. Our results suggest that exposure of endothelial cells to hypoxia cause apoptosis and consequently endothelial dysfunction and that this can be counterbalanced by treatment with either sildenafil or Epo and that sildenafil, but not Epo alone, can induce the switch to an angiogenic phenotype. Additionally, the combination of the two drugs allows us to reduce the dose of the drugs while obtaining an even better outcome than with either drug alone. We are convinced that this observation will provide the basis

### Table 2. VEGF and VEGF receptor

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<th>Control</th>
<th>Epo, 5 U/ml</th>
<th>Epo, 20 U/ml</th>
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<th>Sildenafil, 100 nM</th>
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<td>196 ± 102†</td>
</tr>
<tr>
<td>Hx</td>
<td>631 ± 324†</td>
<td>591 ± 349†</td>
<td>584 ± 254†</td>
<td>485 ± 79†</td>
<td>597 ± 197†</td>
<td>909 ± 656†</td>
</tr>
<tr>
<td>VEGF R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nx</td>
<td>1.0 ± 0.0</td>
<td>0.93 ± 0.09</td>
<td>0.89 ± 0.13</td>
<td>1.14 ± 0.30</td>
<td>0.95 ± 0.11</td>
<td>0.88 ± 0.14</td>
</tr>
<tr>
<td>Hx</td>
<td>0.98 ± 0.08</td>
<td>0.99 ± 0.10</td>
<td>1.11 ± 0.29</td>
<td>1.10 ± 0.25</td>
<td>1.09 ± 0.45</td>
<td>0.92 ± 0.22</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05 compared with Hx, C. †P < 0.05 compared with Nx, C. Boldface values represent those of statistical significance. Epo, erythropoietin.
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for further experiments and possibly the identification of a novel therapeutic approach using well-known drugs.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


