PGE₁ analog alprostadil induces VEGF and eNOS expression in endothelial cells

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Haider, Dominik G., Robert A. Bucek, Aura G. Giurgea, Gerald Maurer, Helmut Glogar, Erich Minar, Michael Wolzt, Mohammad R. Mehrabi, and Mehrdad Baghestanian. PGE₁ analog alprostadil induces VEGF and eNOS expression in endothelial cells. Am J Physiol Heart Circ Physiol 289: H2066–H2072, 2005. First published June 10, 2005; doi:10.1152/ajpheart.00147.2005.—Endothelial nitric oxide synthase (eNOS), VEGF, and hypoxia-inducible factor 1-α (HIF-1α) are important regulators of endothelial function, which plays a role in the pathophysiology of heart failure (HF). PGE₁ analog treatment in patients with HF elicits beneficial hemodynamic effects, but the precise mechanisms have not been investigated. We have therefore assessed the influence of alprostadil on protein and mRNA expression of eNOS, VEGF, and HIF-1α in human umbilical vein endothelial cells (HUVEC) using RT-PCR and immuno-blotting under normoxic and hypoxic conditions. In addition, we studied protein expression by immunohistochemical staining in explanted hearts from patients with end-stage HF, treated or untreated with systemic alprostadil. Alprostadil causes an upregulation of eNOS and VEGF protein and mRNA expression in HUVEC and decreases HIF-1α. Hypoxia potently increased eNOS, VEGF, and HIF-1α synthesis. The alprostadil-induced upregulation of eNOS and VEGF was prevented by inhibition of MAPKs with PD-98056 or U-0126. Consistently, the expression of eNOS and VEGF was increased, and HIF-1α was reduced in failing hearts treated with alprostadil. The potent effects of alprostadil on endothelial VEGF and eNOS synthesis may be useful for patients with HF where endothelial dysfunction is involved in the disease process.

endothelial nitric oxide synthase, prostaglandin E mitogen-activated protein kinases, vascular endothelial growth factor

ENDOTHELIAL DYSFUNCTION in chronic heart failure (HF) is associated with an increased mortality risk (17). An important aspect of this impairment is the inability to generate vasodilatory nitric oxide (NO) from endothelial NO synthase (eNOS). NO has been demonstrated to upregulate the synthesis of VEGF (15, 27, 39), which is essential for functional restoration of the capillary circulation and prevention of excess myocardial remodeling (10). VEGF is decreased in the coronary and pulmonary circulation of patients with chronic HF. An important regulator of VEGF synthesis is hypoxia, mediated by the hypoxia-inducible factor-1α (HIF-1α) as the key transcription factor (23, 35).

PGE₁ is produced by vascular cells as a protective response to different stimuli. Intravenous administration of the PGE₁ analog alprostadil has been reported to improve hemodynamic parameters in patients awaiting heart transplantation (25). In addition to acute vasodilatory properties, alprostadil may exert salutary effects by altered endothelial cell (EC) protein expression.

We have therefore assessed the influence of alprostadil on protein and mRNA expression of eNOS, VEGF, and HIF-1α in human umbilical vein endothelial cells (HUVEC) and the involvement of MAPK under normoxic and hypoxic conditions. To correlate these results with clinical conditions, the expression pattern of these proteins was investigated in explanted heart tissue of patients with end-stage HF undergoing transplantation, patients who were included in a chronic ambulatory alprostadil treatment program.

METHODS

HUVEC Experiments

Isolation and culture of ECs. Umbilical cords were obtained at delivery after informed consent had been given by mothers. HUVEC were isolated by using collagenase type IA (Sebak) and cultured as described earlier until the third passage (2). Alprostadil (Pfizer) was incubated with HUVEC for 8 h at 0.1, 1.0, 5.0, 10.0, or 50.0 ng/ml under normoxic conditions in parallel experiments. Cells were then exposed in the presence of alprostadil to either normoxic or hypoxic conditions. The potent effects of alprostadil on endothelial VEGF and eNOS synthesis may be useful for patients with HF where endothelial dysfunction is involved in the disease process.

Western blot analysis. HUVEC were grown to 95% confluence. Cells were solubilized in Tris-lys-sis buffer (20 mM, pH 8.2) containing 1% Nonidet P-40 (Sigma), 140 mM NaCl, 2 mM EDTA, 1 mM iodoacetamide, 1 mM PMSF, and aprotonin and leupeptin (both at 10 µg/ml, all from Sigma). Lysates were kept on ice for 30 min during the reaction with the SDS sample buffer. Proteins were separated on 12–15% SDS polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were cut, and strips were incubated with mouse anti-human VEGF (1:1,000, 22 kDa, Labvision), mouse eNOS (final dilution 1:1,000, 135 kDa, Ab-1, Labvision), and mouse anti-human VEGF (1:1,000, 22 kDa, Ab-7, Labvision), mouse anti-human VEGF (1:1,000, 22 kDa, Ab-7, Labvision), mouse anti-human VEGF (1:1,000, 22 kDa, Ab-7, Labvision), mouse anti-human VEGF (1:1,000, 22 kDa, Ab-7, Labvision), mouse anti-human VEGF (1:1,000, 22 kDa, Ab-7, Labvision), mouse anti-human VEGF (1:1,000, 22 kDa, Ab-7, Labvision), mouse anti-human VEGF (1:1,000, 22 kDa, Ab-7, Labvision), mouse anti-human VEGF (1:1,000, 22 kDa, Ab-7, Labvision), mouse anti-human VEGF (1:1,000, 22 kDa, Ab-7, Labvision).
anti-human HIF-1α (1:500, 120 kDa, ab8366, Abcam), rabbit anti-human phosphor ERK1/2 (1:1000, 42/44 kDa, KAP-MA 021; Stressgen Biotechnologies; Victoria, Canada), and rabbit anti-human phosphor MAPK-ERK (MEK)1/2 (45 kDa; 1:2,000, Cell Signalling Technology, Beverly, MA). Strips were washed, and binding of primary MAb was revealed as described by Jaksits et al. (11). Each lane was loaded with 10 μg protein. Film exposure time was 40 s for all experiments.

**End-Stage HF Specimen**

Hearts were obtained from patients undergoing orthotopic heart transplantation. The sample comprised 42 patients with idiopathic dilative cardiomyopathy (CMPdil, n = 20) or ischemic cardiomyopathy (CMPisch, n = 22) with end-stage HF (Table 1). The study was approved by the local Ethics Committee. Ten patients with CMPdil and eleven with CMPisch were treated with continuous ambulatory alprostadil infusions (Pfizer) for severe HF with reduced cardiac index and increased pulmonary capillary wedge pressure. Alprostadil therapy was performed without interruption before heart transplantation at a dose of 8 ± 1 ng·kg⁻¹·min⁻¹ for 97 ± 76 and 114 ± 74 days in patients with CMPisch and CMPdil, respectively.

**Immunohistochemistry.** Longitudinally oriented transmural sections were prepared from the middle part of the left ventricle. Samples for immunohistochemical analysis were fixed in 7.5% formaldehyde (in PBS, pH 7.2) immediately after removal. Tissue sections, dried at 55°C for 2 h, and then deparaffinized in xylene for 10 min, followed by dehydration through graded alcohols. Afterward, tissue proteolysis was performed by pretreatment with an autoclave. Sections were then immersed in PBS (pH 7.6) and incubated with the following antibodies for 1 h in the appropriate dilution at room temperature: eNOS Ab-1 (dilution 1:100) and VEGF Ab-6 (1:100) from Labvision (Fremont, CA) and HIF-1α (1:75) from Abcam (Cambridge, UK). CD34 staining (1:1,000, Labvision) was used to identify ECs (21). The detection of antibodies was performed with a Labvision immunohistochemistry kit.

**Location of capillaries, vessels, and cells.** Twenty-five fields were defined and studied under a magnification of ×10. The fields were chosen using systematic random sampling. Smooth muscle cells (SMC) and cardiomyocytes were identified by their characteristic morphology. The number of SMC and ECs stained positive for eNOS, VEGF, and HIF-1α were counted in both groups under a light microscope at a magnification of ×40. Cells were evaluated with a camera resolution of 1,300 × 1,030 pixels in a visual field of 400 μm × 360 μm. Pictures were taken under a magnification of ×20. Analysis and pictures were performed by using the Axiocam S100 station (Zeiss; Jena, Germany). Cell counting was performed by three independent investigators, and the mean value was calculated.

**Statistical analysis and data presentation.** Statistical analysis was performed by using SPSS 10.0.5 (SPSS; Chicago, IL). Differences within and between groups were assessed by using nonparametric tests, and P < 0.05 was considered significant.

**RESULTS**

**HUVEC Experiments**

Exposure of HUVEC to hypoxia increased eNOS, VEGF, and HIF-1α mRNA and protein compared with normoxic conditions (all P < 0.001, Figs. 1 and 2). Incubation with alprostadil dose-dependently upregulated mRNA expression of eNOS and VEGF under normoxia (P < 0.001) and downregulated HIF-1α expression (P < 0.001, Fig. 1). Corresponding results were obtained for protein expression by immunoblotting (Fig. 2). Under hypoxic conditions, the effect of alprostadil was additive and resulted in a net upregulation of eNOS and VEGF and a downregulation of HIF-1α expression, respectively (all P < 0.001, Fig. 1).

Alprostadil activated the MAPK signaling cascade as demonstrated by increased phosphorylated MEK1/2 or ERK1/2 protein (Fig. 2). The MAPK-inhibitor PD-98059 had no effect on mRNA or protein expression (Fig. 3). Coincubation of PD-98059 or U-0126 with alprostadil completely prevented the upregulation of mRNA and protein expression of eNOS and VEGF but had no effect on HIF-1α. This effect was demonstrable under normoxic and hypoxic conditions (Fig. 2).

**End-Stage HF Specimen**

Alprostadil treatment was associated with a 1.5- to 2-fold increase of eNOS and VEGF expression and reduced HIF-1α synthesis in ECs and SMC (all P < 0.001, Table 2 and Fig. 4). These differences in expression pattern were not different between patients with CMPdil and CMPisch, treated or untreated with alprostadil (data not shown).

**DISCUSSION**

The myocardium and its vascular system are a critical target for therapeutic interventions in HF (5). This is the first study demonstrating an influence of the PGE₁ analog alprostadil on

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### Table 1. Characteristics of patients with idiopathic CMPdil or CMPisch and heart failure treated or untreated with alprostadil

<table>
<thead>
<tr>
<th></th>
<th>CMPdil</th>
<th>No Alprostadil</th>
<th>CMPisch</th>
<th>Alprostadil</th>
<th>No Alprostadil</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>54 (SD 11)</td>
<td>50 (SD 20)</td>
<td>52 (SD 9)</td>
<td>59 (SD 9)</td>
<td></td>
</tr>
<tr>
<td>Men/women</td>
<td>8/2</td>
<td>7/3</td>
<td>10/1</td>
<td>10/1</td>
<td></td>
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<tr>
<td>Cardiac index, l·min⁻¹·m⁻²</td>
<td>1.7 (SD 0.3)</td>
<td>2.5 (SD 0.3)</td>
<td>1.7 (SD 0.2)</td>
<td>2.4 (SD 0.2)</td>
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<tr>
<td>Concomitant drug therapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ACE inhibitors</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>β-Blockers</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td>Diuretics</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>11</td>
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</table>

Patients (n = 10) with dilative cardiomyopathy (CMPdil) in both treated and untreated groups, respectively, and patients (n = 11) with ischemic cardiomyopathy (CMPisch) in both treated and untreated groups, respectively. ACE, angiotensin-converting enzyme.
Fig. 1. Endothelial nitric oxide synthase (eNOS), VEGF, and hypoxia-inducible factor-1α (HIF-1α) mRNA expression in human umbilical vein endothelial cells (HUVEC) under normoxic and hypoxic conditions is influenced by alprostadil. Hypoxia increased mRNA expression of eNOS (A), VEGF (B), and HIF-1α (C) in the absence or presence of alprostadil. eNOS and VEGF mRNA was concentration dependently upregulated by alprostadil [means (SD), n = 3 experiments; densitometric analysis; A, top, and B, top]. In contrast, alprostadil caused down-regulation of HIF-1α mRNA [means (SD), n = 3 experiments; C, top]. This effect of alprostadil was confirmed by RT-PCR under normoxic and hypoxic conditions (Wm, weight marker; A–C, bottom).
the expression of eNOS, VEGF, and HIF-1α in isolated ECs, and it confirms previous data in explanted human hearts (21). Although control heart tissue specimens were not available in this study, alprostadil treatment was associated with a similar expression pattern of cardiac ECs and SMC for eNOS, VEGF, and HIF-1α as HUVEC incubated with the PGE1 analog. This consistent finding corroborates our results.

Altered eNOS, VEGF, and HIF-1α expression is detectable in patients with HF (5, 6, 21, 31). Chronic hypoxic conditions and altered shear stress decrease eNOS and VEGF and increase HIF-1α as HUVEC incubated with the PGE1 analog. This consistent finding corroborates our results.

Altered eNOS, VEGF, and HIF-1α expression is detectable in patients with HF (5, 6, 21, 31). Chronic hypoxic conditions and altered shear stress decrease eNOS and VEGF and increase HIF-1α expression in the vascular endothelium (20, 30, 34). In our experiments the incubation of HUVEC with alprostadil resulted in an upregulation of eNOS and VEGF mRNA and protein expression in HUVEC. This was detectable under normoxic and hypoxic conditions, supporting the hypothesis that the effects of PGE1 treatment are not limited to hypoxic stress. It has been suggested that enhanced NO bioactivity may influence the clinical outcome of HF (5, 9, 22, 32). Targeted eNOS overexpression attenuates cardiac and endothelial dysfunction and dramatically improves survival in severe HF (13, 14). Substances like nebivolol and carvedilol have been shown to increase eNOS or VEGF expression and favor the clinical outcome of HF (4, 19). Thus our results emphasize that PGE1 may represent a therapeutic option to increase both eNOS and VEGF expression in patients with chronic HF. This effect on the endothelium is additive to the acute vascular action of PGE1 and might improve the clinical condition of subjects treated with alprostadil.

The expression pattern of eNOS was paralleled by VEGF in our experiments, which may relate to the interaction between NO and VEGF synthesis (37). eNOS is a common convergence pathway for VEGF-induced changes in arteriolar diameter and microvascular permeability (1), and eNOS is activated in ECs by VEGF (12). Thus this close relationship between eNOS and VEGF could emphasize the importance for further targeted improvement of endothelial function in cardiovascular diseases for favoring clinical outcomes. Data obtained by using eNOS immunostaining in human heart tissue biopsies agreed well with cell culture experiments. The expression of eNOS by vascular SMC is at variance with results from human aortic SMC where eNOS mRNA was not detectable (36). However, eNOS expression of vascular SMC was also demonstrated in vascular SMC isolated from patients with coronary artery disease by real-time PCR (29). These differences may therefore be due to the sources of cells used rather than to the cross-reactivity of antibodies with other NOS isoforms.

Surprisingly, alprostadil treatment was associated with a downregulation of HIF-1α in HUVEC and in human heart tissue. This strongly argues that hypoxia is not the prime mechanism for altered eNOS and VEGF expression in HF,
which is also evidenced by the additive effect of alprostadil in hypoxic HUVEC. Furthermore, this finding may imply a role of HIF-1α in HF as a functional antagonist to eNOS and VEGF activity. In addition to its action as a modulator of VEGF regulation, HIF-1α has been reported as a critical link to inflammation (16). As a further aspect, PGs such as PGE-2 have been reported to decrease HIF-1α (38). This functional effect on HIF-1α expression is similar to that observed for alprostadil.

Our results clearly demonstrate that MAPK is a central pathway to transmit the actions of alprostadil on eNOS and VEGF. This was demonstrated in HUVEC using two MAPK inhibitors with different inhibitory specificity and potency on the family of MAPK isoenzymes (3). The expression of eNOS and VEGF is upregulated by mitogenic growth factor and proinflammatory cytokines that have been shown to activate different sets of MAPK pathways and play a pivotal role in neoangiogenesis. This is compatible with data showing that PGE-2 signaling on eNOS and VEGF is also MAPK dependent (8, 26). In contrast, regulation of HIF-1α by alprostadil does not involve MAPK pathways.

The mechanisms of how ECs sense hypoxia are largely unknown. The effect of 18 h of hypoxia was less than that of 12 h of hypoxia for eNOS and VEGF mRNA, but this difference was not seen for HIF-1α. This observation cannot be explained by our experiments. We have compared two etiologies of HF for potential discrepancies in the protein expression profile. Our results revealed no difference of eNOS and VEGF between hearts from patients with CMP ischemic and CMP dilated. Interestingly, higher protein expression levels of HIF-1α were detectable inCMP ischemic. Further trials are required to test whether this finding is due to statistical chance or hypoxia associated with CMP ischemic.

A limitation of our study is the fact that human heart specimens were collected according to a cross-sectional design only. Thus the results have to be interpreted with caution due to differences between groups, e.g., higher cardiac output in patients without alprostadil therapy, and due to multiple testing. Furthermore, alprostadil was already incubated with HUVEC for hypoxia studies. It is unclear whether treatment of hypoxic cells rather than preventive drug administration would have resulted in similar findings. However, the clinical specimens clearly demonstrate the capacity of alprostadil in cells with preexisting chronic damage.

We have demonstrated an upregulation of eNOS and VEGF and a downregulation of HIF-1α by alprostadil treatment in vitro and in vivo. The potent effects of alprostadil on endothelial VEGF and eNOS synthesis may be useful for patients with HF where endothelial dysfunction is involved in the disease process.

Table 2. Cells stained positive for eNOS, VEGF, and HIF-1α protein in explanted hearts of patients with heart failure untreated or treated with alprostadil

<table>
<thead>
<tr>
<th></th>
<th>No Alprostadil Treatment</th>
<th>Alprostadil Treatment</th>
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<tbody>
<tr>
<td>eNOS EC</td>
<td>1,553 (1,353–1,666)</td>
<td>2,694 (2,387–2,884)</td>
</tr>
<tr>
<td></td>
<td>2,493 (1,103–2,874)</td>
<td>2,689 (2,205–2,992)</td>
</tr>
<tr>
<td>VEGF EC</td>
<td>808 (668–942)</td>
<td>2,270 (1,964–2,560)</td>
</tr>
<tr>
<td></td>
<td>196 (171–226)</td>
<td>404 (329–462)</td>
</tr>
<tr>
<td>HIF-1α EC</td>
<td>3,079 (2,077–3,564)</td>
<td>1,592 (1,033–2,166)</td>
</tr>
<tr>
<td></td>
<td>2,015 (1,557–2,899)</td>
<td>901 (616–1,314)</td>
</tr>
</tbody>
</table>

Cell counts are presented as medians with ranges in parentheses. Positive cells were counted in 25 fields at a magnification of 10; n = 21 patients in both treated and untreated groups, respectively. P < 0.001 between endothelial cells (EC) and smooth muscle cells (SMC) within each group: endothelial nitric oxide synthase (eNOS), VEGF, and hypoxia-inducible factor-1α (HIF-1α).

Fig. 4. Immunohistochemical staining of eNOS, VEGF, and HIF-1alpha in explanted hearts of patients treated or untreated with alprostadil. Staining of eNOS (A), VEGF (B), and HIF-1alpha (C) in hearts of patients with alprostadil-treated ischemic cardiomyopathy (CMP ischemia/CMP dil), alprostadil-treated dilative cardiomyopathy (CMP dil/1–C–3), nonalprostadil-treated CMP dil/2–C–3, and nonalprostadil-treated CMP dil/2–C–4. Staining of different cell types for eNOS and VEGF was enhanced in patients treated with alprostadil and mitigated for HIF-1alpha, respectively.


