

Growth hormone induces eNOS expression and nitric oxide release in a cultured human endothelial cell line

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Abstract Growth hormone deficiency is linked to cardiovascular disease and particularly increased peripheral vascular resistance. Surprisingly, its role in endothelial nitric oxide (NO) synthetase (eNOS) regulation and NO release is basically unknown. We therefore studied the effects of different doses of somatotropin in cultures of a human endothelial cell line (EAhy926). We investigated expression and activity of eNOS, as well as other target genes known to be deregulated in cardiovascular disease including E-selectin and the lectin-like oxidized low density lipoprotein receptor. Treatment of cultured human endothelial cells with somatotropin resulted in significant ($P < 0.05$) increases of eNOS gene and protein expression, as well as NO release, whereas production of intracellular reactive oxygen species was significantly reduced, at the highest somatotropin dose level. The enhanced eNOS gene/protein expression and enzyme activity correlate well. Our findings are suggestive for a novel role of growth hormone in endothelial biology, and particularly NO production.

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1. Introduction

Growth hormone (GH) might play an important role in cardiovascular biology and indeed, growth hormone deficiency (GHD) is associated with risk for cardiovascular disease and increased peripheral vascular resistance [1]. Loss of GH production in deficient patients leads to decreased systemic nitric oxide (NO) levels and accelerated progression of arteriosclerosis [1,2]. Consequently, treatment of patients with recombinant GH (i.e. somatotropin) results in reduced total peripheral resistance, enhanced cardiac output, as well as reduced plasma cholesterol concentrations [2,3]. GH also regulates the synthesis of insulin-like growth factor (IGF-1), which enhances release of NO as well [4].

Notably, little is known about the role of GH in the regulation of endothelial NO synthetase (eNOS). We thus studied the effects of GH on the expression of eNOS and of other target genes known to be deregulated in GHD and/or athero-

sclerosis, including genes encoding the adhesion molecules ICAM-1, E-selectin, thrombomodulin, von Willebrand factor (vWF), as well as endothelin-1, endothelin-converting enzyme (ECE) and the lectin-like oxidized low density lipoprotein (oxLDL) receptor (LOX-1) [5]. Further, free radicals react with NO [6] and therefore, knowledge of intracellular reactive oxygen production after treatment of endothelial cells with increasing doses of recombinant GH is desirable. Overall, we investigated the role of GH on endothelial NO production.

2. Materials and methods

2.1. Cell culture

EAhy926 cells were the kind gift of Dr. Edgell (University of North Carolina at Chapel Hill, NC, USA) and are well characterized for their endothelial biology [7]. Cells were cultured in six well plates with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and L-glutamine (2 mM) until confluence (about 2 million cells/dish). At least 3 days after confluence, medium was changed to FCS-free DMEM and cells were immediately treated with increasing doses of somatotropin (Pharmacia and Upjohn, Germany; 10, 100, 1000 ng/ml) for 1–48 h and were harvested thereafter. All experiments were done at least in triplicate.

2.2. Quality assurance

Cultured endothelial cells were examined by inverse phase contrast microscopy before and after treatment with somatotropin (magnification 20-fold). Expression of the endothelial-specific membrane protein platelet endothelial cell adhesion molecule-1 (PECAM-1) and viability of endothelial cells by staining with propidium iodide was determined by fluorescence-activated flow cytometry, as described previously [8].

2.3. RNA and cDNA

RNA was isolated from endothelial cells using a total RNA Isolation kit (Macherey-Nagel, Germany) according to the manufacturer's recommendation. Quality and quantity of isolated RNA were checked using capillary electrophoresis (Agilent Bioanalyzer 2100) following the manufacturer's instructions. 2 µg total RNA from each sample was used for reverse transcription, as described previously [9]. The resulting cDNA was frozen at -20°C until further experimentation.

2.4. Thermocycler reverse transcription polymerase chain reaction (RT-PCR)

PCR reactions were carried out in a thermal cycler (T3, Biometra, Germany) using the melting, annealing and extension cycling conditions shown in Table 1. DNA contamination was checked for by direct amplification of RNA extracts prior to conversion to cDNA. Contamination of RNA extracts with genomic DNA could be excluded, as no template could be amplified. PCR reactions were done within the linear range of amplification and amplification products were separated using a 1.5% agarose gel and stained with ethidium bromide. Gels were photographed on a transilluminator (Kodak image station 440; see Fig. 1a) and amplicons were quantified using the Kodak 1D 3.5 network software.

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Table 1
Oligonucleotides used in gene expression studies

Accession number	Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Length (bp)	PCR cycles
NM_021130	cyclophilin	tttcgtgctctgagcactgg	cttgccatttcctggacccaa	347	27
AF519768	eNOS	gctgcgcagggctctcaccttc	ggctgcagccctttgctctcaa	554	29
AF068236	iNOS	cccttcaatggctggtacat	ttttccaggcctctacctga	391	37
XM_006947	vWF	gctgcagtatggaagcatca	tcgtagcgatctccaattcc	300	29
M16552	thrombomodulin	ccatgggagctggttagaaa	agaccttgacagacctgtgt	293	32
XM_165812	endothelin-1	ggcagagagctgtccaagtc	ctgtttctggagctccttgg	355	34
NM_001397	ECE	cttctctgctggaagtcttg	gggtcatgctccactgatct	400	27
NM_000450	E-selectin	agcccagagccttcagtgta	aactgggatttgctgtgtcc	244	37
NM_002543	LOX-1	ttgctgcacacaatctagca	cgagcatcaagatggagaca	394	36
XM_003896	GHR	ctcaccagctgcccataatt	gccctgtggggactgtacta	419	37
NM_000875	IGF-1 receptor	ggatgcggtgtccaataact	tggcagcactcattgttctc	212	31
X56773	IGF-1 α	ggaggctggagatgtattgc	actcgtgcagagcaaaaggat	217	33
X56774	IGF-1 β	tggatgctcttcagttcgtg	gatgtgtctttggccaacct	294	36

PCR conditions: predenaturation 60 s, 95°C, then denaturation 30 s, 94°C, annealing 60 s, 55°C, extension 60 s, 72°C; final extension step 420 s, 72°C.

2.5. Real-time semi-quantitative PCR

Real-time RT-PCR measurement were done with the Lightcycler (Roche Diagnostics, Germany), as described previously [9]. Exact quantification was achieved by a serial dilution with cDNA produced from endothelial total RNA extracts using 1:5 dilution steps. Gene expression levels were then given as the ratio of the gene of interest (numerator) versus a well known housekeeping gene (cyclophilin A, denominator).

2.6. Western immunoblotting

Cells were treated with somatotropin for 4 or 24 h (10–1000 ng/ml), harvested, pelleted, and resuspended with Tris/Triton X lysis buffer (1 mM Tris containing 0.1% Triton X (Sigma, Germany)). The cell lysate was incubated for 45 min on ice and centrifuged for 15 min at 13000 $\times$ g at 4°C. Protein concentration was determined according to Smith et al. [10] and the lysate was snap-frozen and stored at –80°C until further analysis. Western blotting was done as described previously [11] and a polyclonal eNOS antibody (Santa Cruz Biotechnology, Germany, dilution 1:200) was used. Immunoreactive proteins were visualized with a chemiluminescence reagent kit (NEN, Germany) according to the manufacturer's instructions, and bands were scanned with the Kodak Image Station CF 440 and analyzed using the Kodak 1D 3.5 imaging software (Eastman Kodak Company, USA).

2.7. Intracellular reactive oxygen species (ROS) measurements

ROS measurements were done according to Royall et al. [12]. Briefly, 2',7'-dichlorofluorescein diacetate (DCFH-DA) diffuses into cells and is hydrolyzed into non-fluorescent DCFH. Intracellular ROS oxidizes the non-fluorescent DCFH to highly fluorescent dichlorofluorescein. The increased fluorescent signal corresponds to ROS production. After treatment of cell cultures with 10–1000 ng/ml somatotropin for 4 or 24 h, cells were incubated with 40 μ M DCFH-DA for 30 min at 37°C. Then, cells were harvested, centrifuged for 5 min at 4°C and 1200 rpm, washed in phosphate-buffered saline (PBS) and resuspended with 800 μ l PBS buffer. After addition of propidium iodide (1 μ g/ml), fluorescence emission was measured at 530 $\pm$ 30 nm (fluorescein) and 585 $\pm$ 42 nm (propidium iodide) after excitation of cells at 488 nm using flow cytometric analysis (Becton Dickinson, USA).

2.8. Measurement of eNOS activity

Cells were treated for 4 or 24 h with 10–1000 ng/ml somatotropin. Then, 15 N-labelled arginine (5 mM) was added to culture media for 24 h and eNOS activity was assayed by determining the conversion of L-[guanidino- 15 N]arginine to [15 N]nitrite and [15 N]nitrate using a validated and established gas chromatography/mass spectrometry method [13].

2.9. Data analysis

Statistical significance was computed by analysis of variance followed by a Wilcoxon signed rank test. Differences were determined to be significant at $P < 0.05$.

3. Results

3.1. Cell culture

Examination by phase contrast light microscopy did not provide an indication for morphological changes or signs of abnormalities in control or somatotropin-treated cell cultures. In agreement with its morphological appearance, >95% of EAhy926 cells expressed PECAM-1. This is a well established endothelial-specific adhesion molecule and an accepted marker for endothelial differentiation [8]. Notably, treatment of cultures with somatotropin did not change PECAM-1 expression and cell viability was >98% in all treatment groups, as judged by propidium iodide staining and flow cytometry analysis (data not shown).

3.2. Gene expression studies

We observed a statistically significant ($P < 0.05$) 1.9–2.3-fold increase in eNOS gene expression after 4 h of somatotropin treatment (dose levels 100 and 1000 ng/ml). Dosing of cell cultures with 10 ng/ml of somatotropin resulted in an up to 2.5-fold ($P < 0.05$) induction 24 h post treatment (see Fig. 1b). No change in transcript levels of vWF, thrombomodulin, endothelin-1, ECE, GH receptor (GHR) and IGF-1 receptor was observed, but a dose-dependent and statistically significant ($P < 0.05$) reduction of E-selectin and LOX-1 gene expression to approximately 50% and 20% of control was noted (Fig. 1a,c). Notably, inducible NOS (iNOS) mRNA expression was below the limit of detection, as was IGF-1 α and IGF-1 β in total RNA extracts of EAhy926 cells. The latter genes were strongly expressed in human liver, which served as positive control (see Fig. 1a).

3.3. eNOS protein expression and enzyme activity

Protein expression of eNOS was increased in cells treated with 10–1000 ng/ml somatotropin after 4 and 24 h of treatment. In particular, eNOS protein expression peaked at 4 h when the higher somatotropin dose level (100 or 1000 ng/ml) was used, whereas dose levels of 10 ng/ml resulted in significantly enhanced protein expression after 24 h of treatment (see Fig. 2a). Further, eNOS activity was determined by assaying conversion of L-[guanidino- 15 N]arginine to [15 N]nitrite and [15 N]nitrate. Cultures treated with 1000 ng/ml of somatotropin resulted in significant ($P < 0.05$) 2.8-fold increases in eNOS activity 4 h post treatment, whereas a three-fold in-

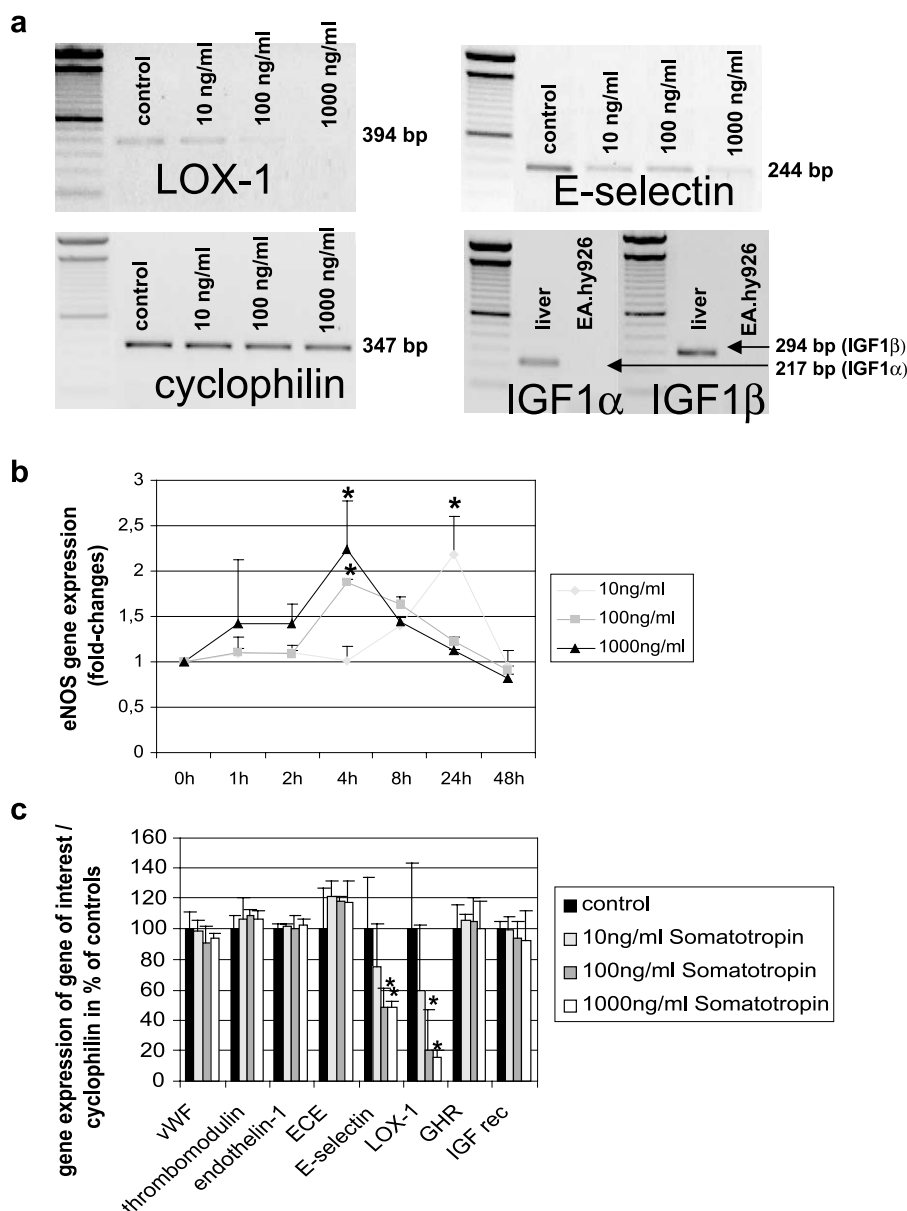


Fig. 1. a: mRNA expression of GH receptor, E-selectin, LOX-1, cyclophilin, IGF-1 α and IGF-1 β in control and somatotropin (10, 100 and 1000 ng/ml; 24 h)-treated EAhy926 cells. b,c: Semiquantitative RT-PCR analysis. Gene expression of eNOS (b), endothelial-specific proteins (c) and GHR and IGF receptor (c) in human endothelial cells upon treatment with somatotropin (10, 100 and 1000 ng/ml; 24 h). Results are presented as ratios of gene of interest/cyclophilin in percent. Data represent mean $\pm$ S.D. of $n = 3$ different cultures with approximate 2 million cells per culture dish. * $P < 0.05$.

creased activity was determined after treatment with the 10 ng/ml dose 24 h post treatment (see Fig. 2b).

3.4. Intracellular ROS production

After 4 h of somatotropin treatment no change in intracellular ROS production was noted at dose levels of 10 and 100 ng/ml, but an approximately 60% reduction was observed in cultures of endothelial cells treated with 1000 ng/ml of somatotropin. Twenty-four hours post treatment ROS production was unchanged with the lower dose level (10 ng/ml), but repressed to 80% and 40% of controls using the 100 and 1000 ng/ml dose levels, respectively (see Fig. 3).

4. Discussion

Patients with low levels of GH due to GHD have a reduced NO availability [1,2]. GH replacement therapy of deficient patients improved NO levels, recovered urinary nitrate and cGMP production, lowered abnormally elevated vascular peripheral resistance and improved cardiac output [2]. Here, we show somatotropin dosing of endothelial cells to result in increased NO production through eNOS activation. Dosing with somatotropin also repressed intracellular ROS production, as detailed below.

There is evidence from animal models of heart failure for

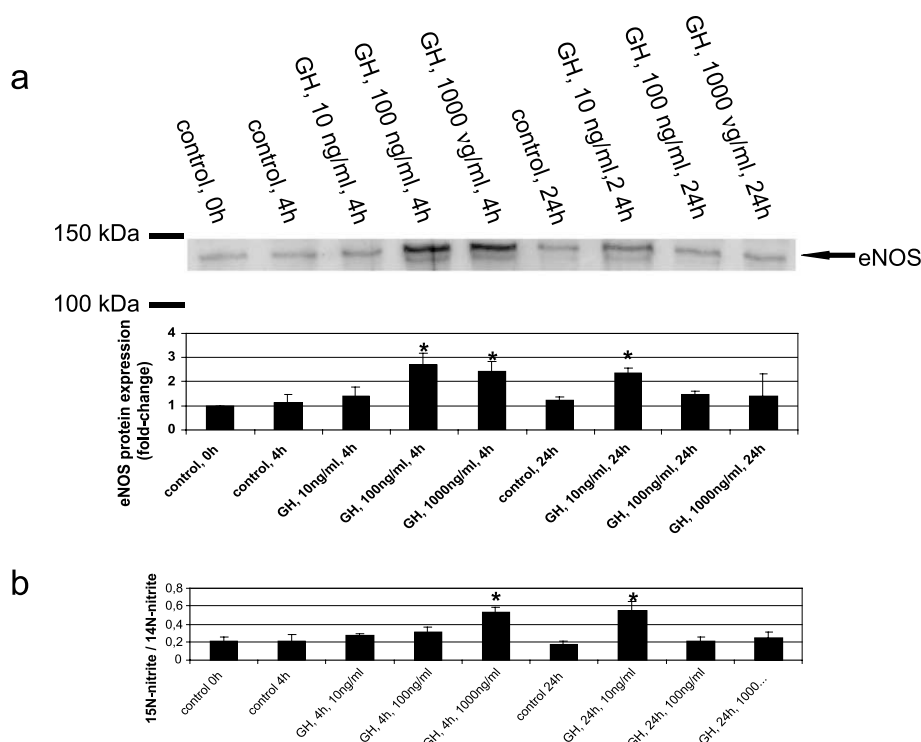


Fig. 2. a: Western immunoblotting of eNOS after treatment of cells with 10–1000 ng/ml of somatotropin for 4 h and 24 h. b: $^{15}\text{N}/^{14}\text{N}$ ratio of nitrite after incubation of EAhy926 cells with [^{15}N]arginine (5 mM) for 24 h. * $P < 0.05$.

GH administration to improve cardiac function [14]. Similarly, endothelium-dependent vascular function is also improved after GH therapy in patients with diagnosed heart failure [2]. There are additional clinical observations, which show beneficial effects of GH therapy in GHD, but no explanation has been put forward.

Vasodilatory effects of GH are mediated, at least in part, by IGF-1. It was shown that activation of IGF-1 contributes to enhanced NO release in cultured endothelial cells [4,15]. We did not detect transcripts of IGF-1 α and/or IGF-1 β in cultures of endothelial cells. The effect of GH are therefore unlikely to be mediated by local IGF production.

We observed dose- and time-dependent effects of somatotropin on eNOS expression and activity. At the higher dose levels (100–1000 ng/ml) we measured significantly enhanced eNOS mRNA expression ($P < 0.05$, 1.9–2.3-fold; Fig. 1b), protein expression ($P < 0.05$, 2.3–2.8-fold; Fig. 2a), whereas NO levels in culture media were significantly increased only at the highest dose level ($P < 0.05$, 2.6-fold; Fig. 2b). This discrepancy can be explained by a dramatic reduction of ROS production in endothelial cells treated with 1000 ng/ml somatotropin, whereas dosing with the lower levels (10–100 ng/ml) had no effect (Fig. 3). Therefore, the enhanced bioavailability of NO after treatment with 1000 ng/ml for 4 h is likely the result of both enhanced eNOS protein expression and reduced ROS production. In strong contrast, we only observed significantly increased eNOS mRNA ($P < 0.05$, 2.3-fold, Fig. 1b) and protein expression ($P < 0.05$, 2.1-fold, Fig. 2) in endothelial cells treated for 24 h with low dose somatotropin (10 ng/ml). Consequently, NO levels were highest ($P < 0.05$, 3.0-fold) at this dose level (10 ng/ml). As detailed above, the bioavailability of NO is dependent, at least in part, on production by NOS and the presence of ROS, which can diminish NO in a

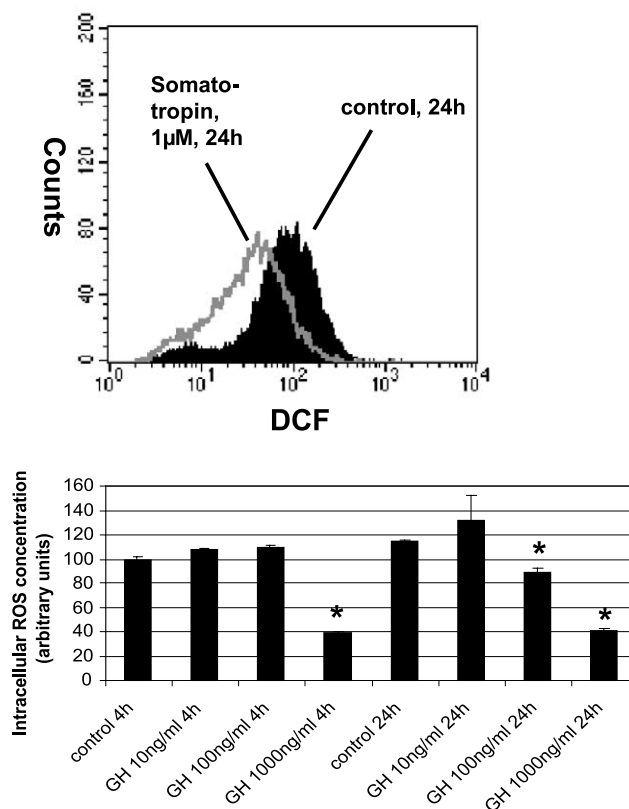


Fig. 3. Flow cytometric analysis of intracellular ROS production in control and somatotropin (10 to 1000 ng/ml, 4 and 24 h)-treated EAhy926 cells. Control (4 h) was set to 100%. * $P < 0.05$.

reaction where peroxynitrite is formed [6]. We observed significantly reduced ROS levels in cells treated with higher somatotropin dose levels (100–1000 ng/ml; Fig. 3). Surprisingly, at these dose levels NO availability was only slightly increased, whereas a significant three-fold induction was obvious at the 10 ng/ml dose. Again, ROS levels were not reduced at this particular dose. One explanation for the observed effect could be that the contribution of eNOS regarding total NO bioavailability is much higher than the NO-consuming reaction with ROS. Further, we observed transient and non-linear effects of somatotropin regarding eNOS expression and activity. This could be due to the considerably short half-life of eNOS mRNA (90–360 min) and protein (80–270 min), as recently shown by Ramet et al. [16].

Arnold and Weigent [17] demonstrated overexpression of GH in EL4 lymphoma cells to foster NO production as evidenced by enhanced iNOS protein expression, whereas eNOS and nNOS protein expression were unchanged. Notably, we did not observe similar findings in cultures of EAhy926 cells, as somatotropin treatment resulted in enhanced eNOS expression and enzyme activity. Potentially, GH's mode of action is cell type-specific. Further evidence stems from the study of Wickman et al. [18], who show GH treatment of hypophysectomized female rats to result in increased gene expression of eNOS in endothelium of the aorta, but not in the heart. The latter investigators did not, however, investigate eNOS activity or NO availability.

GH signaling is dependent on the level of GHR expression [19]. So far, only two studies have been published on GHR expression levels, in endothelial cells of ovarian vessels [20] and capillaries of tumor tissue [21]. We report transcript levels of GHR and of the IGF-1 receptor in a human endothelial cell line, but did not observe a treatment-related effect in the gene expression of these receptors. The strong expression of GHR observed in capillaries of liposarcoma [21] points to a novel role of this receptor in tumor angiogenesis and therefore, GH therapy must be viewed with great caution.

Recently, Elhadd et al. reported GHD patients to carry elevated plasma levels of vWF, ICAM-1, thrombomodulin and E-selectin [5], which are accepted biomarkers of endothelial dysfunction. We observed significant ($P < 0.05$) repression of E-selectin mRNA levels after GH treatment. However, further studies are needed to confirm the role of GH in the pharmacotherapy of endothelial dysfunction.

Additionally, GH plays a major role in lipoprotein metabolism as therapy of GHD patients with somatotropin leads to very significant repression of plasma LDL levels [2]. Further, oxLDL can be internalized by endothelial-specific LOX-1 and enhanced levels of oxLDL result in upregulation of LOX-1 [22]. LOX-1 is therefore associated with the progression of arteriosclerosis [22]. We observed a significant ($P < 0.05$) reduction of LOX-1 mRNA expression upon GH supplementation. We speculate for an important role of GH in the transcriptional regulation of the oxLDL receptor and further studies are on the way to investigate the potential cross-talk between the GH and the LOX-1 receptor.

As detailed above, GHD patients suffer from enhanced oxidative stress [23], which is frequently accompanied by reduced NO availability [6]. We show high dosing of cultured human endothelial cells with GH to reduce intracellular ROS production, presumably due to reduced metabolic deregulation and

improved antioxidative status. This is of importance as ROS may diminish NO availability and may also alter expression of eNOS [24]. Reduction of ROS production would provide an additional benefit for GH therapy.

In conclusion, we provide evidence that GH up-regulates eNOS expression and NO production in a cultured human endothelial cell line. GH treatment resulted in reduced intracellular ROS production, E-selectin and LOX-1 gene expression. This points to a novel role of GH in endothelial biology and may provide the rationale for the increased risk in developing cardiovascular disease in patients with acquired GH deficiency.

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