Growth hormone-induced blood pressure decrease is associated with increased mRNA levels of the vascular smooth muscle K\(_{\text{ATP}}\) channel

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Abstract

Growth hormone (GH) deficiency is associated with abnormal vascular reactivity and development of atherosclerosis. GH treatment in GH deficient states restores systemic vascular resistance, arterial compliance, endothelium-dependent and endothelium-independent vasodilation, and may reverse markers of early atherosclerosis. However, very little is known about the molecular mechanisms underlying these effects. In the present study, male Sprague Dawley rats were hypophysectomized and treated for two weeks with GH (recombinant human GH, 2 mg/kg/day) or saline as s.c. injections twice daily. GH decreased aortic systolic blood pressure compared with saline-treated animals, while the diastolic blood pressure was not significantly changed. GH treatment increased cardiac output as determined by Doppler-echocardiography and the calculated systemic vascular resistance was markedly reduced. In order to identify GH-regulated genes of importance for vascular function, aortic mRNA levels were analyzed by the microarray technique and correlated to the systolic blood pressure levels. Using this approach, we identified 18 GH-regulated genes with possible impact on vascular tone and atherogenesis. In particular, mRNA levels of the inwardly rectifying potassium channel Kir6·1 and the sulfonylurea receptor 2B, which together form the vascular smooth muscle ATP-sensitive potassium channel, were both upregulated by GH treatment and highly correlated to systolic blood pressure. Our findings establish a major role for GH in the regulation of vascular physiology and gene expression. Increased expression of the ATP-sensitive potassium channel, recently shown to be crucial in the regulation of vascular tone, constitutes a possible mechanism by which GH governs vascular tone.


Introduction

In addition to its growth-promoting and metabolic effects, growth hormone (GH) has an important impact on cardiovascular physiology. Besides affecting cardiac structure and contractility (Fazio et al. 1997, Longobardi et al. 2000, Barreto-Filho et al. 2002), both experimental and clinical GH deficiency has been associated with increased systemic vascular resistance (Fazio et al. 1997, Longobardi et al. 2000) and abnormal vascular reactivity (Rossoni et al. 1999, Evans et al. 2000, Capaldo et al. 2001). Accordingly, an increased prevalence of hypertension (Barreto-Filho et al. 2002) and atherosclerotic disease (Pfeifer et al. 1999) has been reported among patients with GH deficiency, and hypopituitary patients show increased cardiovascular morbidity and mortality (Rosen & Bengtsson 1990). GH treatment in GH deficient states has been shown to normalize systemic vascular resistance (Boger et al. 1996, Longobardi et al. 2000), arterial stiffness (Smith et al. 2002), endothelial and/or endothelium-independent vasodilation (Rossoni et al. 1999, Evans et al. 2000, Capaldo et al. 2001), and may reverse markers of early atherosclerosis (Pfeifer et al. 1999).

Despite the data supporting a physiological role for GH in the vascular system, very little is known about the molecular mechanisms underlying its effects, and it is currently unknown how GH treatment affects vascular global gene expression. We therefore assessed the effects of GH on aortic gene expression in GH-deficient hypophysectomized rats. In order to identify genes of importance
for vascular physiology, aortic mRNA levels were correlated to systolic blood pressure levels. Using this approach, we identified GH-regulated genes with potential impact on vascular tone and atherogenesis.

Materials and Methods

Animals
Male Sprague Dawley rats (170–190 g), were hypophysectomized (Hx) by the vendor (Iffa Credo, L’Arbresle Cedex, France). One week after hypophysectomy, replacement therapy with hydrocortisone (400 µg/kg/day; Solu-Cortef, Pharmacia Sweden AB, Stockholm, Sweden) and levothyroxine (20 µg/kg/day; Nycomed, Lidingö, Sweden) as daily s.c. injections was started in our laboratory. The animals were housed in a temperature- and humidity-controlled room with a 0600–1800 h light cycle and standard rat pellets and tap water were available ad libitum. Anesthesia was performed with isoflurane (Baxter Medical AB, Kista, Sweden) and all procedures were approved by the ethics committee at Göteborg University and conformed to the Universities Federation for Animal Welfare Handbook on the Care and Management of Laboratory Animals.

Study protocol
Two weeks after hypophysectomy, an echocardiographic examination was performed (‘baseline’). The rats were then treated for two weeks with GH (recombinant human GH, 2 mg/kg/day, Genotropin, Pharmacia Sweden AB, n=11) or saline (0·9% NaCl, n=11). The treatments were given as s.c. injections twice daily. After the treatment period, a second echocardiographic examination was performed (‘2 weeks’) and invasive blood pressure was recorded. The last injections of GH/saline were given 10 and 3 h before killing the rats. Blood was collected from the arterial catheter and the animals were killed by excision of the heart. The thoracic aorta was dissected free from blood and surrounding tissue, rinsed and frozen.

Echocardiography
Cardiac ultrasound studies were performed using a commercially available ultrasonograph (ATL, HDI 5000 SonocT, Philips Ultrasound, Bothell, Seattle, WA, USA) equipped with a 15 MHz linear array transducer. Pulsed-wave Doppler signals (5 MHz) and velocity time integrals were obtained from the pulmonary artery and the pulmonary artery diameter was measured. Stroke volume was calculated as the product of the mean velocity time integral and the corresponding pulmonary artery area (Baily et al. 1993). Multiplying stroke volume by heart rate yielded cardiac output. Cardiac index was calculated as cardiac output divided by body weight and systemic vascular resistance was calculated as mean arterial pressure (Christensen et al. 1990)/cardiac index.

Blood pressure measurements
During anesthesia, a PE50 catheter was inserted into the aortic arch via the left carotid artery. Blood pressure variables were measured using a pressure transducer (P23DC, Gulton Statham Transducers Inc, Costa Mesa, CA, USA) connected to a Grass model 7D polygraph (Quincey, MA, USA). Blood pressure values were calculated as means of 12 samples taken during 60 s.

Microarray analysis and bioinformatics
Total RNA was extracted from the aorta of 5–6 animals per treatment group (saline and GH respectively) with Trizol Reagent (Invitrogen AB, Carlsbad, CA, USA) according to the manufacturer’s instructions. The RNA from the individual animals was reverse transcribed into cDNA, labeled and analyzed by DNA microarray (Rat genome U34A array; Affymetrix, Santa Clara, CA, USA). The array represents approximately 7000 known rat genes and approximately 1000 uncharacterized expressed sequence tag (EST) clusters. Preparation of labeled cRNA and hybridization were carried out according to the Affymetrix Gene Chip Expression Analysis manual (for more information, see www.affymetrix.com). Scanned output files were analyzed using Affymetrix Microarray Suite Version 5·0 software. To allow comparison of gene expression, the gene chips were globally scaled to an average intensity of 100. A significant detection P-value in all of the saline– and GH-treated individuals was demanded for each probe set. The probe sets classified as present using this procedure (n=2563) were included in further analyses.

Using the signal intensity of the probe set of each individual, a t-test was performed for the comparison of saline (n=5) versus GH (n=6). Probe sets showing a P-value of <0·05 and a regulation of ≥2·5-fold (corresponding to 50% up-regulation or 33% down-regulation) were then called GH regulated. To identify GH-regulated probe sets of potential importance for vascular function, correlation coefficients (Pearson’s r) were calculated for the association between the signal intensity of each GH-regulated probe set and systolic blood pressure. An arbitrary limit of r of ≥0·75 or ≤ −0·75 was chosen. All probe sets with a correlation coefficient compared with systolic blood pressure of ≥0·75 or ≤ −0·75 were significantly correlated (P<0·05) to systolic blood pressure according to the non-parametric Spearman rank order correlation test.

Real-time PCR
The PCR analysis was performed using the ABI Prism 7000 Sequence Detection System (PE Applied
Biosystems, Stockholm, Sweden) using a FAM-labeled probe specific for the Kir6·1 transcript (PE Applied Biosystems). Forward primer, reverse primer and probe corresponded to the base coordinates 752–770, 820–801 and 773–798 of the rat Kir6·1 mRNA (accession number D42145). Pre-designed primers and a VIC-labeled probe for 18S rRNA were used as internal standard. The cDNA was amplified (50 °C/2 min and 95 °C/10 min, followed by 40 cycles at 95 °C/15 s and 60 °C/1 min) and the mRNA amount of each gene was calculated using the Standard Curve Method (User Bulletin #2, PE Applied Biosystems) and adjusted for the expression of 18S rRNA.

**Analyses of serum insulin-like growth factor-I (IGF-I) and plasma nitrate**

Serum was assayed for IGF-I by an IGF binding protein-blocked radioimmunoassay using a commercial kit (Mediagnost, Reutlingen, Germany) and plasma nitrate levels were determined by gas chromatography and mass spectrometry as previously described (Ringqvist et al. 2000).

**Statistical analysis**

All values are expressed as the mean ± S.E.M. Student’s t-test and correlation coefficients (Pearson’s r) were used for the selection of microarray data (as described above). For all other variables, statistical significance was assessed by the Mann–Whitney U-test and the Spearman rank order correlation test. For variables assessed before and after treatment (i.e. body weight and echocardiographic variables), statistical analysis was performed on the delta values. A value of \( P < 0.05 \) was considered statistically significant.

**Results**

**Effects of GH on body weight gain and serum IGF-I**

As expected, GH treatment of Hx rats for 2 weeks markedly increased body weight gain (78.8 ± 1.6 versus 3.5 ± 1.7 g in the saline group, \( P < 0.0001 \)). Likewise, serum IGF-I levels, measured at the end of the treatment period, were increased by GH (792 ± 32 versus 29 ± 2 ng/ml in saline-treated rats, \( P < 0.0001 \)).

**Effects of GH on blood pressure, cardiac output and systemic vascular resistance**

To assess the effect of GH on blood pressure, invasive blood pressure measurements were performed after 2 weeks of treatment. GH decreased systolic blood pressure, while the diastolic blood pressure was not significantly affected (Fig. 1A,B). Doppler-echocardiographic measurements of cardiac output were performed before and after treatment (Table 1). GH treatment did not cause any detectable changes in heart rate, but cardiac output was increased by treatment with GH and the calculated body weight-adjusted systemic vascular resistance was markedly reduced by GH (Table 1).

**Effects of GH on aortic gene expression**

DNA microarray analyses were performed on RNA samples from the aorta of individual saline- or GH-treated Hx rats (n=5 and 6 per treatment group respectively) to investigate if treatment with GH altered the vascular global gene expression pattern. Using the criteria of an up- or down-regulation of at least 1·5-fold and a P-value of <0·01, 94 probe sets were identified as GH regulated. These 94 probe sets represented 67 known genes, 15 duplicates and 12 ESTs (see supplementary table in the online version of Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol183/issue1/).

**Correlation between gene expression and systolic blood pressure**

In order to identify GH-regulated genes of relevance for vascular function, aortic mRNA levels were correlated to systolic blood pressure. For each of the 94 GH-regulated probe sets, a correlation coefficient was calculated between the signal intensity in the microarray analysis and the aortic systolic blood pressure level. Twenty-nine probe sets, representing 18 known genes (Table 2) with 5 duplicates, 5 unassigned ESTs and 1 non-specific sequence (see supplementary table in the online version of Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol183/issue1/), showed a correlation coefficient \( r \) of \( 0.75 \) or greater. The 18 known genes could be sorted into different functional categories, i.e. vascular tone, anti-oxidation, cell adhesion/inflammation, fibrinolysis, nerve growth factor (NGF)-associated genes, cytoskeleton/extracellular matrix, amino acid transport and intracellular signaling/processing (Table 2).

Interestingly, the inwardly rectifying potassium channel Kir6·1, which has been shown to be critical for the
regulation of vascular tone (Miki et al. 2002), was up-regulated by 103% by GH treatment and showed a strong negative correlation to systolic blood pressure (Fig. 2A, B). Kir6.1 mRNA expression was also strongly correlated to systemic vascular resistance ($r = 0.75$). Together with the sulfonylurea receptor 2B (SUR2B), Kir6.1 forms the vascular smooth muscle ATP-sensitive potassium (KATP) channel (Miki et al. 2002). Having established that GH treatment increases vascular Kir6.1 mRNA levels, we therefore looked specifically for the SUR2B among the

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Corr, correlation coefficient. P-values are based on Student’s t-test. *P<0.01, **P<0.005, ***P<0.001, ****P<0.0001, *****P<0.00001.

Table 2 GH-regulated genes highly correlated to systolic blood pressure. Genes regulated by GH ($P$-value in a t-test vs saline of $<0.01$ and an up- or down-regulation of $\geq 1.5$-fold) with a correlation coefficient vs systolic blood pressure of $\geq 0.75$ or $\leq -0.75$
microarray data. Indeed, although not identified initially using our strict criteria of regulation, there was a significant increase in the mRNA levels of the SUR2B (accession number AF019628/AF087839), and the mRNA levels showed a negative correlation to systolic blood pressure (Fig. 2C, D). Furthermore, glutathione S-transferase was up-regulated and a large group of NGF-associated genes was down-regulated by GH.

As mRNA levels of I-kappaB, the inhibitor of nuclear factor (NF)-kappaB, were decreased by GH treatment, we looked specifically for a possible regulation of NF-kappaB. Moreover, we searched for a possible regulation of vascular cell adhesion molecule-1 (VCAM-1), an important adhesion molecule involved in atherogenesis (Cybulsky et al. 2001) and known to be regulated by the NF-kappaB system (Kunsch & Medford 1999). The NF-kappaB mRNA was classified as present but was not regulated by GH (data not shown). However, GH significantly decreased the mRNA levels of VCAM-1 (−32%, \( P=0.007 \), accession number X63722), and VCAM-1 mRNA was strongly correlated to systolic blood pressure (r=0.75, \( P=0.045 \)).

**Real-time PCR confirmation of Kir6.1 regulation**

In order to confirm the results from the microarray analyses, we performed a real-time PCR assay to quantify

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Figure 2 Quantification of Kir6.1 mRNA by microarray (A; \( n=5–6 \) per group) and correlation between Kir6.1 mRNA and systolic blood pressure (B; \( P=0.0095 \) according to the Spearman rank order correlation test) in saline- and GH-treated Hx rats. Quantification of SUR2B mRNA by microarray (C; \( n=5–6 \) per group) and correlation between SUR2B mRNA and systolic blood pressure (D; \( P=0.028 \)) in saline- and GH-treated Hx rats. *\( P<0.05 \), **\( P<0.001 \) compared with saline, based on a t-test.
Kir6·1 mRNA levels. Quantification by real-time PCR (Fig. 3A) gave comparable results to the microarray analysis of Kir6·1 mRNA levels (Fig. 2A). In addition, the negative correlation seen for Kir6·1 mRNA quantified by microarray was confirmed when the real-time PCR data were correlated to systolic blood pressure (Fig. 3B).

Effects of GH on plasma nitrate levels
To evaluate if the GH-induced reduction of vascular resistance was associated with increased formation of nitric oxide, we studied the effect on plasma nitrate levels as a marker of systemic nitric oxide production (Ringqvist et al. 2000). However, there was no difference in plasma nitrate levels between GH- and saline-treated rats (23±0.1±0 and 23±1±1 µmol/l respectively).

Discussion
In this report, we have explored the molecular mechanisms underlying the actions of GH on vascular function by examining aortic mRNA levels assessed by microarray. We have demonstrated that GH regulates the mRNA levels of a large number of genes in vascular tissue, paralleling a decrease in systolic blood pressure. Our most important finding is that GH regulates the mRNA levels of the two subunits of the KATP channel, known to be critical in the regulation of vascular tone (Miki et al. 2002).

Decreased vascular tone by GH – a role for the Kir6·1/SUR2B channel
Several studies have consistently demonstrated decreased systemic vascular resistance in association with increased cardiac output by GH treatment of normal or GH-deficient rats (Cittadini et al. 1996, Longobardi et al. 2000, Tivesten et al. 2001). These effects remain after adjustment for body size. Blood pressure levels may be decreased, as in the present study, or unchanged (Longobardi et al. 2000, Tivesten et al. 2001, Smith et al. 2002). Given the permissive role of the heart in adjusting cardiac output to systemic vascular resistance in order to maintain normal blood pressure levels, it is reasonable to suggest that a large part of the hemodynamic effects of GH is mediated through an effect on vascular tone. The effect on vascular tone may be a direct effect of GH, or mediated by local or circulating IGF-I (Tivesten et al. 2002).

In the present study, GH administration up-regulated aortic mRNA levels of the inwardly rectifying potassium channel Kir6·1, and the mRNA levels of Kir6·1 were strongly correlated to systolic blood pressure. Kir6·1 forms the vascular smooth muscle KATP channel together with the SUR2B (Fujita & Kurachi 2000). Looking specifically for SUR2B in our array data, we found that GH also up-regulated the SUR2B component of the KATP channel. A recent study has shown that the phenotype of Kir6·1-null mice is characterized by coronary vasoconstriction, suggesting that the Kir6·1-containing KATP channel is critical for the regulation of vascular tone (Miki et al. 2002). The vascular KATP channel is also the target for the anti-hypertensive potassium channel opening drugs, which potently reduce systemic blood pressure levels (Fujita & Kurachi 2000). We therefore propose that an up-regulation of the KATP channel expression may result in a decreased vascular tone and thus constitutes a possible mechanism by which GH governs vascular resistance.

Stimulation of beta-adrenoreceptors leads to vascular dilation through a mechanism involving activation of the KATP channel (Fuji et al. 1999). GH has been shown to
improve the aortic relaxation of aged rats in response to beta-adrenergic stimulation (Castillo et al. 2003). In view of our present findings, this result may be attributable to a GH-induced increase in vascular K$_{ATP}$ channel expression.

GH treatment of GH deficient patients increased urinary levels of nitrate, suggesting an increased vascular production of nitric oxide (Boger et al. 1996). In the present study, GH did not increase plasma nitrate levels, arguing against a major role of increased nitric oxide production for the observed blood pressure effect. Several studies have shown that GH, or IGF-I, may cause vaso-relaxation through endothelium-independent mechanisms (Hasdai et al. 1998, Capaldo et al. 2001). In line with these data, our finding of increased vascular Kir6·1 mRNA levels by GH supports the notion of an endothelium-independent vasodilatory mechanism activated by GH.

We chose to correlate aortic gene expression to aortic systolic blood pressure, which is composed of a pulsatile and a steady component with different determinants (Safar et al. 2003), e.g. compliance of large arteries and systemic vascular resistance. For practical reasons, it was not possible to dissect these components within this study and this will be a challenge for future studies.

In the present study, we used human GH which may activate both the GH receptor and the prolactin receptor in the rat (Goffin et al. 1996). Therefore, an effect of prolactin receptor activation on gene expression cannot be excluded. However, prolactin has been reported to increase, rather than decrease, blood pressure in rats (Mills & Woods 1985) and thus the blood pressure-lowering effect of GH in the present study is likely to be an effect of GH receptor stimulation.

**Regulation of genes implicated in atherogenesis**

It is well known that inflammatory processes are involved in atherogenesis and a large body of evidence has implicated enhanced oxidative stress in the atherogenic process (Kunsch & Medford 1999). Oxidative stress, measured as lipid-derived free radicals, was elevated in a cohort of GH deficient patients but fell significantly during GH replacement therapy, paralleled by improvement of endothelial function (Evans et al. 2000). In the present study, we found that the alpha type of glutathione S-transferase was up-regulated by GH. The glutathione S-transferases have been proposed as an important defence in the vascular media, protecting blood vessels against oxidative stress (He et al. 1998). Thus, increased expression of glutathione S-transferase by GH may promote anti-oxidation and thereby neutralize atherogenic stimuli.

VCAM-1 is a vascular inflammatory gene considered to have a key role in the early phases of atherosclerosis by monocyte recruitment to atherosclerotic lesions (Cybulsky et al. 2001). In the present study, GH down-regulated VCAM-1, which should create an anti-atherogenic vascular milieu. The regulation of VCAM-1 expression occurs through a reduction/oxidation-sensitive mechanism involving the transcription factor NF-kappaB (Kunsch & Medford 1999). We could not detect any change in NF-kappaB mRNA levels, while GH down-regulated its inhibitor, I-kappaB alpha. Given the complexity of the NF-kappaB/I-kappaB system (Ting & Endy 2002), further studies should address the interactions between GH and NF-kappaB/I-kappaB in the vascular wall.

**NGF-associated genes**

The neurotropins are a family of related growth factors, including neurotropin-3 and NGF. Neurotropin-3, also called NGF-2, and several genes known to be induced by NGF, such as NGF-inducible anti-proliferative secreted protein, peptidylglycine alpha-hydroxylating monooxygenase and NGF-induced gene A, were down-regulated by GH and showed a positive correlation to systolic blood pressure levels.

Messenger RNA of neurotropin-3 is co-expressed with NGF within the cardiovascular system (Scarisbrick et al. 1993). While data on vascular effects of neurotropin-3 are sparse, NGF has been shown to decrease vascular tone and modulate sympathetic innervation and vascular smooth muscle growth (Zetterl et al. 1991, Emanuelli et al. 2002). The neurotropins have also been shown to be regulated in the response to vascular injury and stretch of vascular smooth muscle cells (Clemow et al. 2000). Taken together, these findings suggest that the neurotropins play an important role in vascular biology. In the current setting, we suggest that the decrease in neurotropin mRNA levels may be secondary to the unloading of the arterial wall during GH treatment.

**Summary**

Despite the extensive data supporting a physiological role for GH in the vascular system and that attention recently has been focused on GH as a new therapeutic alternative in the treatment of heart failure (Isgaard et al. 1998), little is known about the molecular mechanisms underlying the vascular effects of GH. In the present study we have identified a number of vascular genes of potential importance for the action of GH. In particular, we suggest that the vascular smooth muscle K$_{ATP}$ channel is of importance for the GH-induced regulation of vascular tone.

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