EXPERIMENTAL STUDY

IGF-I and GH stimulate Phex mRNA expression in lungs and bones and 1,25-dihydroxyvitamin D₃ production in hypophysectomized rats

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Abstract

Objective: X-linked hypophosphatemia, a renal phosphate (Pi)-wasting disorder with defective bone mineralization, is caused by mutations in the PHEX gene (a Pi-regulating gene with homology to endopeptidases on the X chromosome). We wondered whether changes in Phex and neprilysin (NEP) (another member of the family of zinc endopeptidases) mRNA expression could be observed in relation to vitamin D and Pi metabolism during GH- and IGF-I-stimulated growth of hypophysectomized rats.

Design: Animals were infused s.c. for 2 days with vehicle, 200 mU (67 µg) GH or 300 µg IGF-I/rat per 24 h. We determined serum osteocalcin and osteocalcin mRNA in bone, Phex mRNA in bone and lungs, serum 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) and serum Pi levels, and renal expression of 25-hydroxyvitamin D₃-1α-hydroxylase (1α-hydroxylase), of 25-hydroxyvitamin D₃-24-hydroxylase (24-hydroxylase) and of the Na-dependent Pi-cotransporter type I and II (Na_dPi-I and -II).

Results: As compared with vehicle-treated controls, body weight and tibial epiphyseal width significantly increased in GH- and IGF-I-treated animals. Serum osteocalcin and osteocalcin mRNA levels in bone, Phex mRNA in bone and lungs, serum 1,25-(OH)₂D₃ and renal 1α-hydroxylase mRNA rose concomitantly, whereas expression of NEP in lungs was barely affected and renal 24-hydroxylase mRNA decreased. Na_dPi-I and -II gene expression in the kidney and serum Pi levels remained unchanged.

Conclusions: Our findings suggest a coordinate regulation of Phex mRNA expression in lungs and bone and vitamin D metabolism during GH- and IGF-I-stimulated growth.

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Introduction

X-linked hypophosphatemia (XLH) is the most common inherited disorder of renal phosphate (Pi) wasting (with an incidence of 1 in 20 000). It is a classic example of rachitis, osteomalacia and vitamin D resistance in which Pi depletion predominates (1–3). The disease is characterized by an abnormal proximal renal tubular function resulting in an increased renal clearance of Pi and hypophosphatemia, by the failure to generate elevated circulating serum levels of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) during hypophosphatemia (4), and by defective bone mineralization. The understanding of XLH has been facilitated by a murine model, the Hyp mouse, which exhibits many of the phenotypic features of XLH (5, 6). More recently, the discovery of the PHEX gene by positional cloning (7) has provided

insight into novel regulatory mechanisms of Pi homeostasis, bone mineralization and vitamin D metabolism. PHEX exhibits high homology to a family of zinc metallopeptidases whose function involves the activation or inactivation of bioactive peptides. This function has been particularly investigated for neprilysin (NEP), another member of this family, which is widely distributed in tissues including lung and kidney. PHEX/Phex mRNA expression has been found mainly in bones, teeth and lungs (8–12), but the physiological substrate(s) and product(s) of the PHEX protein have remained unknown. Inactivating mutations of the PHEX gene cause XLH. In the Hyp mouse, decreased renal Pi reabsorption is due to a reduction in mRNA and protein of the Na-dependent Pi-cotransporter type II (Na_dPi-II) (13, 14). The serum level of 1,25-(OH)₂D₃ is not increased in Hyp

mice despite significant hypophosphatemia (15, 16). The latter is an important signal for increased synthesis of $1,25\text{-(OH)}_2\text{D}_3$ in the mammalian kidney (17). Hyp mice exhibit an inappropriate response of $1,25\text{-(OH)}_2\text{D}_3$ synthesis to a low Pi (18), but not to a low calcium challenge (19). The regulation of renal $1,25\text{-(OH)}_2\text{D}_3$ catabolism by the C-24 oxidation pathway is also disturbed in Hyp mice. 25-Hydroxyvitamin D_3 -24-hydroxylase (24-hydroxylase), the first enzyme in the vitamin D degradative pathway, is upregulated by low Pi diet in Hyp mice (16), and this increase in Pi-deprived Hyp mice is associated with a dramatic fall in serum $1,25\text{-(OH)}_2\text{D}_3$ levels. In contrast, Pi deprivation does not upregulate 24-hydroxylase activity in normal mice and leads to an elevation of serum $1,25\text{-(OH)}_2\text{D}_3$ (20). Transplantation of bone marrow from wild-type to Hyp mice has been recently shown to provide a source of progenitors for normal Phex-expressing cells, to increase serum Pi levels and renal $\text{Na}_d\text{Pi-II}$ gene expression, and to suppress the elevated renal 24-hydroxylase gene expression in the recipient Hyp mice (21).

Growth and bone development require Pi and calcium retention in the body. A high renal Pi reabsorption threshold is characteristic for the entire period of longitudinal bone growth during childhood and puberty in humans (22). In contrast to humans, rodents keep growing throughout life, their epiphyses remain open, and their serum Pi remains at a high level after puberty and sexual maturation. Hypophysectomized rats offer the possibility to examine the relationship between Pi metabolism, kidney function and growth (23, 24). These rats lack growth hormone (GH), insulin-like growth factor-I (IGF-I) and all other pituitary-dependent hormones, and they stop growing. Growth is resumed by GH and IGF-I treatment (25), and decreased serum Pi levels are restored towards normal (12). Production of $1,25\text{-(OH)}_2\text{D}_3$ is decreased in hypophysectomized rats (23), and renal 1α -hydroxylase activity fails to increase in response to low Pi (24). GH administration restores the response of serum $1,25\text{-(OH)}_2\text{D}_3$ to low Pi diet (17, 24). $1,25\text{-(OH)}_2\text{D}_3$, in turn, stimulates absorption of calcium and Pi through the gut. Whether a downregulation of 24-hydroxylase or an upregulation of 25-hydroxyvitamin D_3 - 1α -hydroxylase (1α -hydroxylase) gene expression contributes to the rise in serum $1,25\text{-(OH)}_2\text{D}_3$ levels after GH administration has not been determined.

The present study was undertaken in order to test whether Phex mRNA expression, $1,25\text{-(OH)}_2\text{D}_3$ production, and renal Pi absorption are regulated in a coordinate manner during GH- and IGF-I-stimulated growth of hypophysectomized rats. We, therefore, examined the effects of GH and IGF-I on Phex mRNA expression in bone and lungs, i.e. the main Phex-expressing tissues, on NEP mRNA expression in the lungs, on renal 24-hydroxylase, 1α -hydroxylase and

$\text{Na}_d\text{Pi-I}$ and -II cotransporter gene expression, and on serum Pi, osteocalcin and $1,25\text{-(OH)}_2\text{D}_3$ following a 2 day s.c. infusion.

Materials and methods

Animals

All animal experiments were approved by the Institutional Animal Welfare Committee. Hypophysectomy was carried out in 7-week-old male TifRAI rats (courtesy of Mr M Cortesi, Novartis, Basel, Switzerland) before the onset of puberty. They were kept at 25°C on a 12 h light:12 h darkness cycle and had free access to food (normal diet 3431, 0.82% Pi content, provimi; Klüber AG, Kaiseraugst, Switzerland) and drinking water. Animals with a weight gain of less than 2 g/week during 4 weeks after the operation were selected for infusion. Alzet mini-osmotic pumps (model 2001; Alza, Palo Alto, CA, USA) were filled with vehicle (0.1 M acetic acid), recombinant human (rh) IGF-I (provided by Dr K Müller, Novartis) dissolved in vehicle, or rhGH (Novo Nordisk, Gentofte, Denmark) dissolved in the provided solvent. The minipumps were implanted s.c. at the site of the abdomen during ether anesthesia. Groups of six hypophysectomized rats (170–190 g) were infused for 2 days with vehicle, rhIGF-I (300 μg per rat per 24 h), or rhGH (200 mU (67 μg) per rat per 24 h), doses which have been shown to exert maximal effects on body weight (BW) gain and tibial epiphyseal width (25). Food and water intake as well as BW were measured daily at 0830 h during the infusion period. After 2 days of infusion, the rats were anesthetized with 0.2 ml/100 g BW Innovar Vet (Pitman Moore, Washington Crossing, NJ, USA) and bled by aortic puncture. Organs were rapidly excised, blotted on filter paper, and weighed. They were immediately frozen in liquid nitrogen and stored at -80°C until RNA was isolated. Blood was kept on ice for 30 min and centrifuged for 15 min at 1000 *g* at 4°C . Serum was stored in 1 ml aliquots at -20°C for further analysis.

Determination of BW and of tibial epiphyseal width

BW was measured daily and weight gain (in g/2 days) was calculated. The tibia test was performed according to Greenspan *et al.* (26). Silver-stained tibiae were photographed under a stereomicroscope (Wild, Heerbrugg, Switzerland) at a $25\times$ magnification. Ten different measurements of the tibial epiphyseal width were taken on the photograph, and the mean value for each animal was calculated.

RNA isolation

Calvarial bones were quickly removed and placed in Petri dishes containing 1% SDS, 5 mmol/l EDTA,

10 mmol/l Tris, pH 7.5. They were cleaned from adherent tissue including the periosteum, and the edges were cut (27). The pieces of bone and the other frozen tissues (0.5–1 g) were homogenized in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) at 4 °C in 3 ml ice-cold 4 mol/l guanidine isothiocyanate containing 5 mmol/l sodium citrate (pH 7.0), 0.1 mol/l β-mercaptoethanol, and 0.5% sarcosine. Bone homogenates were filtered through a Millex-HA filter unit (0.45 µm pore size; Millipore, Molsheim, France) (28). Total RNA from all the examined tissues was obtained by high-speed sedimentation through a cesium chloride cushion (29). The RNA samples were dissolved in diethylpyrocarbonate-treated H₂O, concentrations were determined spectrophotometrically, and the samples were stored at –80 °C until assayed. Isolated total RNA from calvarial bones was pooled (for each treatment: two pools of three parietal bone pairs).

cDNA synthesis

cDNA was synthesized from 5 µg total RNA (from calvarial bone and kidney) using 200 U SuperScript II reverse transcriptase (Life Technologies AG, Basel, Switzerland) essentially according to the recommendations of the manufacturer as follows: reverse transcription was performed in 20 µl 50 mmol/l Tris–HCl, pH 8.3, containing 50 mmol/l KCl, 8 mmol/l MgCl₂, 0.5 mmol/l dNTPs, 10 mmol/l dithiothreitol and 1 µl (0.5 µg) of oligo(dT)-primer, at 42 °C for 55 min. After termination at 70 °C for 15 min the samples were incubated with 1 µl (2 U) of RNase H at 37 °C for 20 min. The cDNA was stored at –20 °C until processed.

Real-time PCR: determination of *Phex* expression in bone

For each of the corresponding target genes, three oligonucleotides, two primers and an internal oligonucleotide probe were selected using Primer Expression software (PE Biosystems, Foster City, CA, USA; <http://www.pebio.com>).

For the sequences studied, the sense and the antisense primer were placed in two consecutive exons of the gene; the probe spanned the junction of the two exons between the sequences covered by the two primers as described by Heid *et al.* (30) and Leutenegger *et al.* (31). The primer and probe sequences are listed in Table 1.

Amplification was performed by addition of 12 µl of a dilution series of the cDNA in a total volume of 25 µl (50 mmol/l KCl, 10 mmol/l Tris–HCl, pH 8.3, 5 mmol/l MgCl₂, 100 µmol/l of each dNTP and 600 nmol/l of each primer) containing 0.5 U of Taq DNA polymerase (Sigma, Buchs, Switzerland). Amplification was carried out as follows: 2 min held at 50 °C, denaturation for 10 min at 95 °C, followed by 40 cycles of 15 s each at 95 °C and 1 min each at 60 °C.

Table 1 Real-time PCR primers and probes for the detection of *Phex* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Oligonucleotides are given in 5'–3' orientation. f: forward primer; r: reverse primer; p: probe.

GAPDH	
GAPDH.f	TGTGTCCGTCGTGGATCTGA
GAPDH.r	CCTGCTTCACCACTTCTTGA
GAPDH.p	^a CCGCCTGGAGAAACCTGCCAA ^b
<i>Phex</i>	
<i>Phex</i> .1115f	CCAGAATTCCAAACCTCAGCA
<i>Phex</i> .1206r	CCACTGAGGCAGCAGAGTTG
<i>Phex</i> .1185p	^a TGTCCCCTGGATTACCTTGAGAATTCCA ^b

^aNucleotide to which the reporter dye FAM is coupled.

^bNucleotide to which the quencher dye TMARA is coupled.

Gene signals were quantified by normalizing these signals against the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal. Thermal cycling and fluorescence detection were carried out in an automated fluorometer (ABI Prism 7700 Sequence Detection System; PE Biosystems).

Northern blot analysis

Denatured RNA (2 or 15 µg) from several tissues was electrophoresed on a 1% agarose gel containing 2 mol/l formaldehyde, transferred to a nylon membrane (Hybond-N, Amersham International, Amersham, Bucks, UK) by capillary blotting, and fixed by UV cross-linking according to standard procedures (32). Prehybridization and hybridization were performed as described earlier (12). The following cDNAs were used for hybridization: rat *Phex* corresponding to the cDNA sequence (cloned in our laboratory) between nucleotide 1220 and 1944 (12), rat NEP cDNA (nucleotide 135–837, GenBank Accession No: M15944), rat Na₂Pi-I cDNA (nucleotide 578–1004, GenBank Accession No: U28504), rat Na₂Pi-II cDNA (nucleotide 366–1202, GenBank Accession No: L13257), rat 24-hydroxylase cDNA (nucleotide 1048–1586, GenBank Accession No: X 59506) and rat osteocalcin cDNA (33) (520 bp; a gift from Dr G Rodan and Dr M Noda, West Point, PA, USA). mRNA levels were quantified by scanning densitometry using a Bio-Rad video densitometer (Bio-Rad, Richmond, CA, USA). Stripping of the membranes to remove bound cDNA probes was performed for 1 h at 98 °C. Variations of gel loading were corrected against 18S ribosomal RNA values.

Semiquantitative RT-PCR analysis: determination of 1α-hydroxylase expression in kidneys

Five micrograms of total RNA from rat kidney was reverse transcribed to cDNA in 20 µl reaction mixture using SuperScript II reverse transcriptase (Life Technologies) as recommended by the manufacturer. Two microliters of each RT reaction were then added to a

standard 50 µl PCR mixture (Boehringer Mannheim AG, Rotkreuz, Switzerland), and first-strand cDNA was amplified by 30 cycles of PCR consisting of 2 min of denaturing at 95 °C, 40 s of annealing at 55 °C, and 1 min extension at 72 °C, with the following set of primers: 3A/3B (3A: nucleotide 828–843, 3B: nucleotide 2129–2143) for 1α-hydroxylase (GenBank Accession No: AB001992). Previous experiments had established that under these conditions the amounts of PCR product semiquantitatively reflect the examined transcript levels (12). The same samples were amplified with the primers U2/L1 (U2: 5' AAGGCTGAGAATGGG 3', nucleotide 205–219 and L1: 5' ACCCTGTTGCTGT-AGCC 3', nucleotide 979–995, GenBank Accession No: M17701) for rat GAPDH as an internal control. Signals were measured by densitometry and normalized against the GAPDH control.

Serum parameters

Urea, creatinine, calcium and Pi serum levels were measured by routine methods in the Clinical Chemistry Division of the University Hospital of Zurich. 1,25-(OH)₂D₃ serum levels were measured by RIA (Immunodiagnostic Systems, Boldon, UK), osteocalcin serum levels by ELISA (Biomedical Technologies Inc., Stoughton, MA, USA). Two different RIAs were used to measure endogenous rat IGF-I and infused rhIGF-I as described earlier (34). In the human IGF-I RIA, rat IGF-I does not crossreact at the performed dilutions. The values obtained in this RIA, therefore, reflect only the concentration of the infused rhIGF-I. In the rat IGF-I RIA, human IGF-I crossreacts five to six times better than rat IGF-I so that endogenous IGF-I concentrations cannot be determined in the animals infused with rhIGF-I. In animals infused with GH and in the vehicle-infused animals (absence of human

IGF-I) the RIA values represent endogenous rat IGF-I concentrations.

Statistical analysis

The results are expressed as means ± S.D. or S.E.M. Statistical significance was determined by ANOVA. $P < 0.05$ was considered to indicate a significant difference.

Results

Effects of IGF-I and GH on serum IGF-I, growth indices and on indices of bone formation

Two days of IGF-I or GH treatment significantly increased serum IGF-I, BW and tibial epiphyseal width (Table 2). Total serum IGF-I levels were higher in GH-infused than in IGF-I-infused rats in agreement with earlier findings (35). However, total IGF-I levels in these two experimental settings are not readily comparable: IGF-I infused into hypophysectomized rats circulates mainly in the form of a 40 kDa IGF-I-binding protein complex whereas IGF-I induced by GH is predominantly bound within a 150 kDa heterotrimeric binding protein complex (36). In contrast to the 40 kDa form, the bioavailability of IGF-I in the 150 kDa form is largely restricted by its limited capillary permeability (37, 38). GH effects are, therefore, rather mediated by local IGF-I production at the tissue level and thus reflect autocrine/paracrine IGF-I actions in contrast to the systemic actions of infused IGF-I.

Serum osteocalcin levels were higher in hormone-treated than in vehicle-treated rats (Table 2), and osteocalcin mRNA expression in calvarial bones increased

Table 2 Growth parameters and indices of bone formation and kidney function in vehicle-, IGF-I- and GH-treated hypophysectomized rats. Values are means ± S.D. ($n = 6$), except for osteocalcin mRNA levels ($n = 2$: two pools with parietal bones from three rats each), where the original data are shown in Fig. 1. For all other determinations differences were evaluated for statistical significance by ANOVA.

	Vehicle	IGF-I	GH
Growth and bone formation			
Serum rat IGF-I (ng/ml)**	94 ± 37	—	1177 ± 166*
Serum rhIGF-I (ng/ml)**	—	431 ± 64*	—
Body weight gain (g/2 days)	−2.3 ± 1.8	8.9 ± 2.2*	13.2 ± 4.8*
Epiphyseal width (µm)	173 ± 11	211 ± 17*	249 ± 24*
Osteocalcin mRNA (expression relative to vehicle treatment)	1 (1.1, 0.9)	6.7 (9.0, 4.4)	4.5 (6.1, 2.9)
Serum osteocalcin (ng/ml)	67 ± 7	90 ± 9*	78 ± 9
Kidney function			
Serum creatinine (µmol/l)	69 ± 3	56 ± 2*	63 ± 2
Serum urea (mmol/l)	10.0 ± 1.2	4.0 ± 1.1*	4.5 ± 0.4*
Serum calcium (mmol/l)	2.41 ± 0.07	2.42 ± 0.06	2.49 ± 0.05*
Serum Pi (mmol/l)	2.04 ± 0.19	1.99 ± 0.16	1.94 ± 0.24
Serum 1,25-(OH) ₂ D ₃ (pg/ml)	19 ± 6	125 ± 60*	73 ± 24*

* $P < 0.05$ vs vehicle.

**For explanation see Materials and methods section.

6.7- and 4.5-fold respectively in IGF-I- and GH-treated, relative to the expression in vehicle-treated, hypophysectomized rats (Fig. 1 and Table 2).

Effects of IGF-I and GH on Phex mRNA expression in bones and lungs and on NEP mRNA expression in lungs

To determine the influence of IGF-I and GH treatment on the expression of Phex mRNA in calvarial bones, we performed real-time PCR. IGF-I and GH treatment resulted in a mean 4.4- and 3.7-fold increase respectively of relative Phex expression as compared with vehicle-treated hypophysectomized rats (Table 3).

Northern blot analysis was performed to investigate the effects of IGF-I and GH on Phex mRNA levels in the lungs (Fig. 2, upper panel). Quantitative evaluation of the Phex mRNA levels after treatment with IGF-I or GH showed a 3.9 ± 1.0 -fold and 1.9 ± 0.5 -fold increase respectively in the relative expression of the 6.6 kb transcript as compared with vehicle-treated controls (Fig. 2, lower panel). After stripping, the membrane was re-hybridized with an NEP probe (Fig. 3, upper panel), resulting in hybridization signals of 4.2 and 6.5 kb. Quantitative evaluation of the NEP mRNA levels after

Table 3 Relative levels of Phex cDNA from RNA of parietal bones from IGF-I- or GH-treated compared with vehicle-treated hypophysectomized rats, normalized for GAPDH cDNA. Relative expression levels of Phex cDNA from total RNA in two pools of three parietal bone pairs. Parietal bones of three rats had to be pooled in order to obtain sufficient amount of RNA. Each pool was assayed three times by real-time PCR and normalized for GAPDH expression (see also Materials and methods). The numbers represent the mean value of the two RNA pools relative to control, those in parentheses the mean values of the three RT-PCR determinations in each of the two pools.

Treatment	Phex cDNA
Vehicle	1
IGF-I	4.4 (3.2, 5.6)
GH	3.7 (2.9, 4.5)

treatment with IGF-I or GH showed a non-significant 1.55 ± 0.45 -fold and 1.42 ± 0.45 -fold increase respectively in the relative expression of the two transcripts (analyzed in combination) as compared with vehicle-treated controls (Fig. 3, lower panel).

Effects of IGF-I and GH on parameters of kidney function

IGF-I and GH produced significant effects on parameters of kidney function (Table 2). Serum creatinine

Figure 1 Osteocalcin mRNA expression in calvarial bones of vehicle-, IGF-I- and GH-treated hypophysectomized rats. Two micrograms of total RNA prepared from parietal bones (two pools of three parietal bone pairs) of hypophysectomized rats treated for 2 days with vehicle, IGF-I or GH were separated on a 1% agarose formaldehyde minigel, stained with ethidium bromide (lower panel) to confirm equal loading, transferred to a nylon membrane and hybridized with a ³²P-labeled rat osteocalcin cDNA probe as described in Materials and methods. The 0.6 kb osteocalcin signals were detected by autoradiography. Exposure time to visualize signals was 1 day.

Figure 2 Phex mRNA expression in lungs of vehicle-, IGF-I- and GH-treated hypophysectomized rats. Upper panel: 15 µg total RNA extracted from the lungs of hypophysectomized rats treated for 2 days with vehicle, IGF-I or GH were separated on a 1% agarose formaldehyde gel, stained with ethidium bromide to confirm equal loading, transferred to a nylon membrane and hybridized with a ³²P-labeled rat Phex cDNA probe as described in Materials and methods. The 6.6 kb Phex signals were detected by autoradiography. Exposure time to visualize signals was 10 days. Lower panel: relative expression levels of Phex mRNA (means $\pm$ S.E.M., $n = 6$ animals) after treatment with vehicle, IGF-I or GH, normalized for equal loading (18S ribosomal RNA values) and quantified by scanning densitometry. * $P < 0.05$, vs vehicle-treated.

Figure 3 Neprilysin (NEP) mRNA expression in lungs of vehicle-, IGF-I- and GH-treated hypophysectomized rats. Upper panel: 15 µg total RNA extracted from the lungs of hypophysectomized rats treated for 2 days with vehicle, IGF-I or GH were separated on a 1% agarose formaldehyde gel, stained with ethidium bromide to confirm equal loading, transferred to a nylon membrane and hybridized with a 32 P-labeled rat NEP cDNA probe as described in Materials and methods. The 4.2 and 6.5 kb NEP signals were detected by autoradiography. Exposure time to visualize signals was 3 days. Lower panel: relative expression levels of NEP mRNA (means $\pm$ S.E.M., $n = 6$ animals) after treatment with vehicle, IGF-I or GH, normalized for equal loading (18S ribosomal RNA values) and quantified by scanning densitometry.

fell significantly after IGF-I treatment, while GH caused a smaller, statistically not significant decrease. Serum urea fell after treatment with both IGF-I and GH. Serum calcium levels were significantly increased only after GH, and there were no significant changes in serum Pi levels in response to either treatment (Table 2). Northern blot analysis for renal Na₄Pi-I and -II transporter mRNA transcripts showed hybridization

Table 4 Relative mRNA expression levels of 1 α -hydroxylase in the kidneys of IGF-I- or GH-treated compared with vehicle-treated hypophysectomized rats. Total RNA was prepared from kidneys of hypophysectomized rats ($n = 6$) treated for 2 days with vehicle, IGF-I or GH. Relative expression of 1 α -hydroxylase transcripts was measured by quantifying (by scanning densitometry) 1 α -hydroxylase PCR products in reverse-transcribed RNA samples normalized for GAPDH transcript levels. The specific primers used are described in Materials and methods. Means $\pm$ S.D. of three experiments are given.

Treatment	1 α -Hydroxylase mRNA
Vehicle	1
IGF-I	1.55 $\pm$ 0.14*
GH	1.54 $\pm$ 0.26*

* $P < 0.05$ vs vehicle.

signals of 2.3 and 2.7 kb respectively, but did not show differences between the three treatment groups (data not shown).

Both IGF-I and GH markedly raised serum 1,25-(OH)₂D₃ levels (Table 2). We also examined 1 α -hydroxylase gene expression in the kidneys by semi-quantitative RT-PCR. Both IGF-I and GH stimulated relative 1 α -hydroxylase expression (corrected for GAPDH expression), 1.55 $\pm$ 0.14-fold and 1.54 $\pm$ 0.26-fold respectively (Table 4) and downregulated 24-hydroxylase mRNA expression as estimated by Northern analysis: relative expression of the 4 kb transcript decreased to 0.55 $\pm$ 0.09 with IGF-I ($P = 0.09$) and to 0.18 $\pm$ 0.02 ($P = 0.01$) with GH (Fig. 4).

Discussion

Administration of GH or IGF-I during 2 days to hypophysectomized rats fed a normal diet with a Pi content of 0.82% caused a number of changes with respect to growth, bone metabolism and kidney function: BW, tibial epiphyseal width, serum osteocalcin levels and bone osteocalcin mRNA expression were increased. Phex mRNA expression in parietal bones and in lungs was stimulated, and serum 1,25-(OH)₂D₃ concentrations rose together with renal 1 α -hydroxylase mRNA expression. At the same time, renal 24-hydroxylase mRNA expression was suppressed.

The increase in tibial epiphyseal width and the increase in serum osteocalcin and in osteocalcin mRNA in parietal bone reflect anabolic actions of GH and IGF-I on cartilage and bone respectively. As observed earlier during infusion experiments in hypophysectomized rats with the same doses of GH and IGF-I as used in the present study, steady-state growth effects on tibial growth plate cartilage are already maximal after 2 days of infusion (35). Thus, the 2 day infusion period chosen for the present study allows analysis of a state where growth of the animals is maximally accelerated. Osteocalcin, the most abundant non-collagenous protein in bone, is synthesized by osteoblasts. Its increase in serum and increased osteocalcin mRNA in bone reflect enhanced bone formation. Previous studies have shown that systemically administered IGF-I rapidly stimulates bone collagen and alkaline phosphatase mRNA expression in hypophysectomized rats (27, 39). More recently, analysis of transgenic mice with overexpression of IGF-I targeted to osteoblasts showed that locally produced IGF-I increased the activity of osteoblasts (bone formation, bone mineral density and bone volume), without a marked increase in precursor proliferation (40).

Parietal bones were investigated for Phex mRNA expression rather than other bones since the cellular composition of the latter is more heterogeneous. Phex is expressed as a low-abundance transcript of about 6.6 kb in the rat. Even in bone, considered to be the

Figure 4 24-Hydroxylase mRNA expression in kidneys of vehicle-, IGF-I- and GH-treated hypophysectomized rats. Upper panel: 15 µg total RNA extracted from the kidneys of hypophysectomized rats treated for 2 days with vehicle, IGF-I or GH were separated on a 1% agarose formaldehyde gel, stained with ethidium bromide to confirm equal loading, transferred to a nylon membrane and hybridized with a ³²P-labeled rat 24-hydroxylase cDNA probe as described in Materials and methods. The ~4 kb 24-hydroxylase signals were detected by autoradiography. Exposure time to visualize signals was 10 days. Lower panel: relative expression levels of 24-hydroxylase mRNA after treatment with vehicle, IGF-I or GH normalized for equal loading (18S ribosomal RNA values) and quantified by scanning densitometry (means ± S.E.M., n = 6 animals). *P < 0.05 GH-treated vs vehicle-treated.

main Phex-expressing tissue in rats, mice and humans, Phex mRNA levels are far below those of GAPDH mRNA. Our *in vivo* study shows for the first time that both GH and IGF-I do not only enhance expression of Phex mRNA in bone but also in the lungs. Lungs yielded higher amounts of RNA than parietal bones, so that Phex transcripts could be detected by Northern analysis of total RNA from individual rats. The physiological relevance of Phex expression in the lungs is currently unknown. PHEX/Phex shares a high degree of amino acid homology with other type II integral endopeptidases expressed in the lung, such as NEP and endothelin-converting enzyme-1 (41–43). These enzymes control the activity of bioactive peptides targeting blood vessels and the kidney. Therefore, it is not unlikely that PHEX in the lungs serves a similar purpose. It is not yet known whether Phex expression in the lungs is regulated by Pi and/or by 1,25-(OH)₂D₃ and other hormones, or whether GH and IGF-I stimulate Phex expression directly in lung cells. Nevertheless, the upregulation by IGF-I and GH of Phex gene expression in the lungs, shown here for the first time, seems to be specific, since NEP gene expression in lungs (like in the kidney, not shown) was not significantly affected by the hormone treatment. Stimulation of both Phex and NEP gene expression by GH was moderate and failed to reach statistical significance for the latter. For IGF-I, the

clear-cut 3.9-fold increase of Phex mRNA is in marked contrast to the 1.55-fold 'stimulation' of NEP mRNA. The specific cell type(s) expressing Phex mRNA in the lung remain(s) to be identified. *In situ* hybridization and immunocytochemical studies in mice showed Phex mRNA and protein expression in odontoblasts, differentiated osteoblasts and osteocytes, but in contrast to our findings in rats, both Northern and immunoblot analysis of extracts from lungs were reported to be negative (44). According to our observations in rats, PHEX substrates or their products might play a local role in the lung or contribute to the regulation of Pi homeostasis.

In bone, PHEX/Phex appears to play a role in the mineralization process. Inactivating mutations in the PHEX/Phex gene as found in XLH in man (7) or in the Hyp mouse (9) lead to impaired bone mineralization and to osteomalacic bone disease. The bone phenotype in XLH is the result of both hypophosphatemia, secondary to the renal Pi leak, and an intrinsic mineralization defect (44). *In vitro*, Phex mRNA expression in mouse osteoblasts is correlated with the expression of late osteoblast markers such as osteocalcin (6, 45), and with the formation of bone nodules (46). These data are in agreement with our *in vivo* findings that GH- and IGF-I-stimulated Phex mRNA expression in bone is accompanied by increased osteocalcin mRNA expression in bone. The effects of GH and IGF-I occurred without changes in serum Pi. This may be explained as follows: accelerated transfer of food-derived Pi and calcium through the gut would be expected due to the increase in serum 1,25-(OH)₂D₃, which enhances intestinal Pi and calcium absorption (47–49). This would be required for increased Pi retention at bone-forming sites during bone formation. Bone formation may be directly stimulated by GH and IGF-I and then immediately followed by mineralization, in which Phex could play a role.

Whereas decreased serum creatinine levels during GH and IGF-I treatment mainly reflect increased glomerular filtration rate (GFR), decreased urea levels reflect both increased GFR and positive nitrogen balance, consistent with an anabolic state, i.e. growth resumption of the animals.

Both GH and IGF-I significantly increased renal 1α-hydroxylase expression and serum 1,25-(OH)₂D₃ levels. IGF-I tended to be more effective on 1,25-(OH)₂D₃ than GH. Possibly, the slight increase in serum calcium after GH administration counteracted and thereby reduced (maybe together with a fall in serum parathyroid hormone (PTH)) the stimulatory action of GH. We cannot exclude that changes in PTH and/or calcitonin may contribute to enhanced mRNA expression of 1α-hydroxylase as reported by Shinki *et al.* (50). However, GH and IGF-I may well have stimulated renal 1α-hydroxylase expression directly, since both hormones have been shown to increase 1,25-(OH)₂D₃ production by mammalian

kidney cells *in vitro* (51). Our findings indicate that the well-documented *in vitro* stimulation of vitamin D metabolism by IGF-I (52, 53) could be relevant *in vivo*. No circulating mediators would be required for this renal action of IGF-I. *In vivo*, the stimulatory effects of IGF-I on 1,25-(OH)₂D₃ occurred in the face of unchanged serum calcium and Pi levels, as also observed in GH-deficient human subjects (54).

The stimulation of 1 α -hydroxylase expression by GH and IGF-I was accompanied by an inhibition of renal 24-hydroxylase mRNA expression. 24,25-(OH)₂D₃, a second dihydroxylated vitamin D metabolite produced by the kidney, represents the major circulating 25-(OH)D₃ metabolite. GH and IGF-I have been reported to decrease the conversion of 25-(OH)D₃ to 24,25(OH)₂D₃ in hypophysectomized rats (55). It was also reported that GH treatment of GH-deficient children decreased serum levels of 24,25-(OH)₂D₃ (56). Accordingly, 24-hydroxylase mRNA expression was suppressed in our GH-treated hypophysectomized animals and tended to be lower also during IGF-I treatment. At the same time, 1,25-(OH)₂D₃ serum levels were increased. Apparently, 25-(OH)D₃ is preferentially hydroxylated at the 1 position due to the reciprocal regulation of the 1 α - and the 24-hydroxylase by GH and IGF-I.

In conclusion, our data suggest a coordinate regulation of PheX mRNA expression in lungs and bone of serum 1,25-(OH)₂D₃ levels and of 1 α - and 24-hydroxylase during GH- and IGF-I-stimulated growth of rats, consistent with physiological roles of the PheX and vitamin D system in Pi homeostasis during the process of growth.

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