Local action of exogenous growth hormone and insulin-like growth factor-I on dihydroxyvitamin D production in LLC-PK1 cells

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Abstract

Previous in vivo studies have shown that growth hormone (GH) affects vitamin D and mineral metabolism. Insulin-like growth factor-I (IGF-I) was recently reported to be a regulator of renal 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) production, suggesting that it mediates the effects of GH on vitamin D metabolism. However, there is no direct evidence to support this. The present study was designed to investigate the in vitro effects of GH and IGF-I on the renal production of 1,25-(OH)2D3 and 24,25-dihydroxyvitamin D3 (24,25-(OH)2D3) in a pig kidney cell line, LLC-PK1. Confluent cells were preincubated in serum-free medium with hormone (GH or IGF-I) or vehicle, and then incubated with 25-[3H]OHD3. The levels of 1,25-[3H](OH)2D3 and 24,25-[3H](OH)2D3 produced were determined after lipid extraction and HPLC purification. Production of 1,25-(OH)2D3 and 24,25-(OH)2D3 was increased after both IGF-I and GH preincubation in a dose-dependent manner. Significant increases were found after preincubation with 13 nmol/l IGF-I (1,25-(OH)2D3, 1.8-fold; 24,25-(OH)2D3, 1.5-fold) or 0.9 or 9 nmol/l GH (1,25-(OH)2D3, 1.3-fold and 1.5-fold; 24,25-(OH)2D3, 1.4-fold and 1.5-fold respectively). Furthermore, the effect of 9 nmol/l GH on 1,25-(OH)2D3 and 24,25-(OH)2D3 production was blocked in the presence of IGF-I receptor monoclonal antibody. These results confirm that IGF-I acts on renal tubules, resulting in induction of 1,25-(OH)2D3 and 24,25-(OH)2D3 production, and the findings suggest that GH stimulates 1,25-(OH)2D3 and 24,25-(OH)2D3 production by increasing local IGF-I production in the kidney.

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Introduction

It is well established that the bioactivation of vitamin D involves two successive steps of hydroxylation, the first of which is catalysed by 25-hydroxylase in the liver, yielding 25-hydroxyvitamin D3 (25-OHD3). 25-OHD3 is physiologically converted into 1α- or 24-dihydroxyvitamin D3 (1,25-(OH)2D3 and 24,25-(OH)2D3) mostly in kidney proximal tubular cells in non-pregnant mammals (1–3). The direct modulators of the production of 1,25-(OH)2D3, the most active form of vitamin D3, are parathyroid hormone (PTH), 1,25-(OH)2D3 itself, calcitonin and the extracellular concentrations of calcium and phosphate (1–3). 1,25-(OH)2D3 and PTH were found to be modulators of 24-hydroxylation (2–4), the biological significance of which is controversial (5, 6). Growth hormone (GH) has been demonstrated to act on mineral metabolism, increasing intestinal absorption and urinary phosphate reabsorption (7–9). The similarity between vitamin D and GH with regard to mineral metabolism suggests that GH exerts, at least in part, its effects by modulating vitamin D metabolism.

A number of observations seem to support this hypothesis. GH has been shown to restore the formation of 1,25-(OH)2D3 which is reduced by hypophysectomy in rats (10–12). Treatment with rat GH normalized the conversion of 25-OHD3 to 24,25-(OH)2D3, which was markedly increased in hypophysectomized rats (12). Similar results have been found in pigs (13). It is well established in clinical studies that high-dose GH produces increased serum 1,25-(OH)2D3 levels after both short-term (8, 14, 15) and long-term administration (9, 16). We found that GH therapy caused decreased serum 24,25-(OH)2D3 levels (9), similar to results obtained in animal studies (12, 13). This action of GH has been suggested to be mediated by insulin-like growth factor-I (IGF-I) (17–19). Indeed, recent studies have shown that IGF-I stimulates 1α-hydroxylase (1α-OHase) activity in vitro and in vivo (20–23). However, it has not yet been investigated whether GH directly influences renal vitamin D metabolism.

To elucidate the regulation of 25-OHD3 metabolism in the kidney and the relation between growth and vitamin D metabolism, the present study was designed to define the in...
vitro effects of GH and IGF-I on the renal hydroxylation of 25-OHD$_3$ in a pig kidney cell line, LLC-PK1.

**Materials and methods**

**Materials**

Culture media were purchased from Gibco Laboratories (Grand Island, NY, USA), and plasticware was from Costar Corp. (Cambridge, MA, USA). Sep-Pak silica cartridges were purchased from Waters Associates (Milford, MA, USA). A Gilson HPLC system (Gilson Medical Electronics, Villiers le Bel, France) was used, consisting of a column (10 μm; 8 mm × 100 mm) of µPorasil for the straight phase and a column (10 μm; 8 mm × 100 mm) of Bondapak C$_18$, for the reverse phase. Scintillation counting was performed in an Aloka liquid-scintillation system LSC 700 (Aloka Corp., Tokyo, Japan). A Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) and UV-120–02 spectrophotometer (Shimadzu Corp., Kyoto, Japan) were used for the cell protein assay. All chromatography solvents used were HPLC-grade and purchased from Wako Pure Chemical Industries (Osaka, Japan). A Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) and UV-120–02 spectrophotometer (Shimadzu Corp., Kyoto, Japan) were used for the cell protein assay. All chromatography solvents used were HPLC-grade and purchased from Wako Pure Chemical Industries (Osaka, Japan).

1.25-(OH)$_2$D$_3$ and 24,25-(OH)$_2$D$_3$ were gifts from Teijin Pharmaceutical Ltd (Tokyo, Japan). 25-Hydroxy[26(27)-methyl-1$^3$H]cholecalciferol (15 Ci/mmol, TRK 396) was purchased from Amersham International (Amersham, Bucks, UK). Human recombinant IGF-I was purchased from Fujisawa Pharmaceutical Ltd (Tokyo, Japan). IGF-I receptor monoclonal antibody (αIR3) was from Calbiochem (Cambridge, MA, USA). Recombinant porcine GH was from BresaGen Limited (Adelaide, Australia).

**Cell culture**

LLC-PK1 cells were originally obtained from Health Science Research Resources Bank (Osaka, Japan). Cells were grown in 10 cm plastic trays in medium containing 1:1 (v/v) Dulbecco’s modified Eagle’s medium and Ham’s F-12, supplemented with 5% fetal bovine serum. This medium contains 1 mmol/l phosphate and 1 mmol/l calcium. All cultures were maintained at 37°C under 5% CO$_2$/95% air. Confluence was generally reached within 6–7 days, and the assays were performed on the 7th or 8th day.

**Metabolism of 25-OH$D_3$**

On the day before the experiment, the culture medium was changed and the cells were preincubated for 24 h in serum-free medium with vehicle or hormone (IGF-I or GH).

After preincubation, the medium was removed and the monolayers were rinsed with Ca/Mg-free PBS. The cells were harvested with trypsin/EDTA and washed with fresh medium. The resuspended cells (in 4 ml medium) were transferred to glass tubes containing 75 pmol 25-[1$^3$H]OH$D_3$, dried under nitrogen, provided as substrate. Incubation was continued in a shaking water bath at 37°C for 4 h unless otherwise indicated. The reaction was terminated by the addition of 3 ml methanol/chloroform (1:1, v/v). Vitamin D metabolites were extracted and separated by previously described methods (24) as follows.

Briefly, the chloroform phases were dried under nitrogen, and the residues redissolved in 1 ml hexane/isopropanol (96:4, v/v) and applied to Sep-Pak silica cartridges prewashed successively with 5 ml methanol, 5 ml chloroform, 5 ml hexane and 5 ml hexane/isopropanol (96:4, v/v). The 25-[1$^3$H]OH$D_3$ fraction was removed by washing the cartridge with 11 ml hexane/isopropanol (96:4), and the mixed 24,25-[1$^3$H](OH)D$_2$ and 1,25-[1$^3$H](OH)D$_3$ fractions were eluted with 10 ml hexane/isopropanol (80:20, v/v). The 24.25-(OH)$_2$D$_3$ and 1,25-(OH)$_2$D$_3$ fractions were dried under nitrogen, redissolved in the chromatographic solvent, and applied to a straight-phase HPLC system equilibrated with hexane/isopropanol (92:8, v/v) (flow rate 2 ml/min). Portions of the 24.25-(OH)$_2$D$_3$ and 1,25-(OH)$_2$D$_3$ fractions from the straight-phase HPLC system were rechromatographed by reverse-phase HPLC at a flow rate of 2 ml/min with methanol/water (80:20, v/v) as eluent.

1α- and 24-OHase activities were estimated by determining the radioactivity of 1.25-[1$^3$H](OH)$_2$D$_3$ and 24,25-[1$^3$H](OH)D$_3$ produced by the cells during the incubation. The radioactivities of the 24.25-(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$ fractions yielded by the straight-phase and continuous straight-reversed phase HPLC systems showed good correlation (data not shown). Therefore enzyme activities are expressed using radioactivity values yielded by straight-phase HPLC.

The intra-assay coefficient of variation was less than 10%, and the interassay coefficient of variation was less than 15% for 1,25-(OH)$_2$D$_3$ and 24,25-(OH)$_2$D$_3$ in this assay system. All experiments were performed in duplicate. The results are expressed as pmol/mg protein.

**Statistics**

Statistical analysis was performed using ANOVA (Fisher’s PLSD) in the computer program Statview J 4.02 (Abacus Concepts, Berkeley, CA, USA). Results are given as the mean±S.E.M. P<0.05 was considered significant.

**Results**

**Metabolism of 25-OH$D_3$ by LLC-PK1 cells**

The untreated cells were able to convert 25-OH$D_3$ into more polar metabolites. The elution profile of the straight-phase HPLC is shown in Fig. 1a. The major polar metabolite (peak I) was co-eluted with synthetic
24,25-(OH)\(_2\)D\(_3\), and a small peak (peak II) was detected at the same elution position as synthetic 1,25-(OH)\(_2\)D\(_3\). These two fractions were rechromatographed by reverse-phase HPLC. Figure 1b shows the profile of the reverse-phase HPLC of peak I, which was co-eluted with synthetic 24,25-(OH)\(_2\)D\(_3\). The profile of peak II on the reverse-phase column showed a single peak which was co-eluted with synthetic 1,25(OH)\(_2\)D\(_3\) (Fig. 1c).
Effect of assay incubation time on 1,25-(OH)2D3 and 24,25-(OH)2D3 production

The time course of 24,25-(OH)2D3 and 1,25-(OH)2D3 synthesis from 25-OHD3 is shown in Fig. 2. The incubation was performed for 1–5 h, and the production of both metabolites reached a plateau after 4 h of incubation.

Effect of IGF-I and GH on 1,25-(OH)2D3 and 24,25-(OH)2D3 production

Exogenous recombinant human IGF-I increased the amount of 1,25-[3H](OH)2D3 and 24,25-[3H](OH)2D3 detected. These effects of IGF-I required incubation of the cells for more than 4 h. Recombinant porcine GH at concentrations between 0.09 and 9 nmol/l in a 24 h preincubation with the cells also stimulated 1,25-(OH)2D3 and 24,25-(OH)2D3 production. Significant stimulation occurred at 0.9 and 9 nmol/l GH (Fig. 5). Furthermore, the maximal stimulation caused by preincubation with 9 nmol/l GH was completely blocked by the addition of 1 µg/ml IGF-I receptor antibody for both 1,25-(OH)2D3 and 24,25-(OH)2D3 production (Fig. 5).

Discussion

We demonstrate the modulation of dihydroxyvitamin D3 production in a mammalian kidney cell
The LLC-PK1 cells were originally derived from the kidneys of Hampshire pigs (25), and are shown to express 1,25-(OH)₂D₃ receptors and 25-OHD₃ 24-OHase in response to 1,25-(OH)₂D₃ (26). The study of Condamine et al. (21) showed that LLC-PK1 cells express 25-OHD₃ 1α-OHase activity, and the present study obtained similar findings. The cells were observed to convert 25-OHD₃ into two main metabolites, which corresponded to synthetic 24,25-(OH)₂D₃ and 1,25-(OH)₂D₃. No difference was found in HPLC elution profiles between the present results and those obtained with other highly specific substrates, e.g. 25-[³H]OHD₃ (25-hydroxy[23,24(n)-³H]cholecalciferol (110 Ci/mmol, TRK558)) (data not shown). LLC-PK1 cells proved to be useful models for studying 25-OHD₃ 1α- and 24-OHase enzymatic systems.

GH and/or IGF-I have been observed in previous in vivo studies to increase serum 1,25-(OH)₂D₃ levels and 1α-OHase activity in humans and animals (8–20, 23). IGF-I is suggested to mediate the GH-dependent increase in serum 1,25-(OH)₂D₃, as it can restore the increase in serum 1,25-(OH)₂D₃ induced by restriction of dietary phosphorus to the same degree as GH (18). Further studies are required to determine whether similar results are also observed in vitro.

Recent in vitro studies have shown stimulatory effects of IGF-I on renal 1,25-(OH)₂D₃ production. Condamine et al. (21) observed a local action of exogenous IGF-I on 1,25-(OH)₂D₃ production in LLC-PK1 cells, which may act by different mechanisms on phosphate deprivation. Menaa et al. (22) reported that the stimulatory effect of IGF-I on 1,25-(OH)₂D₃ production is a calcium-dependent effect in primary mouse kidney cells, unlike that induced by PTH, which involves the cAMP and/or the protein kinase C pathway. The present study also confirmed that IGF-I is an upregulator of renal 1α-OHase. In addition, the stimulatory effect on 1,25-(OH)₂D₃ production was also observed in GH-treated cells. This effect occurred in a dose-dependent manner, like that induced by IGF-I, with significant stimulation observed with large doses of GH (0.9 and 9 nmol/l). IGF-I receptor antibody (clone aIR3) recognizes the α-subunits of the IGF-I receptor, and blocks the signal transduction of IGF-I in a wide series of cell lines (27–29). In the present study, we used it to block IGF-I binding to its receptor in LLC-PK1 cells. The stimulatory effect of GH on 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃ production was completely blocked by this IGF-I receptor antibody. IGF-I expression in pig kidney has previously been demonstrated (30). Thus previous reports plus the present observations strongly suggest that GH indirectly affects 1,25-(OH)₂D₃ production in the kidney by increasing local production of IGF-I. Furthermore, we suggest that the elevated serum levels of 1,25-(OH)₂D₃ induced by GH administration in vivo are secondary to increased IGF-I production in the liver and other tissues. To confirm these suggestions, mRNA expression must be determined, which requires cloning of the enzyme of this cell line.

24,25-(OH)₂D₃ production was also observed in this study, and also a stimulating effect on the 24-OHase system by IGF-I and GH treatment. In contrast, we have previously observed decreased serum levels of 24,25-(OH)₂D caused by GH administration in GH-deficient children (9), similar to results obtained in animal studies (12, 13). Wu et al. (31) observed decreased 24-OHase mRNA levels after administration of GH and IGF-I in hypophysectomized rats fed a high phosphate diet, suggesting that the condition of the supplied phosphate may play a key role in the regulation of 24-OHase activity by GH and/or IGF-I in vivo. Iida et al. (32) indicated that vitamin D receptors in renal proximal tubular cells are downregulated when renal production of 1,25-(OH)₂D₃ is stimulated, and therefore
1,25-(OH)₂D₃-induced 24-OHase is certainly decreased. Thus, theoretically, the production of one of these two metabolites, but not both, is increased. The discrepancy between the present and previous studies can be explained as follows. First, in no study have these two hydroxylases been simultaneously observed in this cell line until now. Either the ‘switching’ role played by the vitamin D receptor has been lost in our experimental system or the study period of 4 h is too short to ‘switch’ it on if it does exist. Secondly, changes in other factors caused by GH administration in vivo that affect vitamin D metabolism (such as PTH and phosphate) may also play a role in the variation in serum 24,25-(OH)₂D₃ levels. We suspect that the upregulation of 24,25-(OH)₂D₃ production in LLC-PK1 cells is, at least in part, a direct stimulatory effect of IGF-I (GH) on 24-OHase, and not only due to induction of 24-OHase by 1,25-(OH)₂D₃, because the appearance of the two metabolites occurs at the same time.

In summary, the present observations in a pig kidney cell line confirm that IGF-I acts on renal tubules, resulting in induction of 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃ production. We demonstrate that a large dose of GH also stimulates 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃ production by increasing local IGF-I production in the kidney. The present results suggest a mechanism of in vivo effects of GH on vitamin D metabolism.
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References


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