1,25-Dihydroxyvitamin D_3 production in the isolated perfused rat kidney in response to changing perfusate phosphorus concentrations and insulin-like growth factor

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We used the isolated perfused rat kidney as a model to assess the direct, acute effect of perfusate phosphorus and insulin-like growth factor (IGF-1) (somatomedin-C) on renal 25-hydroxyvitamin D_3 1-α-hydroxylase (1-α-hydroxylase) activity. 1-α-hydroxylase activity was assessed by measuring the conversion of 25-hydroxyvitamin D_3 (25(OH)D_3) (20 ng/mL of perfusate, total volume 100 mL) to 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2D_3$) using a radioreceptor assay specific for 1,25(OH)$_2D_3$. In experiment 1, all perfusions were performed with constant perfusate calcium (2.5 mmol/L) and four different concentrations of phosphate (2.0, 1.0, 0.5, and 0.25 mmol/L). In experiment 2, perfusions were performed with normal perfusate calcium (2.5 mmol/L) and phosphorous (2.0 mmol/L) and three different levels of IGF-I (0, 1, and 3 μg/mL of perfusate). Analysis of variance showed a significant ($P < 0.05$) difference in 1-α-hydroxylase activity between groups in both experiments 1 and 2. In experiment 2, this difference is shown with increasing time of perfusion. These data indicate that there is an increase in 1-α-hydroxylase activity in response to decreasing perfusate phosphorous concentration and a high concentration of IGF-I.

Keywords: 1-α-hydroxylase activity; 1,25-dihydroxyvitamin D_3; hypophosphatemia; insulin-like growth factor; growth hormone; isolated kidney

Introduction

It is now established that the vitamin D produced in the skin or ingested in the diet is first hydroxylated in the liver to 25-hydroxyvitamin D_3 [25(OH)D_3], which is then further hydroxylated in the kidney to form either 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2D_3$], the most biologically active vitamin D metabolite, or 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2D_3$], whose biological function is not yet clearly known.[1] Several factors have been implicated in the regulation of renal 1-α-hydroxylase activity, with serum phosphorus being recognized as an important primary regulator. Severe phosphorus depletion with associated hypophosphatemia increases 1,25(OH)$_2D_3$ production in vivo.[2,3] However, in vitro studies have failed to show a direct effect of changes in phosphate concentrations on renal 1,25(OH)$_2D_3$ production.[4] In association with hypophosphatemia, growth hormone (GH) has been identified as a secondary modulator of renal 1-α-hydroxylase activity and probably mediates its effect through insulin-like growth factor (IGF-I), also called somatomedin C.[5] Early studies by Beck et al.[6] demonstrated an increase in intestinal calcium absorption in response to GH but failed to establish a mechanism of action. Spencer and Tobiassen[7] first experimentally linked GH to vitamin D metabolism through the use of the hypophysectomized rat as an animal model. They demonstrated that GH promoted the conversion of 25(OH)$_2D_3$ to 1,25(OH)$_2D_3$, a reaction that is blunted in the hypophysectomized animal. In addition, several studies have shown that the activation of renal 1-α-hydroxylase activity by low serum phosphate in vivo can be blocked by hypophysectomy and restored by GH replace-

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ments.[8,9] Although these studies linked GH to vitamin D metabolism, it was not clear whether GH acted indirectly through a GH-dependent factor such as IGF-I or whether GH acted directly to influence real 1-α-hydroxylase activity. Recent studies in hypophysectomized, vitamin D replete rats have shown that systemic IGF-I can restore the increase in serum $1,25(OH)_2D_3$ induced by dietary phosphate restriction.[9-11]

The aim of the present study is to more clearly define the direct role of phosphorus and IGF-I in regulating renal 1-α-hydroxylase activity through the use of the isolated perfused rat kidney as an in vitro model system.

Materials and methods

Animals

Adult male Sprague-Dawley rats (250–300 g) purchased from SASCO (Omaha, NE) were used for the present study. The animals were maintained at 23° C in controlled lighting and started on a vitamin D-replete, normal calcium diet containing 1.2% calcium, 0.7% phosphorus, and 3.3 IU/g of vitamin D_3.

Vitamin D compounds and insulin-like growth factor (IGF-I)

$25(OH)D_3$ was a generous gift from Dr. M.R. Uskokovic, Hoffman-La Roche, Nutley, NJ USA. [^{3}H-26,27]$1,25(OH)_2D_3$ as a recovery tracer (1,700–2,200 cpm/50 μL) and as an assay tracer (11,500–13,500 dpm/50 μL) was provided as part of a radioreceptor assay kit from INCSTAR Corp., Stillwater, MN USA. Human recombinant IGF-I was provided by Ciba Geigy, Basle, Switzerland and Chiron, Emeryville, CA USA. Its purification and characterization have been described previously.[12]

Solvents and scintillation fluid

Scintillation fluid (Scintiverse LC) and all solvents were purchased from Fisher Scientific Laboratories, Fairlawn, NJ, USA and were used as obtained.

Perfusion fluid composition

The perfusion solution was a modified Krebs-Henseleit bicarbonate buffer containing bovine serum albumin (6 g/100 mL), all L-amino acids and glucose (5 mmol/L). The solution was filtered through a 0.45 μm pore size Millipore filter. The volume of perfusate used in each experiment was 100 mL. In experiment 1 perfusate calcium concentration was held constant (2.5 mmol/L) with four different concentrations of perfusate phosphorus (group A, 2.0; group B, 1.0; group C, 0.5; group D, 0.25 mmol/L). In experiment 2, perfusate calcium and phosphorus were held constant at 2.5 and 2.0 mmol/L, respectively. The perfusion groups were as follows: group 1, $25(OH)D_3$ only; group 2, $25(OH)D_3$ plus IGF-I (1 μg/mL of perfusate); and group 3, $25(OH)D_3$ plus IGF-I (3 μg/mL of perfusate).

Addition of vitamin D and IGF-I to perfusions

Kidney perfusions were performed with 100 mL of perfusate containing 5.0 nmoles of $25(OH)D_3$ for a period of 6 hours. Five nmoles of $25(OH)D_3$ was dissolved in 123 μL of ethanol and then added to 5 mL of perfusate outside the chamber. This mixture was then introduced into the recirculating pool of perfusate via the venous reservoir after 15 minutes of stabilization of the kidney. Recombinant human IGF-I was dissolved in 1.0 mmol/L acetic acid to make a stock concentration of 1 μg/μL. IGF-I was introduced at the start of the perfusion [5 minutes after the addition of $25(OH)D_3$] into the recirculating pool of perfusate via the venous reservoir to yield perfusate IGF-I concentrations of 1 and 3 μg/mL.

Study of $25(OH)D_3$ conversion to $1,25(OH)_2D_3$ in the isolated perfused rat kidney using the radioreceptor assay

Isolation and perfusion of the kidney were performed exactly as described before in detail.[13,14] After the addition of $25(OH)D_3$ to the perfusate, aliquots of perfusate (2 mL) were taken every hour and perfusions were continued for 6 hours. One mL aliquots of perfusate were analyzed for $1,25(OH)_2D_3$ concentration using the radioreceptor assay (INCSTAR, Stillwater, MN USA), which utilizes a calf thymus receptor that is specific for $1,25(OH)_2D_3$.[15] The radioreceptor assay involves the rapid extraction and purification of the metabolites using a C_{18}OH cartridge. Prior to extraction, 50 μL of [3H-26,27]$1,25(OH)_2D_3$ (1,700–2,200 cpm/50 μL) is added to each sample to monitor recovery. Acetonitrile (1.0 mL) is added to each sample causing precipitation of lipids and proteins. Potassium phosphate (0.4 M) is then added to the supernatant to remove any remaining lipids. The sample is then applied to a C_{18}OH cartridge. Elution of vitamin D metabolites is done through the sequential addition of 90% hexane/10% methylene chloride, which removes both $25(OH)D_3$ and $24,25(OH)_2D_3$, and 99% hexane/1% isopropanol, which removes any remaining $24,25(OH)_2D_3$, leaving only $1,25(OH)_2D_3$ in the cartridge. The final application of 96.5% hexane/3.5% isopropanol elutes purified $1,25(OH)_2D_3$ from the cartridge. The eluted samples were collected and counted in an aqueous scintillation fluid. Radioactivity was measured on a Packard 5650 scintillation counter (Packard Instrument Company, Dowers Grove, IL). The minimum detectable concentration of $1,25(OH)_2D_3$ is 5 pg/mL. The intra- and interassay coefficients of variation are 6.5% and 11.5%, respectively.[16]

Measurement of serum calcium and phosphorous concentrations

Serum calcium was analyzed in the presence of lanthanum chloride by means of an atomic absorption spectrophotometer. Serum phosphorus was determined colorimetrically by an EKTA Chem 100 autoanalyzer in the clinical lab at Arkansas Children's Hospital. The concentrations of both ions were normal.

Results

$1,25(OH)_2D_3$ production in response to changing perfusate phosphorus concentrations

Experiment 1. There was an increase in $1,25(OH)_2D_3$ production in response to a perfusate phosphorus concentration of 0.25 mmol/L. Analysis of variance showed a significant difference ($P < 0.05$) in 1-α-hydroxylase activity as measured by radioreceptor assay between groups A versus D, B versus D, and C versus D at 6

hours of perfusion *(Figure 1)*. There was no significant difference in $1,25(OH)_2D_3$ production between groups A, B, C, and D at 1, 2, and 4 hours; hence, $1,25(OH)_2D_3$ production is shown only at 6 hours.

$1,25(OH)_2D_3$ production in response to increasing perfusate IGF-I concentration

Experiment 2. (*Figure 2*) Renal 1-α-hydroxylase activity increased with both an increased IGF-I concentration (3 μg/mL) and with increasing time of perfusion (6 hours). Analysis of variance showed a significant difference ($P < 0.05$) in $1,25(OH)_2D_3$ production between group 1 and group 3 and group 2 and group 3 at 6 hours of perfusion. This difference is due to the increased IGF-I concentration (3 μg/mL) in group 3 and is significant only after 6 hours of perfusion.

Discussion

Through the use of a radioreceptor assay specific for $1,25(OH)_2D_3$ the present study documents the small amounts of $1,25(OH)_2D_3$ produced in the vitamin D-replete rat kidney under physiological conditions. Past studies[1,4,13] designed to evaluate the effects of specific modulators (calcium, phosphorus, parathyroid hormone) on vitamin D metabolism have used both the vitamin D-replete and deficient animal and radiolabeled $25(OH)D_3$ as a means of monitoring $1,25(OH)_2D_3$ production. From these experiments it was noted that very little (<0.1%) $25(OH)D_3$ is converted to $1,25(OH)_2D_3$ in a vitamin D-replete animal, making it extremely difficult to measure $1,25(OH)_2D_3$ production unless one uses high quantities of radiolabeled $25(OH)D_3$. In a similar fashion, using very high quantities of radiolabeled $25(OH)D_3$ in an isolated perfused rat kidney, Reddy and Thomas have demonstrated the conversion of tritiated $25(OH)D_3$ into tritiated $1,25(OH)_2D_3$ in a vitamin D-replete rat kidney. It was also noted that the tritiated $1,25(OH)_2D_3$ produced in these experiments comigrated with authentic $1,25(OH)_2D_3$ on three different high pressure liquid chromatography systems (unpublished observations). Thus, the small amounts of $1,25(OH)D_2D_3$ produced in the vitamin D-replete rat kidney can be demonstrated only by the use of high quantities of radiolabeled $25(OH)D_3$. Therefore, a radioreceptor assay, specific for $1,25(OH)_2D_3$, was developed in our laboratory and was used in the present study to detect trace amounts of $1,25(OH)_2D_3$ produced under conditions of vitamin D repletion.

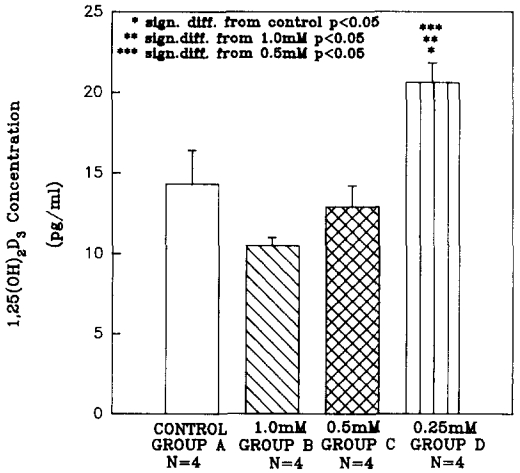

Figure 1 Effect of perfusate phosphorus concentration on the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ in isolated perfused kidneys taken from vitamin D-replete adult male rats. Kidneys were perfused for 6 hrs. with either 2.0 mmol/L (group A), 1.0 mmol/L (group B), 0.5 mmol/L (group C), or 0.25 mmol/L (group D) perfusate phosphorus and normal perfusate calcium (2.5 mmol/L) in all groups. Data are presented as mean ± S.E. P value < 0.05 by analysis of variance (ANOVA) in group A versus D, B versus D, and C versus D.

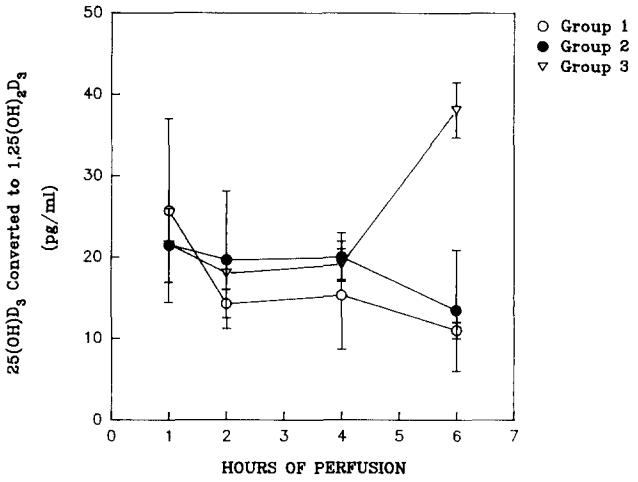

Figure 2 Effect of IGF-I on the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ in isolated perfused kidneys taken from vitamin D-replete adult male rats. Perfusion groups: group 1–$25(OH)D_3$ only, group 2–$25(OH)D_3$ plus IGF-I (1 μg/mL of perfusate) and group 3–$25(OH)D_3$ plus IGF-I (3 μg/mL of perfusate). Results are expressed as mean ± S.E. P value < 0.05 by ANOVA between groups with increasing time of perfusion. This is due to increased 1-hydroxylase activity in group 3 at 6 hours.

Several studies have documented an increase in $1,25(OH)_2D_3$ production in response to dietary phosphate restriction.[2,17] Using the isolated perfused rat kidney as a whole organ model we have demonstrated an increase in $1,25(OH)_2D_3$ production in response to low perfusate phosphorus concentrations. This is in agreement with more recent studies by Condamine et al. and indicative of a direct effect of acute changes in phosphorus concentration on renal $1,25(OH)_2D_3$ production.[18] The increased renal 1-α-hydroxylase activity that we observed in response to the very low perfusate phosphorus (0.25 mmol/L) concentrations may be due to the reduction of renal cortical phosphate to levels sufficient to stimulate 1-α-hydroxylase activity.[19]

Our results also show that there is an increase in $1,25(OH)_2D_3$ production with a perfusate IGF-I concentration of 3 μg/mL. While we recognize that 3 μg/mL of IGF-I is not physiologic,[20,21] our results are again consistent with the more recent study of Condamine

et al.,[18] which indicates that IGF-I does directly regulate the synthesis of $1,25(OH)_2D_3$ from its precursor, $25(OH)D_3$. The high concentration of IGF-I that is required for 1-α-hydroxylase activation may be due to the preexisting high 1-α-hydroxylase activity that is produced by endogenous IGF-I and/or loss of exogenous IGF-I within the perfusion system. Again, as with phosphorus, the use of the isolated whole organ (kidney) demonstrates the direct effect of IGF-I on renal 1-α-hydroxylase activity.

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