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Insulin markedly potentiates the capacity of parathyroid hormone to increase expression of 25-hydroxyvitamin D_3-24-hydroxylase in rat osteoblastic cells in the presence of 1,25-dihydroxyvitamin D_3

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Abstract We have previously shown that insulin alters the renal metabolism of 25-hydroxyvitamin D. To examine the effect of insulin on vitamin D metabolism in bone, we have used UMR-106 osteoblast-like cells to study the regulation of $25(OH)D_3$-24-hydroxylase (24-hydroxylase) expression by insulin. The 24-hydroxylase is an important enzyme in degrading 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$) in target tissues. Insulin alone had no effect on mRNA levels of the cytochrome P450 component (CYP24) of the 24-hydroxylase or on 24-hydroxylase activity itself in UMR cells. However, insulin increased the capacity of parathyroid hormone (PTH) to elevate CYP24 mRNA levels by 3–4-fold and to increase 24-hydroxylase activity by 2-fold in the presence of $1,25(OH)_2D$. Insulin increased the maximal responsiveness of UMR cells to PTH without altering their sensitivity. The action of insulin required the presence of $1,25(OH)_2D$ and was partly dependent on new protein synthesis. Insulin-like growth factor 1 also potentiated the effects of PTH. This marked stimulation of the 24-hydroxylase by PTH and insulin may serve to regulate $1,25(OH)_2D$ action and/or to produce 24,25-dihydroxyvitamin D in bone cells.

Key words: Insulin; Parathyroid hormone; 24-Hydroxylase; Vitamin D; Osteoblast; IGF-1

1. Introduction

The active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$), is synthesized primarily in the kidney by the renal 1-hydroxylase [1–5]. The $1,25(OH)_2D$ acts on its major target tissues such as intestine, kidney, and bone to regulate calcium and phosphate homeostasis [2]. In these tissues, $1,25(OH)_2D$ is further hydroxylated by the 24-hydroxylase. Hydroxylation in the 24-position is thought to be the first step in the degradation of $1,25(OH)_2D$.

The 24-hydroxylase is composed of an electron-transport chain consisting of three proteins – ferredoxin reductase, ferredoxin, and a specific cytochrome P450 – P450cc24 (CYP24) [3]. With the purification and cloning of CYP24 [6,7], it has been possible to study the hormonal regulation of the 24-hydroxylase in intestinal [8], renal [9], and bone cells [10]. In osteoblastic cells, we have found that the 24-hydroxylase is induced by $1,25(OH)_2D$ and that this induction is potentiated by parathyroid hormone (PTH) [10].

Insulin and insulin deficiency (diabetes) have significant effects on calcium and vitamin D metabolism, including loss of bone mineral [11]. We have previously shown that diabetes and subsequent insulin administration markedly alter renal 1-hydroxylase and 24-hydroxylase activity [12]. In diabetic rats, the 1-hydroxylase activity is decreased, and the 24-hydroxylase activity is increased compared to non-diabetic rats. Insulin administration increases 1-hydroxylase activity and decreases 24-hydroxylase activity in the kidney. Insulin appears to work by modulating the action of PTH [13].

In addition to the kidney, insulin may also have effects on vitamin D metabolism in bone cells. To examine this possibility, we have studied the effect of insulin on the expression of the 24-hydroxylase in UMR-106 osteoblast-like cells. The purpose of this study was to determine if insulin alone or in combination with $1,25(OH)_2D$ and PTH had an effect on 24-hydroxylase expression. We measured the effect of these hormones on mRNA levels of the cytochrome P450 component of the 24-hydroxylase (CYP24) and also on 24-hydroxylase enzymatic activity.

2. Materials and methods

The UMR-106 cell line was obtained from the American Type Culture Collection. This cell line is a cloned derivative of a transplantable rat osteogenic sarcoma [14]. Cells were cultured in T25 flasks with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells reached confluence in 3–5 days and were used within 30 passages of receipt as previously described [10].

To study the effects of hormones, confluent cells were deprived of serum for 24 h. Cells were then treated with hormones for 0–36 h, depending on the experiment. The $1,25(OH)_2D$ was a kind gift from Dr. Milan Uskokovic (Hoffmann-LaRoche, Nutley, NJ), and the PTH was rat PTH (1–34) from Bachem (Torrance, CA). Insulin used was either purified from bovine pancreas (Sigma, St. Louis, MO) or was human recombinant insulin (Calbiochem, San Diego, CA). Insulin-like growth factor 1 (IGF-1) used was recombinant human IGF-1 (Calbiochem, San Diego, CA). At the end of the experiment, cells were washed and stored frozen until isolation of total RNA using RNAzol (Tel-Test, Inc., Friendswood, TX).

The CYP24 mRNA levels were measured by dot blot as previously described [10]. The full length clone for rat CYP24 (p108, 3.2 kb) [7], kindly supplied by Drs. Y. Ohyama and K. Okuda (Hiroshima University School of Dentistry, Hiroshima, Japan), was used to generate the radiolabeled probe. Some dot blots were stripped and rehybridized with a probe for beta-actin (Oncor, Inc., Gaithersburg, MD). This checked for uniformity of sample loading and the specificity of the response to $1,25(OH)_2D$. Based on actin rehybridization, sample loading was quite uniform. Therefore, data were routinely normalized to the amount of total RNA applied, which was determined spectrophotometrically [10].

24-Hydroxylase activity was measured using radiolabeled $25(OH)D_3$ as a substrate, as previously described [10]. Briefly, cells were cultured to confluence in T75 flasks, washed twice with serum-free medium, and then pre-incubated for 1 h in serum-free medium. Cells were then incubated with 50 nM radiolabeled $25(OH)D_3$ for 1 h. After lipid extraction, the tritiated vitamin D products were partially

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purified using silica and C18 Sep-paks and then separated out by HPLC. HPLC was performed using a Zorbax-Sil column (0.5×25 cm) and a solvent system of hexane:methylene chloride:methanol (77:17.25:5.75).

Data are reported as the mean ± S.E.M. of the indicated number of flasks. The two-tailed Student's t-test was used to determine significance, and $P < 0.05$ was considered significant.

3. Results

We studied the effect of insulin on the induction of CYP24 mRNA by $1,25(OH)_2D$ and PTH (Fig. 1). Cells were incubated with hormones for 6 h, and mRNA levels for individual flasks were determined by dot blot (Fig. 1A). In the absence of insulin (control), $1,25(OH)_2D$ (100 nM) alone slightly increased CYP24 mRNA levels. PTH (25 nM) alone had no effect, but PTH markedly increased CYP24 mRNA levels in the presence of $1,25(OH)_2D$, as previously reported [10]. In the presence of 10 nM insulin (insulin-treated), the effect of $1,25(OH)_2D$ on mRNA levels was significantly increased compared to $1,25(OH)_2D$ alone. In addition, insulin potentiated the action of PTH in the presence of $1,25(OH)_2D$ by about 3-fold. Insulin and insulin plus PTH had no effect in the absence of $1,25(OH)_2D$. A dot blot of the pooled samples for each experimental condition is shown in Fig. 1B. A Northern blot of pooled samples showed a single band at 3.6 kb, regardless of hormone treatment (Fig. 1C). This is very close to the previously reported size of the CYP24 transcript in renal cells [9]. Insulin and PTH had no effect on beta-actin mRNA levels in the same studies (data not shown).

Because of the large increase, we further characterized the effect of insulin on PTH action with regard to CYP24 mRNA levels. All experiments were performed in the presence of 100 nM $1,25(OH)_2D$, since $1,25(OH)_2D$ was needed to observe an effect of insulin. From 3 to 36 h after addition, insulin (10 nM) significantly increased CYP24 mRNA levels over those seen with PTH (25 nM) alone (Fig. 2). At its maximum, CYP24 mRNA levels were over 3-fold higher in the presence of insulin compared to PTH alone. Insulin increased CYP24 mRNA levels in a dose-dependent fashion over the concentration range of 0.10–100 nM after 3 h (Fig. 3). Finally, insulin significantly increased CYP24 mRNA levels in the concentration range of 0.25–25 nM PTH (Fig. 4). Insulin (10 nM) increased the maximal response to PTH by about 3-fold after 3 h. The half-maximal effective concentration (EC_{50}) of PTH was about 1 nM in both the absence and presence of insulin.

Since insulin had markedly potentiated CYP24 mRNA levels by 3 h (Fig. 2), the effect of insulin on 24-hydroxylase activity was determined after the same time interval (Table 1). PTH (25 nM) markedly increased 24-hydroxylase activity in the presence of 100 nM $1,25(OH)_2D$. Addition of insulin (10 nM) resulted in a further significant increase in activity in

Table 1
Effect of hormones on 24-hydroxylase activity

Condition	24-Hydroxylase activity (pmol/h per mg protein)
Control	1.97 ± 0.15
$1,25(OH)_2D$	2.10 ± 0.16
$1,25(OH)_2D$+insulin	1.91 ± 0.11
$1,25(OH)_2D$+PTH	6.60 ± 0.99[a]
$1,25(OH)_2D$+PTH+insulin	12.4 ± 1.6[b]

Table entries are the mean ± S.E.M. of 4 individual flasks. Cells were treated with 10 nM insulin, 100 nM $1,25(OH)_2D$, and/or 25 nM PTH for 3 h.
[a]Significantly different from control ($P < 0.05$, t-test).
[b]Significantly different from $1,25(OH)_2D$+PTH ($P < 0.05$, t-test).

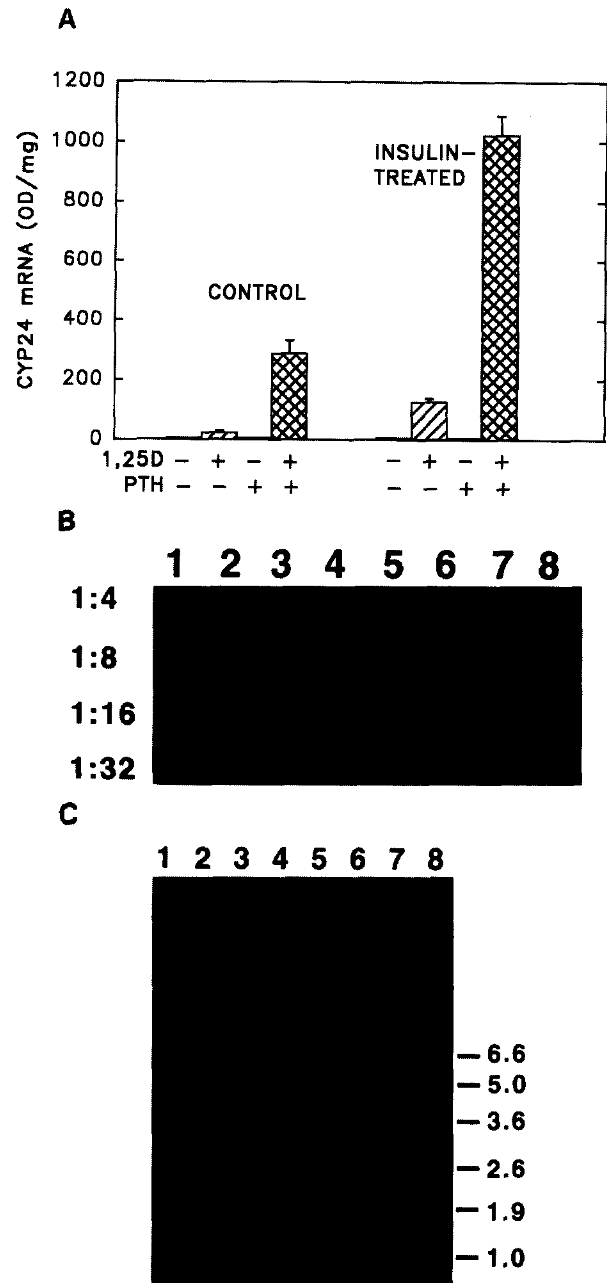

Fig. 1. Effect of insulin on the action of PTH and $1,25(OH)_2D$. Flasks of UMR cells were treated with PTH and/or $1,25(OH)_2D$ (1,25D) in the presence or absence of insulin for 6 h. Hormone concentrations were 25 nM PTH, 100 nM $1,25(OH)_2D$, and 10 nM insulin. CYP24 mRNA levels were quantitated by dot blot. (A) Mean ± S.E.M. of CYP24 mRNA levels in 4 individual flasks. Insulin significantly increased the effect of $1,25(OH)_2D$ and $1,25(OH)_2D$+PTH ($P < 0.05$, t-test) but had no effect in the absence of $1,25(OH)_2D$. (B) Dot blot of pooled RNA samples from each experimental condition: (1) No hormones; (2) 1,25D alone; (3) PTH alone; (4) 1,25D+PTH; (5) insulin alone; (6) 1,25D+insulin; (7) PTH+insulin; and (8) 1,25D+PTH+insulin. (C) Northern blot of pooled RNA samples. Lanes 1–8 contain RNA from cells incubated under the same experimental conditions as (B). Blot was calibrated by running standards of known molecular size in one lane (numbers on right).

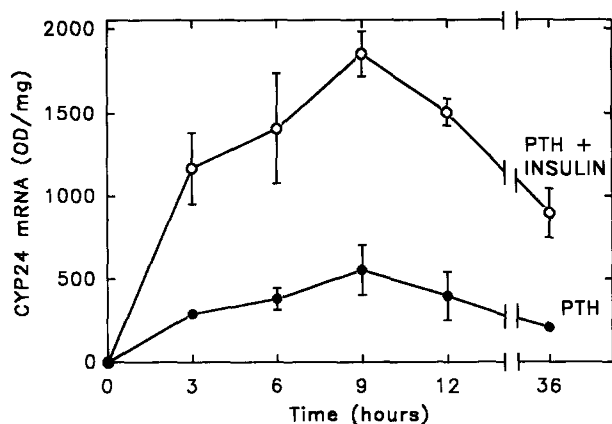

Fig. 2. Time course of insulin action. UMR cells were treated with PTH (25 nM) in the presence or absence of insulin (10 nM) for the indicated period of time. $1,25(OH)_2D$ (100 nM) was present in all experiments. CYP24 mRNA levels were quantitated by dot blot. Data points are the mean ± S.E.M. of 4 flasks. Insulin significantly increased the effect of PTH at all time points ($P < 0.05$, t-test).

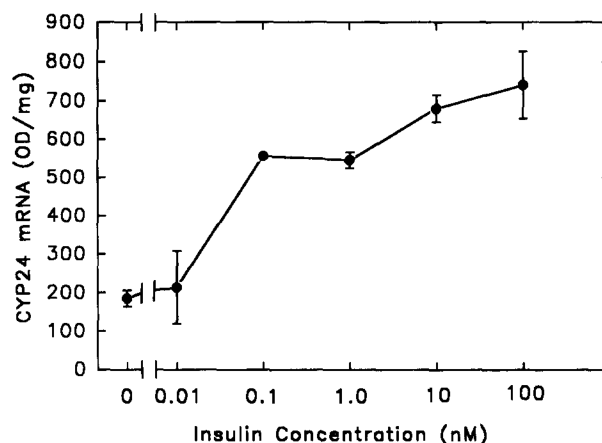

Fig. 3. Dose response of insulin action. UMR cells were treated with the indicated concentration of insulin for 3 h. PTH (25 nM) and $1,25(OH)_2D$ (100 nM) were present in all experiments. CYP24 mRNA levels were quantitated by dot blot. Data points are the mean ± S.E.M. of 4 flasks. Insulin had a significant effect at concentrations of 0.1–100 nM ($P < 0.05$, t-test).

the presence of $1,25(OH)_2D$ and PTH. $1,25(OH)_2D$ alone and $1,25(OH)_2D$+insulin had no measurable effect on hydroxylase activity. This lack of effect may be due to the short incubation time with hormones (3 h).

Since IGF-1 is related to insulin with regard to mechanism of action, the capacity of IGF-1 to potentiate the action of PTH after 3 h was studied. IGF-1 increased CYP24 mRNA levels in a dose-dependent fashion in the presence of 25 nM PTH and 100 nM $1,25(OH)_2D$ (Fig. 5). IGF-1 concentrations as low as 0.1 nM were effective. This is similar to the potency of insulin in this system (Fig. 3), suggesting that insulin and IGF-1 are working through their own specific receptors. The effects of IGF-1 and insulin on CYP24 mRNA levels were not additive (data not shown), suggesting that their signal transduction pathways may converge distal to their receptors.

To determine if the action of insulin was dependent on protein synthesis, experiments were performed with cycloheximide (Table 2). Cycloheximide (30 μg/ml) had no effect on the action of $1,25(OH)_2D$ alone or $1,25(OH)_2D$+PTH. However, the action of insulin in combination with $1,25(OH)_2D$ and PTH was significantly inhibited by 43% in the presence of cycloheximide. Thus, insulin in combination with $1,25(OH)_2D$ and PTH produced an increase in CYP24 mRNA which was partially dependent on new protein synthesis.

Table 2
Effect of cycloheximide on hormone action

Agonist	CYP24 mRNA (OD/mg)	
	−Cycloheximide	+Cycloheximide
Control	0	0
$1,25(OH)_2D$	52 ± 6	144 ± 3
$1,25(OH)_2D$+PTH	176 ± 29	175 ± 7
$1,25(OH)_2D$+PTH+insulin	512 ± 16	294 ± 20[a]

Table entries are the mean ± S.E.M. of 5 flasks. Cells were preincubated with $1,25(OH)_2D$ for 3 h. PTH and insulin were then added, and cells were incubated for an additional 3 h in the presence or absence of cycloheximide. Control cells were incubated for 6 h in the absence of hormones. Concentrations were 30 μg/ml cycloheximide, 25 nM PTH, 100 nM $1,25(OH)_2D$, and 10 nM insulin.
[a]Significantly different from minus cycloheximide ($P < 0.05$, t-test).

4. Discussion

These studies demonstrate that insulin potentiates the action of PTH with regard to the expression of the 24-hydroxylase in osteoblastic cells. Insulin markedly increases the action of PTH with regard to both CYP24 mRNA levels (Fig. 1) and enzyme activity (Table 1). Insulin also has a small effect on the action of $1,25(OH)_2D$ alone. However, the magnitude of the effect on $1,25(OH)_2D$ is much smaller than that on $1,25(OH)_2D$ and PTH combined (Fig. 1).

Insulin alone has no effect on 24-hydroxylase expression (Fig. 1), suggesting that insulin works by enhancing the $1,25(OH)_2D$/PTH stimulatory pathway. This is consistent with the time course and dose-response studies. Insulin magnifies the action of $1,25(OH)_2D$/PTH without changing the time course (Fig. 2). Likewise, insulin alters the maximal response of the UMR cells to PTH without significantly altering their sensitivity to PTH (Fig. 4).

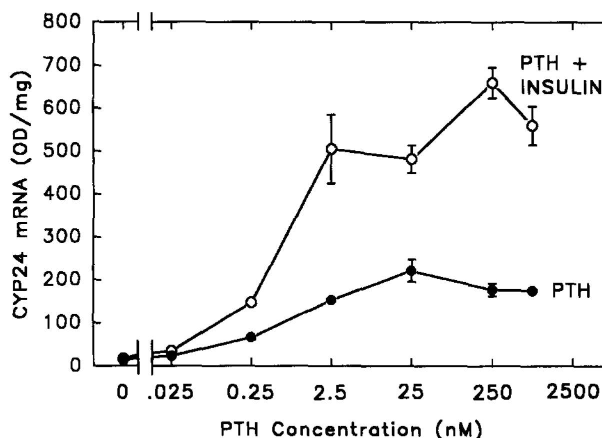

Fig. 4. Effect of insulin on PTH dose response. UMR cells were incubated with the indicated concentration of PTH in the presence or absence of 10 nM insulin for 3 h. $1,25(OH)_2D$ (100 nM) was present in all experiments. CYP24 mRNA levels were quantitated by dot blot. Data points are the mean ± S.E.M. of 4 flasks. Insulin significantly increased the effect of PTH at PTH concentrations of 0.25–2500 nM ($P < 0.05$, t-test).

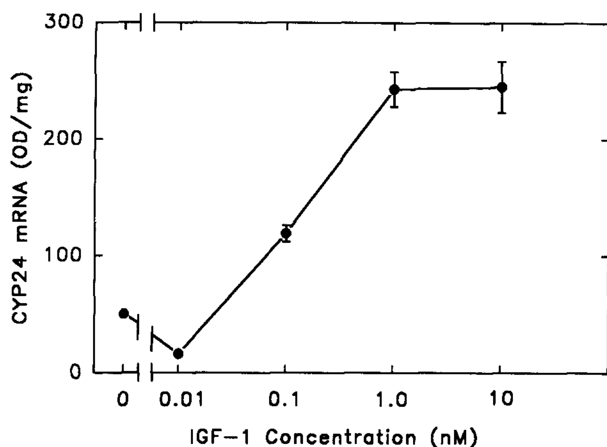

Fig. 5. Dose response of IGF-1 action. UMR cells were treated with the indicated concentration of IGF-1 for 3 h in the presence of PTH (25 nM) and $1,25(OH)_2D$ (100 nM). CYP24 mRNA levels were quantitated by dot blot. Data points are the mean ± S.E.M. of 4 flasks. IGF-1 had a significant effect at concentrations of 0.1–10 nM ($P < 0.05$, *t*-test).

Insulin may act via multiple pathways [15] to potentiate the action of $1,25(OH)_2D$ and PTH. Presumably, insulin is acting via the insulin receptor which has been characterized in UMR cells [16]. Part of the effect of insulin requires new protein synthesis while part does not (Table 2). This is in contrast to the action of PTH, which does not require new protein synthesis. The insulin pathway requiring new protein synthesis may involve such things as the induction of immediate early genes or the synthesis of new vitamin D receptors. Insulin and PTH increase c-fos mRNA levels in these cells [17], and growth factors can increase vitamin D receptor gene expression in cultured cells [18]. The component of insulin action not requiring protein synthesis may involve phosphorylation of existing proteins. Insulin may alter the phosphorylation state of proteins such as the cAMP response element binding protein (CREB) [19], ferredoxin [20], or the vitamin D receptor [21].

Insulin and IGF-1 are associated with bone growth or a decreased rate of bone loss [11,22]. The present studies suggest several ways by which insulin and IGF-1 may reduce bone loss. One possibility is that the stimulation of 24-hydroxylase activity in bone cells by insulin and IGF-1 inhibits the action of $1,25(OH)_2D$ in bone. It has been suggested that 24-hydroxylation is the first step in the degradation of $1,25(OH)_2D$ by target tissues [23,24]. Another mechanism by which insulin could increase bone density involves the production of $24,25(OH)_2D$ from circulating 25(OH)D by bone. $24,25(OH)_2D$ has been reported to be essential for bone formation [25].

It is of interest that insulin decreases 24-hydroxylase activity in the kidney [12] but increases it in bone cells(Table 1) in the presence of PTH. This may reflect the fact that a major function of the 24-hydroxylase (and the 1-hydroxylase) in the kidney is to hydroxylate 25(OH)D to $24,25(OH)_2D$ (and $1,25(OH)_2D$). In bone, the major function of the 24-hydroxylase may be to inactivate $1,25(OH)_2D$. Thus, from a physiological standpoint it may be appropriate for insulin, which stimulates $1,25(OH)_2D$ production by the kidney, to also inhibit $1,25(OH)_2D$ action in bone via induction of the 24-hydroxylase. On the other hand, lack of insulin (diabetes) could result in decreased 24-hydroxylase activity and increased action of $1,25(OH)_2D$ in bone.

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