Metabolic acidosis up-regulates PTH/PTHrP receptors in UMR 106-01 osteoblast-like cells

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Background. Metabolic acidosis results in skeletal demineralization by multiple mechanisms. One of these involves the inorganic phase of bone by which hydrogen ion is buffered by bone carbonate. In addition, the cellular components of bone participate by the induction and repression of several skeletal genes. Previous studies have suggested that the action of parathyroid hormone (PTH), a major regulator of bone turnover, might be altered by acidosis. The present studies were designed to test directly, in vitro, whether acidosis altered the effects of PTH in UMR 106-01 osteoblast-like cells.

Methods. Studies were conducted in confluent cultures of UMR 106-01 cells in modified Eagle’s medium (MEM) with 5% fetal bovine serum (FBS) at pH values varying from 7.4 to 7.1 by addition of HCl. After time periods of 4 to 48 hours, cells were tested for cyclic AMP generation in response to PTH. PTH binding and PTH/PTHrP receptor mRNA levels were determined by radioligand binding assay and Northern analysis respectively.

Results. After 48 hours, decreases in pH from 7.4 to 7.1 resulted in a progressive increase in PTH-stimulated cyclic AMP generation from 1978 ± 294 to 4968 ± 929 pmol/culture/5 min (P < 0.05). Basal cyclic AMP concentrations were unchanged. PTH binding increased 1.5- to twofold. Competitive inhibition binding revealed an increase in receptor number supported by up-regulation of PTH/PTHrP receptor mRNA up to twofold from control levels.

Conclusions. These findings demonstrate that metabolic acidosis stimulates the response to PTH in UMR 106-01 osteoblast-like cells by a mechanism that involves an increase in the levels of PTH/PTHrP receptor mRNA. Thus, the skeletal response to acidosis that includes an increase in bone resorption may result, at least in part, from an increase in PTH/PTHrP receptors leading to an enhanced effect of PTH on bone.

Metabolic acidosis results in skeletal demineralization by multiple mechanisms. One of these processes involves the physicochemical dissolution of bone as excess protons are buffered by bone carbonate. Studies by Bushinsky et al, using neonatal mouse calvariae, have shown that when the culture medium is acutely acidified by reducing bicarbonate concentration, there is a net influx of protons with concomitant calcium efflux from the bone secondary to dissolution of bone calcium carbonate [1–4]. In addition, the cellular components of bone may participate in the process of skeletal demineralization induced by acidosis. Goldhaber and Rabadjija demonstrated that the calcium release from neonatal mouse calvariae cultured for one week in acidic medium occurred only in live but not dead bone [5]. Histologic examination of the resorbed calvariae demonstrated the presence of numerous osteoclasts in different stages of bone destruction; in addition, thyrocalcitonin, an inhibitor of osteoclastic function abolished the proton-mediated calcium release. This finding established the role of bone cells in skeletal demineralization during chronic metabolic acidosis. Similar findings were reported by Bushinsky [6]. Additional studies on the direct effect of metabolic acidosis on bone cells suggested that metabolic acidosis inhibits osteoblast but stimulates osteoclast function [7, 8]. Krieger, Sessler and Bushisky reported inhibition of osteoblastic collagen synthesis in calvariae incubated in acidified medium while there was enhancement of osteoclastic β-gucuronidase activity [7].

The role of PTH on skeletal demineralization in metabolic acidosis remains elusive. Martin et al studied the effect of acute acidosis on the uptake and action of PTH in isolated perfused canine tibiae. Lowering the pH of the perfusate by addition of HCl resulted in an increase in the arteriovenous difference for intact PTH (iPTH) across the perfused bone. Basal and PTH stimulated cyclic adenosine 3’5’-monophosphate (cAMP) generation also increased significantly under acidic conditions [9]. Using neonatal mouse calvariae, Bushinsky and Nilsson studied the effect of PTH and metabolic acidosis on osteoblast and osteoclast function. The combination of metabolic acidosis and PTH resulted in a greater effect in the stimulation of calcium flux, osteoclastic β-gluc-
uronidase activity and inhibition of osteoblastic collagen synthesis than either treatment alone [10]. These findings suggest that metabolic acidosis may enhance the actions of PTH on bone cells. The present experiments were designed to examine the direct effect of metabolic acidosis on PTH/PTHrP receptors in UMR106-01 osteoblast like cells.

METHODS

Rat PTH 1-34 and Nle521Tyr34-rat PTH [1–34]NH2 were obtained from Bachem (Torrance, CA, USA). Acetonitrile was obtained from EM Science (Cherry Hill, NJ, USA). 125I was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). 32P-CTP was purchased from Amersham Life Science (Arlington Heights, IL, USA). UMR 106-01 osteoblast-like cells were provided by Dr. Nicola Partridge, Robert Woods Johnson Medical School (Piscataway, NJ, USA). All other reagents were purchased from Sigma-Aldrich Company (St. Louis, MO, USA), unless otherwise indicated, and were of the highest grade available.

Cell culture

Studies were performed in cultures of UMR 106-01 osteoblast-like cells between passages 14 and 24. Cells were grown to confluence in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), penicillin and streptomycin. Confluent cultures were incubated in medium containing concentrated HCl (5, 10 and 15 μL per 9 mL of growth medium resulting in a pH of 7.3, 7.2 and 7.1, respectively). The culture medium was changed daily and there was no significant difference in pH during the incubation period. Control cultures were incubated in regular medium, which has a pH of 7.4.

PTH-stimulated cAMP production

The medium was aspirated from confluent 12-well culture plates of UMR 106-01 cells, and the cells were washed three times with Krebs-Henseleit solution. PTH (10−8 mol/L) was added for five minutes in the presence of 1 mmol/L 3-isobutyl-1-methylxanthine in a total volume of 0.5 mL. The reactions were terminated with the addition of 0.1 mL of 1.8 mol/L perchloric acid. After five minutes, the cell extracts were neutralized with 0.1 mL of 3 mol/L KHCO3, the volume was brought to 1 mL with 20 mmol/L MES [2-(n-morpholino) ethanesulfonic acid, pH 6.2], and cAMP was measured by radioimmunoassay as previously described [11].

Iodination of PTH

Five micrograms of Nle521Tyr34-rat PTH [1–34]NH2 were iodinated with 25 μg of chloramine T in the presence of 2 mCi of 125I. The reaction was terminated with 75 μg of sodium metabisulfite and 0.5 mL of 20% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) was added. This was applied to a SepPak C18 cartridge (Waters Chromatography Division, Milford, CA, USA) which had been equilibrated sequentially with 3 mL of 100% ACN, 5 mL of 80% ACN, 0.1% TFA and finally with 5 mL of 20% ACN, 0.1% TFA. Following application of the iodination reaction, the cartridge was washed with 2.5 mL of 20% ACN and 0.1% TFA, and the iodinated hormone was eluted with three successive 0.5 mL aliquots of 50% ACN, 0.1% TFA. The peak tube was diluted in 1% BSA and aliquots were stored at −80°C.

Studies of PTH binding

The medium was removed from confluent cultures of UMR106-01 cells, and the cells were washed twice with MEM containing 5% FBS at 10°C. Following addition of PTH 10−8 mol/L to duplicate wells for determination of non-specific binding, all wells were incubated with 5 × 104 cpm of 125I Nle521Tyr34-rat PTH [1-34]NH2. For studies of competitive inhibition binding, increasing concentrations of PTH were added to duplicate wells in addition to radiolabeled PTH. Incubations were continued for three hours at 10°C. The cells were then washed three times with cold MEM-5% FBS and phosphate-buffered saline (PBS). The cells were solubilized in 0.1 mol/L sodium hydroxide and the radioactivity quantified. Specific binding was corrected for protein content per well.

Studies of PTH/PTHrP-receptor mRNA

Total RNA was isolated from cultured cells using the TRIzol method as described by manufacturer (Life Technologies, Grand Island, NY, USA). PTH/PTHrP receptor mRNA was measured by Northern blotting using a probe consisting of nucleotides 439 to 768 of the rat PTH/PTHrP-receptor cDNA prepared by reverse transcription-polymerase chain reaction (RT-PCR) from rat kidney RNA, cloned into pCRII and sequenced to confirm identity with the published sequence of the cloned rat PTH/PTHrP-receptor cDNA. Northern blot was performed using the Northern Max-Gly™ kit as described by the manufacturer (Ambion, Austin, TX, USA). Briefly, 20 μg of total RNA was mixed 1:1 with Glyoxal Load Dye and the mixture was incubated for 30 minutes in a 50°C water bath. The samples were then applied to 1% Agarose LE gel and electrophoresed at 100 volts for one hour. After transferring to nylon membranes, the RNA was bound by UV cross-linking (Stratalinker; Stratagene Cloning Systems, La Jolla, CA, USA). The filters were prehybridized for one hour at 42°C in Ultrahyb solution. The filters were probed with 32P-CTP radiolabeled cDNA for 16 to 20 hours, washed with 2 × standard sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) for seven minutes at 42°C. The filters were analyzed by

Studies of PTH/PTHrP receptors
Fig. 1. Dose response of the effect of metabolic acidosis on parathyroid hormone (PTH)-stimulated cAMP generation. Cells were exposed to acidic medium with pH ranging from 7.3 to 7.1 for 48 hours. Control cultures were exposed to medium with pH 7.4. cAMP generation following acute stimulation with PTH (10^{-8} mol/L for 5 min) was determined by radioimmunoassay. The values represent the mean ± SEM from 4 to 6 different experiments. Symbols are: (□) basal; (■) PTH stimulated; *P < 0.05 compared to control.

Statistical analysis

Data were analyzed using the Student t test. For analysis of multiple groups, analysis of variance (ANOVA) was used followed by the Student t test with Bonferroni correction. Differences between groups were considered significant when P < 0.05. Data are expressed as mean ± standard error of the mean.

RESULTS

Effect of metabolic acidosis on basal and PTH-stimulated cAMP generation

To assess the effect of metabolic acidosis on PTH-stimulated cAMP generation, confluent cultures of UMR106-01 osteoblast-like cells were exposed to acidic medium with pH ranging from 7.3 to 7.1 for 48 hours. As shown in Figure 1, metabolic acidosis enhanced the ability of the cells to respond to acute stimulation with PTH with no significant difference in the basal cAMP levels. The PTH-stimulated cAMP generation in control cultures (pH 7.4) was 1978 ± 294 pmol/culture/5 min. Cultures exposed to acidic medium demonstrated increased PTH-stimulated cAMP generation with values reaching 3380 ± 333 and 4968 ± 929 pmol/culture/5 min at pH 7.2 and 7.1, respectively (P < 0.05 for the different acidic media as compared to control). Although cAMP generation was higher at pH 7.1 as compared to pH 7.2, the difference did not reach statistical significance (P = 0.058).

Figure 2 shows the time course for the effect of metabolic acidosis on PTH-stimulated cAMP generation. Cells were exposed to medium with pH 7.2 for up to 72 hours. The increase in cAMP generation in response to PTH was evident after 12 hours of exposure to acidic medium and reached a plateau between 48 and 72 hours of exposure.

Effect of metabolic acidosis on PTH binding

To determine if the stimulatory effect of metabolic acidosis on PTH-stimulated cAMP generation was due to an increase in PTH binding to its receptor, we examined the effect of metabolic acidosis on 125I PTH binding in confluent cultures of UMR 106-01 cells. As shown in Figure 3, exposure to acidic medium with progressively decreasing pH resulted in a dose dependent increase in PTH binding. In control cultures (pH 7.4), PTH binding was 4616 ± 308 cpm/mg protein. Incubation with acidic medium increased PTH binding to 7801 ± 859 cpm/mg protein at pH7.2 and 9279 ± 1192 cpm/mg protein at pH 7.1 (P < 0.05 for the different acidic media as compared to control).

Competitive inhibition binding experiments revealed that binding was increased at all concentrations of unlaabeled PTH tested between 0.01 and 10 nmol/L (Fig. 4). Scatchard analysis indicated that the increase in binding was due to an increase in receptor number with no change in affinity.
bolic acidosis demonstrate an enhanced calcemic re-
sponse to PTH [13]. Studies by Bushinsky and Nilsson,
using neonatal mouse calvariae, have also demonstrated
enhanced PTH action in the setting of metabolic acidosis
in terms of calcium flux, osteoclastic β-glucuronidase
activity and inhibition of osteoblastic collagen synthesis
[10]. PTH exerts its effect on target tissues by acting via
a G-protein coupled receptor, the PTH/PTHrP receptor,
which in bone is present in cells of the osteoblastic lin-
eage. The adenylate cyclase/cAMP system serves as one
of the major signal transduction pathways activated by
the PTH/PTHrP receptor. Based on previous findings
suggesting that metabolic acidosis may alter the actions
of PTH by affecting the PTH/PTHrP receptor/adenylate
cyclase system [9, 14], we designed the present studies
binding of 125I Nle8,21Tyr34-rat PTH [1-34]NH2.
Confluent cultures of
UMR-106-01 cells were exposed to acidic medium with pH 7.3 to 7.1 of PTH by affecting the PTH/PTHrP receptor/adenylate
for 48 hours. Control cultures were exposed to medium with pH 7.4. Binding of 125I Nle8,Tyr3-rat PTH [1-34]NH2 was quantitated and cor-
rected for protein. The values represent the mean ± SEM from 6
different experiments. *P < 0.05 compared to control.

Effect of metabolic acidosis on
PTH/PTHrP-receptor mRNA

The effects of metabolic acidosis on the levels of PTH/
PTHrP-receptor mRNA were examined by Northern
analysis. As shown in Figure 5A, exposure to acidic me-
dium with decreasing pH resulted in a progressive in-
crease in PTH/PTHrP-receptor mRNA. Densitometry of the levels of PTH/PTHrP-receptor mRNA normalized by GAPDH showed an increase to 182 and 218% of control at pH 7.2 and 7.1, respectively (Fig. 5B). The
difference in mRNA levels reached statistical signifi-
cance at both pH 7.2 and pH 7.1 as compared to controls
(P < 0.05). Similarly, the difference in mRNA levels
between pH 7.2 and 7.1 also was statistically significant
(P < 0.05).

Figure 6 illustrates the time course for the effect of
acidic medium (pH 7.1) on PTH/PTHrP receptor mRNA
levels. The increase in receptor mRNA levels was evi-
dent after 12 hours and the difference from control val-
ues reached statistical significance after 24 and 48 hours
(P < 0.05 as compared to control in both cases). Simi-
larly, the difference in PTH/PTHrP receptor mRNA lev-
els between the 24- and 48-hour time point also was
statistically significant.

DISCUSSION

It is well accepted that metabolic acidosis inhibits os-
teoblast function while enhancing osteoclast function [7],
ultimately leading to skeletal demineralization. PTH also
is known to enhance osteoclastic activity, a process that
requires the presence of osteoblasts [12]. Several lines
of evidence have suggested that the effects of PTH in
bone may be accentuated by metabolic acidosis. Studies
by Beck and Webster have shown that rats with meta-
role in the regulation of osteoblast-mediated osteoclastic function, and PTH is known to affect this system in a direction that would favor osteoclastic activity [20–23]. However, the effects of acidosis on the individual elements of the RANKL/RANKL/OPG system have not been elucidated at the present time.

The effects of metabolic acidosis on bone remodeling are rather complex. In animal studies, the induction of metabolic acidosis leads to decreased bone mass and increased osteoclastic bone resorption without a mineralization defect [24–29]. Studies in humans have demonstrated an association between metabolic acidosis and osteomalacia [30–38]. A recent study by Domrongkit-

![Fig. 4. Competitive inhibition of equilibrium binding of $^{125}\text{I} \text{Nle}^{8,21}\text{Tyr}^{34}\text{rat PTH [1-34]}\text{NH}_2$. Confluent cultures of UMR 106-01 cells were exposed to control (○; pH 7.4) or acidic (●; pH 7.1) medium. Binding of $^{125}\text{I} \text{Nle}^{8,21}\text{Tyr}^{34}\text{rat PTH [1-34]}\text{NH}_2$ was examined in the presence of increasing concentrations of unlabeled PTH. The inset shows a Scatchard analysis of the data.](image)

![Fig. 5. Dose response of the effect of metabolic acidosis on PTH/PTHrP receptor mRNA (A). Confluent cultures of UMR-106-01 cells were exposed to control medium (pH 7.4) or acidic medium with pH 7.3 to 7.1 for 48 hours. Total RNA was isolated and PTH/PTHrP receptor mRNA levels were examined using Northern analysis. The membranes were stripped and reprobed using a cDNA for GAPDH. Densitometric analysis of the levels of PTH/PTHrP receptor mRNA corrected for GAPDH mRNA (B). The values represent the mean ± SEM from 4 different experiments. *P < 0.05 compared to control.](image)

![Fig. 6. Time course of the effect of metabolic acidosis on PTH/PTHrP receptor mRNA (A). Confluent cultures of UMR-106-01 cells were exposed to control medium (pH 7.4) or acidic medium with pH 7.1 for the time periods indicated. Total RNA was isolated and PTH/PTHrP receptor mRNA levels were examined using Northern analysis. The membranes were stripped and reprobed using a cDNA for GAPDH. Densitometric analysis of the levels of PTH/PTHrP receptor mRNA corrected for GAPDH mRNA (B). The values represent the mean ± SEM from 5 different experiments. *P < 0.05 compared to time 0.](image)
by up-regulating PTH/PTHrP receptors, may potentially worsen the effects of PTH on bone. In this regard, Lefebvre et al studied the effect of bicarbonate therapy on bone in patients on hemodialysis. They found that correction of metabolic acidosis was associated with less progression of hyperparathyroid bone disease. However, bicarbonate administration also was associated with lower levels of PTH [40]; thus, the precise contribution of metabolic acidosis to the pathogenesis of hyperparathyroid bone disease remains to be determined.

In summary, the present studies demonstrate that metabolic acidosis increases PTH-stimulated cAMP regulation as well as PTH binding and PTH/PTHrP receptor mRNA levels in UMR 106-01 cells. The up-regulation of PTH/PTHrP receptors may alter the effects of PTH in bone cells and may play a role in the alterations of bone cell function observed in metabolic acidosis. Future studies are needed to clarify the molecular mechanisms responsible for the abnormalities in bone remodeling associated with metabolic acidosis.

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