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ASPIRIN INHIBITION OF 1α-HYDROXYVITAMIN D_3 OR PARATHYROID HORMONE INDUCED HYPERCALCEMIA *IN VIVO* IN RATS

A MECHANISM INDEPENDENT OF PROSTAGLANDIN BIOSYNTHESIS INHIBITION

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Abstract—The interactions of calcium-regulating hormones, active forms of vitamin D and parathyroid hormone, and aspirin were studied in rats. Aspirin, a prostaglandin biosynthesis inhibitor, abolished the hypercalcemia induced by 1α-hydroxyvitamin D_3 at 20, 50 and 100 mg/kg p.o. in parathyroidectomized or thyroparathyroidectomized rats with or without vitamin D deficiency, and in thyroparathyroidectomized plus nephrectomized rats. Aspirin did not affect the stimulation of intestinal calcium absorption by 1α-hydroxyvitamin D_3. By contrast, indomethacin, another prostaglandin biosynthesis inhibitor, did not affect hypercalcemia or stimulation of intestinal calcium absorption by 1α-hydroxyvitamin D_3. Aspirin also abolished the hypercalcemic action of parathyroid hormone in rats with or without intact thyroparathyroid glands. Moreover, aspirin alone caused hypocalcemia in rats with intact thyroparathyroid glands. Indomethacin had no effect in either of these systems. These data suggest that aspirin may inhibit bone resorption by the active form of vitamin D or parathyroid hormone via a mechanism independent of prostaglandin biosynthesis inhibition.

Parathyroid hormone (PTH†), 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), and calcitonin (CT) play important roles in regulation of calcium homeostasis and constancy of plasma calcium concentration [1]. Regulation of bone resorption is a critical element of the action of these hormones. It has been shown that prostaglandin may also play a role in the regulation of bone resorption [2–4]. In this regard, aspirin has been reported to inhibit bone resorption by PTH *in vitro* [5]. The present study was carried out to examine a possible relationship between the calcemic action of PTH or 1α-hydroxyvitamin D_3 (1α$(OH)D_3$) and prostaglandins, using aspirin, an inhibitor of prostaglandin biosynthesis which has been reported to inhibit bone resorption by PTH *in vitro* [5]. The results show that aspirin inhibited the calcemic effect of both PTH and 1α$(OH)D_3$ *in vivo* independently of prostaglandin biosynthesis.

MATERIALS AND METHODS

Male Wistar rats, fed either a normal or a vitamin D deficient diet [6], were subjected to parathyroidectomy (PTX), thyroparathyroidectomy (TPTX), or TPTX plus nephrectomy (NX), under light ether anesthesia. Nephrectomized rats were used to eliminate possible effects of aspirin in the kidneys which might have affected calcium handling by the kidney. After an overnight fast, rats whose plasma calcium was less than 6 mg/100 ml (normal rats) or 5 mg/100 ml (vitamin D deficient rats) were used; they were given either i.v. PTH (bovine PTH, TCA powder, Sigma Chemical Co., St. Louis, MO, U.S.A.) or intraperitoneal 1α$(OH)D_3$ (Teijin Ltd., Tokyo, Japan). We used 1α$(OH)D_3$ instead of 1,25$(OH)_2D_3$ simply because it was more available in our laboratory. We did similar experiments injecting 1,25$(OH)_2D_3$ or 1,24-dihydroxyvitamin D_3 [6], and the results were the same (data not shown). Aspirin (Yoshida Seiyaku K.K., Tokyo, Japan) or indomethacin (Sumitomo Chemical Co., Osaka, Japan) was given orally 30 min before PTH or 1α$(OH)D_3$ injection. The dosages and the protocols of the experiments are described in Results. Blood samples were taken by orbital puncture at appropriate intervals, and plasma calcium concentration was measured by the OCPC method [7]. Intestinal calcium absorption was determined by the everted gut sac method [6]. The effect of aspirin on the action of PTH and CT (bovine CT, TCA powder, Sigma Chemical Co.) in normal (vitamin D-replete) rats was also studied. It has been suggested that aspirin can chelate calcium, and that this may play a role in the effect of aspirin on calcium homeostasis. To test this possibility, the *in vitro* effect of aspirin on plasma calcium concentration was examined. Blood samples were taken from normal rats via aortic puncture using a heparinized syringe, and aliquots were transferred to test tubes. One of the graded doses of aspirin (0–100 mg/100 ml) was added to each test tube and incubated at 37° for 30 min. Then, plasma

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† Abbreviations: PTH, parathyroid hormone; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1α$(OH)D_3$, 1α-hydroxyvitamin D_3; CT, calcitonin; PTX, parathyroidectomy; TPTX, thyroparathyroidectomy; and NX, nephrectomy.

Table 1. Effect of aspirin on plasma calcium levels in response to $1\alpha(OH)D_3$ in parathyroidectomized rats*

Drugs administered		Changes in plasma calcium (mg/100 ml)
$1\alpha(OH)D_3$ (μg/kg, i.p.)	Aspirin (mg/kg, p.o.)	
0	0	−0.33 ± 0.39 (8)
2.5	0	1.68 ± 0.18† (17)
0	200	−0.69 ± 0.35 (10)
2.5	200	−0.52 ± 0.38 (17)

* Twenty-four hours after parathyroidectomy, rats were given 2.5 μg/kg, i.p., $1\alpha(OH)D_3$, and plasma calcium levels were measured 20 hr after drug administration. Aspirin was administered orally 30 min prior to $1\alpha(OH)D_3$ administration. Values are expressed as mean ± S.E.M. The number of rats used is given in parentheses.

† Significantly different from control, $P < 0.001$.

was prepared for the determination of calcium concentration. Results are expressed as means ± S.E.M. and were analyzed by Student's *t*-test.

RESULTS

Effect on plasma calcium concentration. As shown in Table 1, aspirin, at a dose of 200 mg/kg body weight, completely abolished the elevation of plasma calcium level induced by $1\alpha(OH)D_3$ in PTX rats. The inhibitory effects of aspirin were also evident in TPTX rats with or without vitamin D deficiency (Table 2) as well as in the TPTX plus NX rats (even at 20 mg/kg) (Table 3). At least some of the actions of aspirin may be due to its ability to chelate calcium. Aspirin added to blood *in vitro* (Table 4) caused a slight but dose-dependent decrease in plasma calcium concentration. The magnitude of the decrease was, however, much smaller than that observed when the drug was given *in vivo* (compare with the data in Fig. 2 or 3). This suggests that the inhibitory effect of aspirin on the increase of plasma calcium level by 1α-$(OH)D_3$ may not be due to the chelating activity of calcium.

The inhibitory effect of aspirin on the hypercalcemic action of $1\alpha(OH)D_3$ may be mediated by inhibition of prostaglandin biosynthesis. The possibility of this was examined by observing the effect of indomethacin, another prostaglandin biosynthesis inhibitor, using the same experimental protocols. Indomethacin did not prevent the hypercalcemic effect of $1\alpha(OH)D_3$ (Table 5). The dose of indomethacin (20 mg/kg, p.o.) was chosen so that the inhibitory effects on prostaglandin synthesis, of these two drugs, were comparable or in favor of indomethacin (see Discussion).

The effects of aspirin and indomethacin on the action of another calcemic agent, PTH, were also examined. Aspirin completely inhibited the hypercalcemic effect induced by PTH, while indomethacin had no effect (Fig. 1). The effect of aspirin was dose dependent (data not shown), and a similar effect was

Table 2. Effect of aspirin on calcemic action of $1\alpha(OH)D_3$ in thyroparathyroidectomized rats*

Drugs administered		Changes in plasma calcium (mg/100 ml)
$1\alpha(OH)D_3$ (μg/kg, i.p.)	Aspirin (mg/kg, p.o.)	
Experiment I: Vitamin D-repleted		
2.5	0	1.45 ± 0.23† (7)
2.5	200	−1.21 ± 0.42 (6)
Experiment II: Vitamin D-deficient		
0	0	−0.21 ± 0.12 (5)
0	200	−0.04 ± 0.16 (5)
2.5	0	0.26 ± 0.13† (5)
5	0	0.56 ± 0.17‡ (5)
2.5	200	−0.28 ± 0.21 (5)
5	200	−0.25 ± 0.09 (5)

* Twenty-four hours after thyroparathyroidectomy, rats were given 2.5 μg/kg, i.p., $1\alpha(OH)D_3$, and plasma calcium levels were measured 20 hr later. Aspirin was given orally 30 min prior to the administration of vitamin D sterol. Values are expressed as mean ± S.E.M. The number of rats used is given in parentheses.

† Significantly different from control, $P < 0.05$.

‡ Significantly different from control, $P < 0.001$.

Table 3. Effect of aspirin on the calcemic action of $1\alpha(OH)D_3$ in thyroparathyroidectomized nephrectomized rats*

Drugs administered		
$1\alpha(OH)D_3$ (μg/kg, i.p.)	Aspirin (mg/kg, p.o.)	Changes in plasma calcium (mg/100 ml)
2.5	0	0.39 ± 0.29 (7)
2.5	20	−0.48 ± 0.14† (5)
2.5	50	−0.79 ± 0.43† (4)

* Twenty-four hours after bilateral nephrectomy and thyroparathyroidectomy, rats were given 2.5 μg/kg, i.p. of $1\alpha(OH)D_3$, and plasma calcium levels were measured 8 hr later. Aspirin was given orally to rats 30 min before the administration of $1\alpha(OH)D_3$. Values are expressed as mean ± S.E.M. The number of rats used is given in parentheses.

† Significantly different from the group without aspirin, $P < 0.01$.

observed at 50 mg/kg, i.p., a dose comparable to that used in humans [8].

Since PTH and vitamin D are both critical to the regulation of calcium metabolism, the above data suggest that aspirin may affect plasma calcium concentrations in animals with intact parathyroid glands. As depicted in Fig. 2, aspirin caused a marked decrease in plasma calcium concentration in rats with intact parathyroid glands.

Neither aspirin nor indomethacin had any significant effect on hypocalcemia in response to calcitonin (Fig. 3).

Effect on intestinal calcium absorption. Table 6 shows the effect of aspirin on stimulation of intestinal calcium absorption by $1\alpha(OH)D_3$ in vitamin D deficient rats. In contrast to the effect on the hypercalcemic action of $1\alpha(OH)D_3$, aspirin failed to affect the stimulation by $1\alpha(OH)D_3$ of intestinal calcium absorption. Indomethacin also had no effect on intestinal calcium absorption stimulated by $1\alpha(OH)D_3$ (Table 6).

DISCUSSION

The present study clearly demonstrates that aspirin inhibited the hypercalcemic action of either $1\alpha(OH)D_3$ or PTH in PTX, TPTX, or TPTX plus NX rats. This suggests that the effect of aspirin may not be secondary to effects on the thyroid glands, parathyroid glands, and/or kidneys. Moreover, the plasma calcium level fell in response to aspirin in rats with intact parathyroid glands. Since these rats were either fasted or fed a low calcium diet, it is unlikely that the inhibitory effect of aspirin was due to suppression of intestinal calcium absorption. Consistent with this is the finding that aspirin had no effect on the stimulation of the intestinal calcium absorption by $1\alpha(OH)D_3$. These results strongly suggest that aspirin inhibited bone resorption induced by either $1\alpha(OH)D_3$ or PTH.

Aspirin is a well known inhibitor of prostaglandin biosynthesis. Thus it is possible that the effect of aspirin may be due to the inhibition of prostaglandin biosynthesis. However, indomethacin, another inhibitor of prostaglandin synthesis, did not affect the calcemic action of $1\alpha(OH)D_3$ or PTH, suggesting that the effect of aspirin is not mediated by this mechanism. The dose of indomethacin (20 mg/kg, p.o.) was based on the relative potencies of aspirin and indomethacin, reported for (i) anti-inflammatory effects [9, 10], (ii) both *in vivo* and *in vitro* inhibitory effects of prostaglandin biosynthesis [9, 10] and (iii)

Table 4. *In vitro* effect of aspirin on the plasma calcium concentration*

Aspirin added (mg/100 ml)	Plasma calcium concentration (mg/100 ml)
0	9.8
10^{-4}	9.8
10^{-3}	9.7
10^{-2}	9.7
10^{-1}	9.7
1	9.6
10	9.5
10^{2}	9.4

* Aliquots of blood taken from normal (vitamin D-replete) rats were transferred into test tubes, to each of which one of the graded doses of aspirin was added. The blood samples were incubated at 37° for 30 min, and plasma was prepared for the determination of calcium. Each data represents an average of duplicate assays.

Table 5. Effects of indomethacin on the calcemic action of 1α(OH)D_3 in TPTX vitamin D deficient rats*

Indomethacin (mg/kg, p.o.)	1α(OH)D_3 (μg/kg, i.p.)	Increase in plasma Ca^+ (mg/100 ml) 6 hr	20 hr
0	0	−0.11 ± 0.21	−0.41 ± 0.15
0	2.5	0.52 ± 0.12†	1.24 ± 0.26†
20	2.5	0.35 ± 0.18‡	1.33 ± 0.17†

* Twenty-four hours after thyroparathyroidectomy, rats were given either 1α(OH)D_3 or vehicle, and plasma calcium concentrations were measured 20 hr later. Indomethacin was given 30 min prior to the administration of vitamin D sterol. Data are the results of five rats and are expressed as mean ± S.E.M.

† Significantly different from control, $P < 0.01$.

‡ Significantly different from control, $P < 0.001$.

gastric and intestinal lesions caused by these drugs which are known to correlate with inhibition of prostaglandin biosynthesis [11]. Based on this information, it is likely that indomethacin at 20 mg/kg, p.o., causes an effect on prostaglandin synthesis comparable to, or even more inhibitory than, that of aspirin at 200 mg/kg, p.o. [12]. Moreover, Galasko *et al.* [13] reported that indomethacin at a dosage of 2 mg/kg p.o. per day, inhibits osteoclast proliferation and bone destruction and, also, inhibits tumor prostaglandin biosynthesis in VX2 carcinoma injected rabbits. Humes *et al.* [14] also reported that indomethacin suppresses tumor growth and inhibits tumor prostaglandin biosynthesis by more than 60% in Malony sarcoma virus injected mice. These observations, together with the fact that the effective dose of indomethacin in rats is approximately one-tenth that in rabbits [15], suggest that the 20 mg/kg p.o. dose of indomethacin, may be enough to suppress prostaglandin biosynthesis in bone of rats in this study, particularly in the short duration experiments shown in Figs. 1–3.

Another possible mechanism for the effect of aspirin is chelation of calcium ions to aspirin, which would prevent an increase in plasma calcium in response to either 1α(OH)D_3 or PTH. The addition of aspirin to blood *in vitro*, however, decreased the plasma calcium concentration much less than when the drug was given *in vivo*. The highest concentration, 100 mg/100 ml, is considered to be the maximum possible plasma concentration of aspirin under the conditions of this study (200 mg/kg, p.o.). This suggests that calcium chelation is a minor contribution to the effects of aspirin on plasma calcium levels.

Aspirin caused hypocalcemia in rats with intact

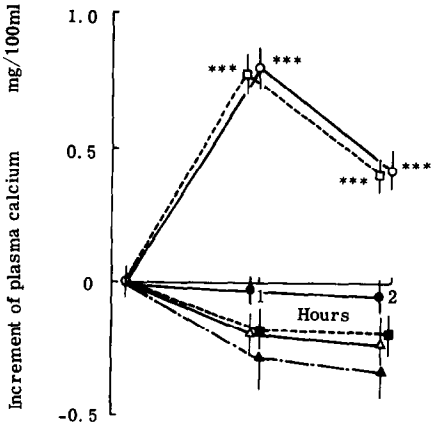

Fig. 1. Effects of aspirin and indomethacin on the elevation of plasma calcium induced by PTH in TPTX rats. Rats were fasted overnight and given either aspirin (200 mg/kg, p.o.), indomethacin (20 mg/kg, p.o.) or vehicle. Thirty minutes later, rats received either PTH (23.8 units/kg, i.v.) or vehicle. Plasma calcium concentration was measured at 1 and 2 hr after PTH administration. Each point and vertical bar represent a mean ± S.E.M. of data obtained in five rats. Key: control (●), PTH (○), indomethacine (■), PTH plus indomethacin (□), aspirin (▲), and PTH plus aspirin (△). The triple asterisk (***) indicates a significant difference ($P < 0.001$) from control.

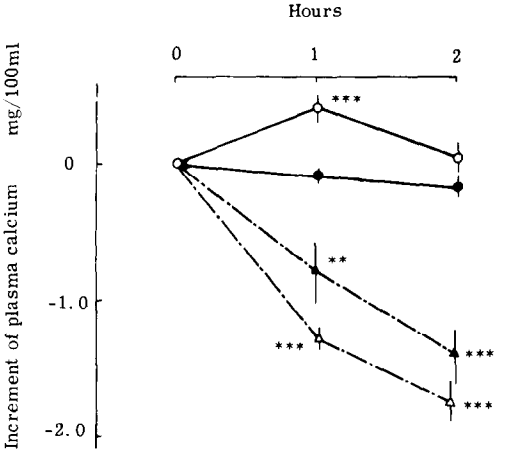

Fig. 2. Effects of aspirin on the calcemic action of PTH in rats with intact parathyroid and thyroid glands. Rats were fasted overnight and given either aspirin (200 mg/kg, p.o.) or vehicle. Thirty minutes later, rats received either PTH (100 units/rat, i.v.) or vehicle. Plasma calcium concentration was measured at 1 and 2 hr after PTH administration. Each point and vertical bar represent a mean ± S.E.M. of data in five rats. The double (**) and triple (***) asterisks indicate significant differences from control at $P < 0.01$ and $P < 0.001$ respectively. Key: control (●), PTH (○), aspirin (▲) and aspirin plus PTH (△).

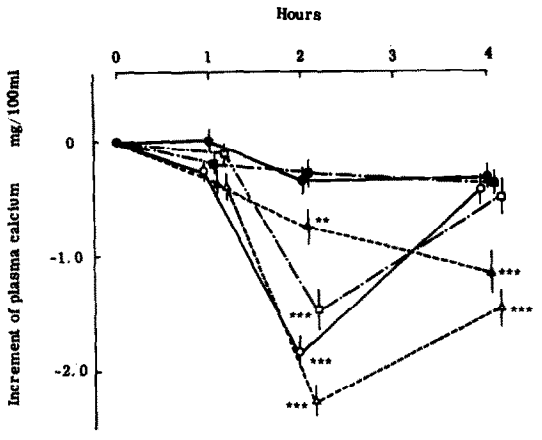

Fig. 3. Effects of aspirin and indomethacin on the hypocalcemic action of calcitonin. Rats with intact thyroparathyroid glands were fasted overnight and given either aspirin (200 mg/kg, p.o.), indomethacin (20 mg/kg, p.o.), or vehicle. Thirty minutes later rats received calcitonin (100 mU, i.v.) and plasma calcium concentration was determined at the indicated times. Each point and vertical bar represent a mean ± S.E.M. of data from five rats. The double (**) and triple (***) asterisks indicate significant differences from control at $P < 0.01$ and $P < 0.001$ respectively. Key: control (●), calcitonin (○), aspirin (▲), aspirin plus calcitonin (△), indomethacin (■), and indomethacin plus calcitonin (□).

parathyroid glands. In contrast, little hypocalcemic effect was observed in TPTX-vitamin D deficient animals. This strongly suggests that aspirin inhibits bone resorption stimulated by $1\alpha(OH)D_3$ (i.e. by $1,25(OH)_2D_3$) or PTH through mechanisms independent of prostaglandin biosynthesis or chelation of calcium ions.

It has been proposed that PTH stimulates bone resorption via cyclic AMP and that the effect of vitamin D is independent of the cyclic AMP system. But the fact that aspirin inhibited the calcemia induced by both PTH and $1\alpha(OH)D_3$ suggests that aspirin affects the bone resorptive process common to these two hormonal agents. It has been suggested that prostaglandin in bone cells plays a role as a mediator of bone resorption. The lack of effect of indomethacin as compared to aspirin, however, seems to rule out the possible role of the prostaglandin system as a mediator of aspirin action.

It has been shown that aspirin is distinct from other cyclooxygenase inhibitors such as indomethacin and phenylbutazone, in that it can acetylate this enzyme [16]. Whether this is involved in the inhibitory action of aspirin or bone resorption remains to be studied.

The dose of aspirin used in this study (200 mg/kg, p.o.) is approximately twice as high as those commonly used in treating various rheumatologic diseases in humans (90–120 mg/kg p.o. per day) [17]. The dose used in treating hypercalcemia in malignancy is in the same range as those used in treating rheumatoid arthritis [18]. However, there are two types of hypercalcemia in malignancy; one is responsive to aspirin or indomethacin [19] and the other is non-responsive to these drugs [20, 21]. Ultimately, the former is a prostaglandin-producing tumor and the latter is not [20, 22]. In an animal model, Galasko and coworkers [13, 23] reported two main phases of bone destruction by mestastases; one is mediated by osteoclasts that are stimulated by prostaglandins and responsive to indomethacin, and in the other osteoclasts disappear despite continued bone destruction, which is non-responsive to indomethacin. In sum, aspirin or indomethacin does inhibit bone resorption via inhibition of prostaglandin biosynthesis in the hypercalcemia of malignancies with prostaglandin-producing tumors, whereas aspirin seems to inhibit bone resorption via a mechanism independent of prostaglandin biosynthesis inhibition in normal physiological conditions, as shown in this study. The mechanism of action of aspirin remains to be studied in the future.

Our results are consistent with the data of Powles and his associates [5] who demonstrated that aspirin

Table 6. Effects of aspirin and indomethacin on the intestinal calcium absorption and plasma calcium levels in vitamin D deficient rats treated with $1\alpha(OH)D_3$*

Drugs administered			
$1\alpha(OH)D_3$ (μg/kg, i.p.)	Aspirin or indomethacin (mg/kg, p.o.)	Intestinal Ca^{2+} absorption $^{45}Ca^{2+}$ (S)/$^{45}Ca^{2+}$ (M)	Plasma Ca concentration (mg/100 ml)
Experiment I: Aspirin			
0	0	2.20 ± 0.30	4.48 ± 0.09
2.5	0	4.59 ± 0.25†	5.74 ± 0.17†
0	200	2.19 ± 0.27	4.14 ± 0.04
2.5	200	4.60 ± 0.19†	4.09 ± 0.07†
Experiment II: Indomethacin			
0	0	2.29 ± 0.15	4.89 ± 0.15
2.5	0	3.61 ± 0.30†	5.63 ± 0.27†
0	10	2.42 ± 0.12	4.80 ± 0.13
2.5	10	3.72 ± 0.29†	5.54 ± 0.27†

* Vitamin D deficient rats were fasted overnight and orally given either aspirin, indomethacin or vehicle. Thirty minutes later rats received either $1\alpha(OH)D_3$ or vehicle. Intestinal calcium absorption and plasma calcium concentration were determined 8 hr after $1\alpha(OH)D_3$ injection. Data are the results of five rats and are expressed as mean ± S.E.M.
† Significantly different from control, $P < 0.01$.

inhibits *in vitro* osteolysis stimulated by PTH and the hypercalcemic effect by $1\alpha(OH)D_3$.

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