SB 242784, a Selective Inhibitor of the Osteoclastic V-H⁺-ATPase, Inhibits Arterial Calcification in the Rat

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Abstract—The present experiments were carried out to further test the hypothesis that arterial calcification is linked to bone resorption by determining whether the selective inhibition of bone resorption with SB 242784, a specific inhibitor of the osteoclastic V-H⁺-ATPase, will inhibit arterial calcification. Treatment for 96 hours with toxic doses of vitamin D caused widespread calcification in the aorta and in the femoral, mesenteric, hepatic, renal, and carotid arteries, and treatment with SB 242784 completely prevented the vitamin D–induced calcification of each of these arteries at a dose of 40 mg/kg per day and significantly reduced calcification at a dose of 10 mg/kg per day. Treatment with vitamin D also caused extensive calcification in the lungs, tracheal cartilage, and kidneys, and treatment with SB 242784 prevented or reduced calcification at each of these sites. Measurement of serum levels of cross-linked N-telopeptides, a specific measure of bone resorption activity, showed that treatment with vitamin D alone produced the expected 2.4-fold increase in bone resorption activity and that concurrent treatment with the 40-mg dose of SB 242784 reduced bone resorption activity to below control levels. With the inclusion of the present results, there are now three types of bone resorption inhibitors (each with an entirely different mode of action on the osteoclast) that share the ability to potently inhibit arterial calcification in the rat, the V-H⁺-ATPase inhibitor SB 242784, the cytokine osteoprotegerin, and the amino bisphosphonates alendronate and ibandronate. (*Circ Res*. 2002;91:547-552.)

Key Words: SB 242784 ■ V-H⁺-ATPase ■ arterial calcification ■ vitamin D ■ bone resorption

We recently proposed the hypothesis that arterial calcification is linked to bone resorption[1,2] to explain the association between increased bone resorption and increased arterial calcification that has been seen in the vitamin D–treated rat,[1] in the osteoprotegerin-deficient mouse,[3] and in patients with postmenopausal osteoporosis.[4–12] One prediction of the hypothesis that arterial calcification is linked to bone resorption is that inhibitors of bone resorption should inhibit arterial calcification.[2] In a previous study, we showed that arterial calcification induced by treatment with warfarin and by treatment with high doses of vitamin D is indeed inhibited by two potent inhibitors of bone resorption, the amino bisphosphonates alendronate and ibandronate, at doses of these drugs known to inhibit bone resorption in the rat.[2,13] In a subsequent investigation, we showed that another potent inhibitor of bone resorption, the cytokine osteoprotegerin, also inhibited arterial calcification at subcutaneous doses of osteoprotegerin that have been shown to inhibit bone resorption in the rat.[14] In the present investigation, we have further tested the hypothesis that arterial calcification is linked to bone resorption by determining the effect of a potent inhibitor of bone resorption with an entirely different mode of action, the small molecule SB 242784, on arterial calcification in the rat.

SB 242784 inhibits bone resorption by potently inhibiting the osteoclastic V-H⁺-ATPase,[15] an enzyme required for the secretion of protons by osteoclasts. This enzyme acidifies the environment between the osteoclast and bone matrix, thus dissolving bone mineral and creating the acidic environment required by proteolytic enzymes to degrade the bone matrix. The importance of the osteoclastic V-H⁺-ATPase for bone resorption is strongly supported by the osteopetrotic phenotype of mice with a targeted deficiency of the osteoclast-specific 116-kDa subunit of the V-H⁺-ATPase.[16] In rats, a dose of 10 mg SB 242784/kg per day has been shown to inhibit retinoid-induced hypercalcemia and ovariectomy-induced osteoporosis.[15] These actions of SB 242784 appear to reflect specific inhibition of bone resorption, and there is no evidence that related V-H⁺-ATPases were affected in the experimental animals.

In the animal model used in the present study to test the efficacy of SB 242784 as an inhibitor of arterial calcification, arterial calcification was induced by treatment with toxic doses of vitamin D,[1,13] a treatment that has been known for >60 years to cause calcification of the elastic lamellae in the arterial media of humans, rats, and other species.[17–19] Although the mechanism by which vitamin D intoxication induces the calcification of arteries and other soft tissues is poorly understood, these vitamin D doses potently stimulate bone resorption and elevate serum calcium by >30%.[13,14]

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Materials and Methods

Materials

Vitamin K_1 (phylloquinone) and vitamin D_3 (cholecalciferol) were purchased from ICN, and warfarin was purchased from Sigma Chemical Co. The SB 242784 used in the present study was a generous gift of Smith-Kline Beecham (Middlesex, England). Stock solutions of vitamin K_1, sodium warfarin, and vitamin D were prepared as described.[14] Stock solutions of SB 242784 were prepared for injection by first dissolving solid SB 242784 with emulphor (alkamuls EL 620, Rhodia, Inc), mixing overnight at room temperature, and then diluting with water to give a final solution of 7% emulphor and 1.65 mg/mL SB 242784. Male Simonsen albino rats (Sprague-Dawley–derived) were purchased from Simonsen Labs (Gilroy, Calif).

Methods

For histological analysis of mineral accumulation, the appropriate tissues were removed within 30 minutes of the death of the animal and were fixed in formalin for at least 24 hours at room temperature. Sectioning and histological staining (hematoxylin and eosin and von Kossa) of formalin-fixed tissues were carried out by the San Diego Pathologists Medical Group, Inc. Alizarin red staining of formalin-fixed tissues was carried out as described.[20,21]

For measurement of mineral accumulation, the appropriate alizarin red–stained tissues were first ashed by heating in air for 8 hours at 600°C, and the ashed tissues were then extracted with 0.15 mol/L HCl for 1 hour at room temperature to dissolve calcium phosphate mineral. The tissues examined were as follows: the abdominal aorta section beginning 1cm above the renal branch and ending at the femoral bifurcation, the segment of trachea obtained by cutting between tracheal rings 12 and 13 (counting from the larynx) and rings 22 and 23, the lungs, and one kidney. Calcium levels in serum and in the acid extracts of tissues were determined colorimetrically using cresolphthalein complexone (Sigma), and phosphate levels in serum and in acid extracts of tissues were determined colorimetrically as described.[22] Serum samples obtained at death were analyzed to determine the level of cross-linked N-telopeptides (OSTEOMARK NTx) by Ostex, Inc, using a specific ELISA.[23]

Maintenance of Animals

In the vitamin D treatment experiments, rats were fed ad libitum a rodent diet (No. 5001, Purina Mills Inc) containing 0.67% phosphorus and 0.95% calcium by weight. In the warfarin treatment experiments, rats were fed ad libitum a chemically defined, synthetic diet containing 0.6% phosphorus and 0.6% calcium by weight (ICN Biochemicals, Inc). The animals were killed by exsanguination while they were under ether anesthesia. All animal experiments were approved by the Animal Subjects Committee at the University of California, San Diego.

Treatment of Animals

For analysis of the effects of SB 242784 on vitamin D–induced soft tissue calcification, 7-week-old male rats received subcutaneous injections of 500 000 IU vitamin D/kg body wt at 0, 24, and 48 hours, and subsets of these animals received subcutaneous injections of SB 242784 at doses of 10 or 40 mg/kg per day beginning 2 days before the first vitamin D injection. Rats were killed 96 hours after the first vitamin D injection, and the appropriate tissues were removed for analysis. For analysis of the effects of SB 242784 on warfarin-induced arterial calcification, 22-day-old male rats were treated with warfarin injections every 12 hours and vitamin K injections every 24 hours as described.[1] Subsets of these animals received subcutaneous injections of SB 242784 at doses of 10 or 40 mg/kg per day beginning at the time of the first warfarin injection. Rats were killed 7 days after the first warfarin injection, and the appropriate tissues were removed for analysis.
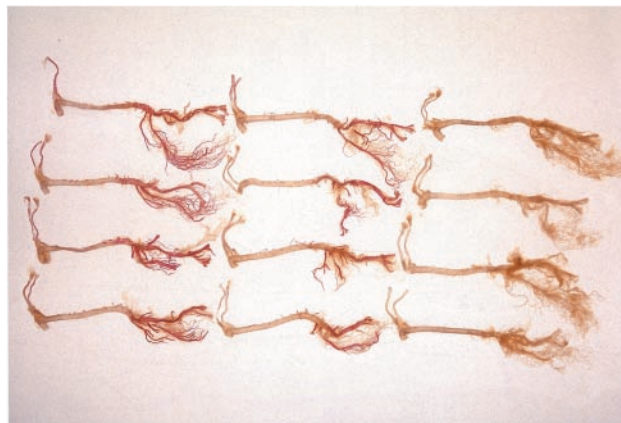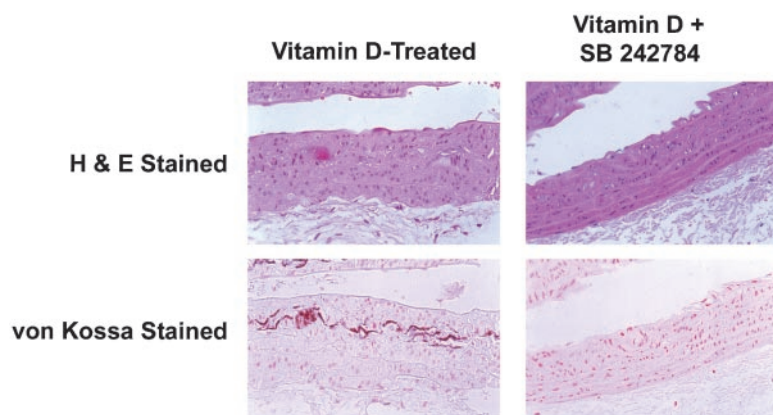

Figure 1. Effect of SB 242784 on vitamin D–induced calcification of arteries. Twelve 7-week-old male rats received subcutaneous injections of 500 000 IU of vitamin D/kg body wt at 0, 1, and 2 days. Beginning 48 hours before the first vitamin D injection, 4 of these animals were also injected subcutaneously with SB 242784 at a dose of 10 mg/kg per day, and 4 were injected with SB 242784 at a dose of 40 mg/kg per day. Animals were killed 96 hours after the first vitamin D injection, and the carotid arteries, aorta, and portions of the pulmonary, mesenteric, hepatic, renal, and femoral arteries were dissected as a unit, fixed in formalin, and stained with alizarin red (see Materials and Methods). Left, Arteries from the 4 animals treated with vitamin D alone (carotid arteries on left). Middle, Arteries from the 4 animals treated with vitamin D plus 10 mg SB 242784/kg per day. Right, Arteries from 4 animals treated with vitamin D plus 40 mg SB 242784/kg per day. (Untreated control rats have no alizarin red staining for calcification in their arteries; see Price et al[14] for example.)

Results

The effect of SB 242784 on arterial calcification was tested in rats treated with 500 000 IU vitamin D/kg for 3 days, a procedure that has been shown previously to induce extensive calcification of arteries, cartilage, kidneys, and lungs. Figure 1 shows the level of alizarin red staining for calcification seen in the arteries of rats treated with vitamin D alone and of rats treated with vitamin D plus 10 or 40 mg SB 242784/kg per day. Note that the higher SB 242784 dose completely prevented vitamin D–induced arterial calcification and that the lower SB 242784 dose significantly reduced arterial calcification. Microscopic examination of von Kossa–stained sections revealed calcification of the elastic lamellae in the media of arteries from the vitamin D–treated animals and an absence of staining in the arteries from animals treated with vitamin D plus the higher dose of SB 242784 (Figure 2). Figure 3 shows the level of alizarin red staining seen in the lungs and trachea of rats treated with vitamin D alone and of rats treated with vitamin D plus 10 mg or 40 mg SB 242784/kg per day. Note that the higher dose of SB 242784 again completely prevented calcification of the lung and reduced calcification of the tracheal ring cartilage.

The calcium content of arteries and other soft tissues was determined to obtain a quantitative measure of the effects of treatment with vitamin D alone and with vitamin D plus SB 242784 on the accumulation of calcium phosphate mineral in these tissues. As shown in the Table,

Figure 2. Effect of SB 242784 on vitamin D–induced calcification of elastic lamellae of arteries. Animals were treated with vitamin D alone or with vitamin D plus SB 242784 at a dose of 40 mg/kg per day as described in the Figure 1 legend. The abdominal aorta was removed immediately after the rats were killed and fixed in 10% buffered formalin, and longitudinal sections of each aorta were stained by von Kossa and by hematoxylin and eosin (H&E). The panels illustrate the typical level of calcification seen in the aorta of animals treated with vitamin D alone (left) and the absence of calcification in the aorta of animals treated with vitamin D plus SB 242784 (right).

treatment with vitamin D plus the higher dose of SB 242784 significantly decreased tissue levels of calcium in the abdominal aorta, kidney, lung, and trachea compared with the levels seen in animals treated with vitamin D alone. In the abdominal aorta, lung, and trachea, the levels of calcium in the animals treated with vitamin D plus the higher dose of SB 242784 were not significantly different from the levels seen in age-matched control animals (Table). Calcium levels measured on serum obtained 96 hours after the first vitamin D injection were as follows: 13.4 ± 1.2 mg/dL for rats treated with vitamin D only (n=4), 14.0 ± 0.9 mg/dL for rats treated with vitamin D plus the 10-mg dose of SB 242784 (n=4) ($P>0.5$ versus vitamin D alone), 10.8 ± 0.5 mg/dL for rats treated with vitamin D plus the 40-mg dose of SB 242784 (n=4) ($P<0.01$ versus vitamin D alone), and 10.9 ± 0.3 mg/dL for untreated control rats (n=4) ($P<0.001$ versus vitamin D alone).

To confirm that SB 242784 did in fact inhibit bone resorption at the doses used in the above experiments, serum samples obtained at death were analyzed to determine the level of cross-linked N-telopeptides, a specific marker for bone resorption activity that is released during the osteoclast-mediated breakdown of bone matrix collagen. As seen in Figure 4, the level of cross-linked N-telopeptides was elevated by 140% in rats treated with vitamin D alone and was reduced to below control levels in rats treated with vitamin D plus the higher dose of SB 242784. This result supports the hypothesis that vitamin D treatment induces arterial calcification by accelerating bone resorption and shows that SB 242784 treatment dose-dependently inhibits bone resorption activity in the vitamin D–treated rat.

The effect of SB 242784 on arterial calcification was also examined in rats treated with warfarin, a treatment that inactivates the calcification-inhibitory activity of ma-

Figure 3. Effect of SB 242784 on the calcification of lungs and trachea induced by vitamin D treatment. In the experiment described in the Figure 1 legend, lungs and attached trachea were dissected as a unit, fixed in formalin, and stained with alizarin red (see Materials and Methods). Top, Lungs from the 4 animals treated with vitamin D alone. Middle, Lungs from the 4 animals treated with vitamin D plus 10 mg SB 242784/kg per day. Bottom, Lungs from the 4 animals treated with vitamin D plus 40 mg SB 242784/kg per day. (Untreated control rats have no alizarin red staining for calcification in their lungs; see Price et al[14] for example.)

Effect of Treatment With SB 242784 on Soft Tissue Calcification in Rats Treated With Vitamin D

Tissue, μmol	Vitamin D Only	Vitamin D+10 mg SB 242784	Vitamin D+40 mg SB 242784	Age-Matched Control
Abdominal aorta	3.1±1.3	1.4±0.5*	0.3±0.03†	0.3±0.02
Kidney	39.6±32.5	20.7±11.9	2.6±0.2‡	1.1±0.2
Lungs	95.5±24.3	26.7±33.6	3.8±0.3§	3.4±0.5
Trachea	2.0±0.2	1.9±0.3	0.9±0.7‖	0.6±0.2

Seven-week-old male rats received subcutaneous injections of 500 000 IU of vitamin D/kg body wt at t=0, 24, and 48 hours. Subsets of these animals were also injected with either 10 or 40 mg SB 242784/kg body wt per day beginning at 2 days before the first injection of vitamin D. Animals were killed 96 hours after the first vitamin D injection, and the abdominal aorta, one kidney, the lungs, and an anatomically uniform 1 cm of trachea were ashed (see Materials and Methods for further details). The ashed tissues were extracted with acid to dissolve minerals, and each acid extract was analyzed for calcium as described in Materials and Methods. Results are given as μmol calcium in the indicated tissue. Vitamin D treatment does not result in any significant increase in the size or wet weight of the tissues analyzed. Therefore, the increase in calcium content seen in the tissues from the vitamin D treatment group cannot be due to changes in the size or mass of the tissues (authors' unpublished observations, 2002).

Data are mean±SD, n=4, for all groups.
*P=0.06 compared with vitamin D only.
†P=0.005 compared with vitamin D only.
‡P=0.063 compared with vitamin D only.
§P=0.0003 compared with vitamin D only.
‖P=0.023 compared with vitamin D only.

trix Gla protein and thereby induces rapid calcification of arteries and cartilage. In the first experiment, weanling rats were treated for 1 week with warfarin alone or with warfarin plus 10 mg SB 242784/kg per day. This experiment showed that the 10-mg dose of SB 242784 did not significantly inhibit arterial calcification as assessed by alizarin red staining. Subsequent analysis of bone resorption activity using the cross-linked N-telopeptide assay showed that the 10-mg dose of SB 242784 also did not

significantly reduce bone resorption activity in the warfarin-treated rats. When this experiment was repeated at the higher dose (40 mg/kg per day) of SB 242784, we observed that the drug strongly inhibited arterial calcification as evaluated by alizarin red staining. However, the dose of 40 mg/kg per day reduced the growth rate of the rats. Because reduced growth rate has been shown to independently reduce the susceptibility to warfarin-induced arterial calcification in the weanling rat,[1] it is not possible to know whether the effect of the higher dose of SB 242784 on arterial calcification is due to the specific inhibition of bone resorption by the drug or is due to a nonspecific effect of the drug on growth. Therefore, these studies were not pursued further.

Discussion

The principal conclusion of the present study is that inhibition of bone resorption using SB 242784, a selective inhibitor of the osteoclastic V-H$^+$-ATPase, is able to potently inhibit arterial calcification induced by treatment with toxic doses of vitamin D. SB 242784 also potently inhibited the calcification of the kidneys, lungs, and tracheal ring cartilage of animals treated with vitamin D.

One mechanism by which SB 242784 could inhibit arterial calcification is the inhibition of bone resorption. We have recently proposed the hypothesis that arterial calcification is linked to bone resorption to explain the association between increased bone resorption and increased arterial calcification, which has been seen in the vitamin D–treated rat, in the osteoprotegerin-deficient mouse, and in patients with postmenopausal osteoporosis.[1,2] One prediction of this hypothesis is that inhibitors of bone resorption should also inhibit arterial calcification. In
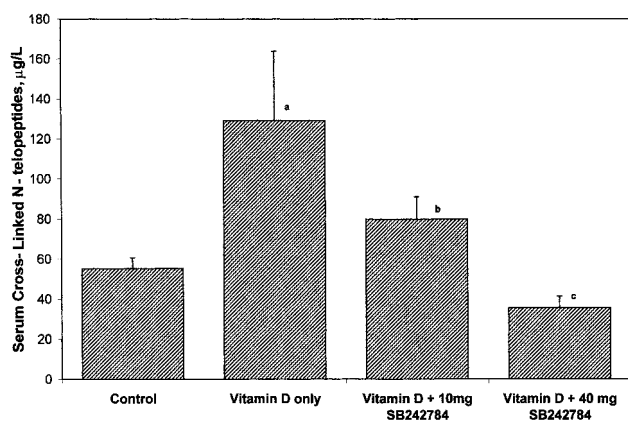
Figure 4. Effect of SB 242784 on bone resorption activity as measured by serum cross-linked N-telopeptides. Serum was obtained from each animal 96 hours after the first vitamin D injection in the experiment described in the Figure 1 legend, and serum was also obtained from 4 age-matched control rats. Each serum sample was analyzed to determine the level of cross-linked N-telopeptides (OSTEOMARK NTx, Ostex), a specific marker for bone resorption activity (see Materials and Methods). Results are presented as mean±SD. a, P<0.01 compared with control; b, P<0.05 compared with vitamin D only; and c, P<0.001 compared with vitamin D only.

previous studies, we showed that arterial calcification induced by treatment with warfarin and by treatment with high doses of vitamin D is indeed inhibited by the amino bisphosphonates alendronate and ibandronate and by the cytokine osteoprotegerin at doses of each drug that inhibit bone resorption in the rat.[2] Therefore, the fact that the doses of SB 242784 required to inhibit bone resorption (Figure 4) are correlated with the doses of SB 242784 required to inhibit calcification of arteries and other soft tissues (Table) is further evidence in support of the hypothesis that arterial calcification is indeed linked to bone resorption. It should be noted that the dose of SB 242784 required to inhibit vitamin D–enhanced bone resorption (40 mg/kg per day) is 4 times higher than the dose of this drug shown to inhibit experimental osteoporosis in the rat.[15] A possible explanation is that higher doses of the drug may be required to inhibit the rapid resorption of bone induced by vitamin D treatment than are required to inhibit the slower resorption induced by ovariectomy. This explanation is supported by the observation that 10-fold higher doses of the bisphosphonates alendronate and ibandronate are required to inhibit the rapid resorption of bone in rats on a calcium-deficient diet than are required to inhibit the slower resorption in rats on a normal diet.[24]

Some investigators believe that arterial calcification is induced by the direct action of vascular smooth muscle cells in the artery, and they would accordingly interpret the observation that a given inhibitor of bone resorption also inhibits arterial calcification as evidence that this inhibitor has a hitherto-unrecognized action on vascular cells; they would not interpret this as evidence of a link between bone resorption itself and arterial calcification. We have addressed this line of argument by testing bone resorption inhibitors with entirely different modes of action on the osteoclast, the amino bisphosphonates alendronate and ibandronate, the cytokine osteoprotegerin, and (the present study) the specific inhibitor of the osteoclast-specific V-H$^+$-ATPase SB 242784. The observation that each of these bone resorption inhibitors is a highly effective inhibitor of arterial calcification is virtually impossible to reconcile with the hypothesis that arterial calcification is induced by the action of vascular cells, because one would need to postulate novel, as-yet-undiscovered, vascular actions for every one of these compounds. The only reasonable explanation for the ability of such diverse bone resorption inhibitors to inhibit arterial calcification is that arterial calcification is indeed linked in some way to bone resorption.

The nature of the biochemical mechanism that is responsible for the putative linkage between bone resorption and arterial calcification is presently unclear. One possibility is that soft tissue calcification could be an entirely passive physicochemical process that is driven by serum levels of calcium and phosphate and that inhibitors of bone resorption exert their effects on soft tissue calcification by reducing the level of calcium and phosphate in serum. This hypothesis is not supported by the observation that the doses of osteoprotegerin and bisphosphonates that inhibit

arterial calcification do not lower serum levels of calcium or phosphate in the warfarin-treated rat and that they do not normalize serum calcium levels in the vitamin D–treated rat. However, it should be noted that the higher dose of SB 242784 did normalize serum calcium levels in the present study; therefore, it is possible that the effect of SB 242784 on arterial calcification could be partially explained by the effect of this drug on serum calcium levels.

Another possibility is that soft tissue calcification is promoted by crystal nuclei generated at sites of bone resorption that travel in the blood and occasionally lodge in soft tissue structures. This hypothesis is supported by the observation that under some circumstances, a complex consisting of a calcium phosphate mineral phase and the proteins fetuin and matrix Gla protein is released from bone and can be detected in the blood[25] and is also supported by the observation that the release of this complex from bone is inhibited by inhibitors of bone resorption.[26]

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