

Osteoprotegerin Inhibits Artery Calcification Induced by Warfarin and by Vitamin D

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Abstract—The present experiments were carried out to test the hypothesis that arterial calcification is linked to bone resorption by determining whether the selective inhibition of bone resorption with osteoprotegerin will inhibit arterial calcification. In the first test, arterial calcification was induced by treating 22-day-old male rats with warfarin, a procedure that inhibits the γ -carboxylation of matrix Gla protein and causes extensive calcification of the arterial media. Compared with rats treated for 1 week with warfarin alone, rats treated with warfarin plus osteoprotegerin at a dose of 1 mg/kg per day had dramatically reduced alizarin red staining for calcification in the aorta and in the carotid, hepatic, mesenteric, renal, and femoral arteries, and they had 90% lower levels of calcium and phosphate in the abdominal aorta ($P < 0.001$) and in tracheal ring cartilage ($P < 0.01$). More rapid arterial calcification was induced by treating 49-day-old male rats with toxic doses of vitamin D. Treatment for 96 hours with vitamin D caused widespread alizarin red staining for calcification in the aorta and the femoral, mesenteric, hepatic, renal, and carotid arteries, and osteoprotegerin completely prevented calcification in each of these arteries and reduced the levels of calcium and phosphate in the abdominal aorta to control levels ($P < 0.001$). Treatment with vitamin D also caused extensive calcification in the lungs, trachea, kidneys, stomach, and small intestine, and treatment with osteoprotegerin reduced or prevented calcification in each of these sites. Measurement of serum levels of cross-linked N-teleopeptides showed that osteoprotegerin dramatically reduced bone resorption activity in each of these experiments ($P < 0.001$). Therefore, we conclude that doses of osteoprotegerin that inhibit bone resorption are able to potently inhibit the calcification of arteries that is induced by warfarin treatment and by vitamin D treatment. These results support the hypothesis that arterial calcification is linked to bone resorption. (*Arterioscler Thromb Vasc Biol.* 2001;21:1610-1616.)

Key Words: osteoprotegerin ■ artery calcification ■ vitamin D
■ bone resorption ■ matrix Gla protein

We have recently proposed the hypothesis that arterial calcification is linked to bone resorption^{1,2} to explain the association between the 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 previous studies, we have shown that arterial calcification induced by treatment with warfarin and by treatment with high doses of vitamin D is indeed inhibited by 2 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 the present investigations, we have determined the effect of another potent inhibitor of bone resorption, osteoprotegerin, on arterial calcification by using subcutaneous doses of this protein that have been shown to inhibit bone resorption in the rat.

Osteoprotegerin is a secreted protein of the tumor necrosis factor family, which regulates bone mass by inhibiting

osteoclast differentiation and activation.^{14,15} In mice, targeted deletion of the osteoprotegerin gene results in an overall decrease in total bone density and a high incidence of fractures,^{3,16} as well as the calcification of the aorta and renal arteries. The early-onset osteoporosis observed in these mice is a result of increased bone resorption associated with increased numbers and activity of osteoclasts. In contrast, overexpression of osteoprotegerin in transgenic mice results in severe osteopetrosis associated with a decrease in osteoclasts in metaphyseal trabecular bone.¹⁴ Osteoprotegerin exerts its inhibitory effects on the osteoclast by binding to osteoprotegerin ligand (OPGL) and thereby inhibiting the interaction between receptor activator of nuclear factor κ B (RANK) and OPGL on osteoclasts and their precursors.^{17,18} Therefore, osteoprotegerin is a secreted inhibitor of the RANK signaling pathway in the osteoclast.

Two animal models have been used to test the efficacy of osteoprotegerin as an inhibitor of arterial calcification in the present study. In the first animal model, arterial calcification was induced by treatment with the vitamin K antagonist

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Figure 1. Effect of osteoprotegerin on arterial calcification induced by warfarin treatment. Eight 22-day-old male rats were treated with warfarin injections every 12 hours and vitamin K injections every 24 hours as described,¹ and 4 of these animals received subcutaneous injections of osteoprotegerin at a dose of 1 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 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. Left, Arteries from 4 rats treated with warfarin alone (carotid arteries on right). Right, Arteries from 4 rats treated with warfarin plus osteoprotegerin. (Untreated control rats have no alizarin red staining for calcification in their arteries; see Figure 2 for example.)

warfarin.^{1,19} This treatment causes arterial calcification by inhibiting the vitamin K–dependent carboxylation of the matrix Gla protein (MGP) and thereby inactivating the calcification inhibitory activity of the protein and does not elevate bone resorption or serum calcium levels. The arterial calcification induced by warfarin treatment is initially associated with the elastic lamellae of the arterial media, a pattern of arterial calcification also seen in the MGP gene knockout mouse.²⁰ MGP deficiency also causes abnormal calcification of cartilage in warfarin-treated rats,¹⁹ in the MGP knockout mouse,²⁰ and in patients with a defect in the MGP gene.^{21,22}

In the second animal model, 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.^{23–25} Although the mechanism by which vitamin D intoxication induces arterial calcification is poorly understood, these vitamin D doses potentially stimulate bone resorption and elevate serum calcium by >30%.

Methods

The Methods section can be accessed online at <http://atvb.ahajournals.org>.

Results

Effect of Osteoprotegerin on Soft Tissue Calcification Induced by Warfarin Treatment

The initial osteoprotegerin test was carried out by use of a warfarin treatment procedure that has been previously shown

to induce rapid calcification of arteries and cartilage in the rat^{1,19} and an osteoprotegerin dose that has been previously shown to inhibit bone resorption.^{14,26,27} As seen in Figure 1, treatment for 1 week with warfarin caused widespread alizarin red staining for calcification in the lower abdominal aorta and the femoral, mesenteric, hepatic, and renal arteries and scattered foci of calcification in the carotid arteries and the thoracic aorta; treatment with osteoprotegerin at a dose of 1 mg/kg per day dramatically reduced the extent of warfarin-induced arterial calcification at each of these locations. Microscopic examination of von Kossa–stained sections revealed calcification of the elastic lamellae in the media of arteries from the warfarin-treated animals and an absence of staining in the arteries from animals treated with warfarin plus osteoprotegerin (figure not shown). Warfarin treatment also caused extensive alizarin red staining for calcification in tracheal ring cartilage, and osteoprotegerin treatment inhibited this calcification (figure not shown).

A repeat experiment was carried out to obtain a quantitative measure of the effects of treatment with warfarin alone and with warfarin plus osteoprotegerin on the accumulation of calcium phosphate mineral in arteries and cartilage. As shown in Table 1, treatment with warfarin alone increased tissue levels of calcium in the abdominal aorta by 105-fold and increased tissue levels of phosphate by 430-fold. Osteoprotegerin treatment markedly reduced abdominal aortic calcification in the warfarin-treated animals, with a 90% reduc-

TABLE 1. Effect of Osteoprotegerin on Calcification of Arteries and Cartilage in Rats Treated With Warfarin

	Warfarin Only (n=6)	Warfarin+ Osteoprotegerin (n=6)	Age-Matched Control (n=6)
Abdominal aorta			
Calcium, nmol	3690±2170	431±240*	35±4
Phosphate, nmol	2600±1380	267±165*	6±1.4
Trachea			
Calcium, nmol	1010±280	480±250†	67±48
Phosphate, nmol	820±230	290±80‡	34±26

Values are mean±SD. Rats aged 22 days received subcutaneous injections of warfarin every 12 hours, and a subset of these rats was also injected with osteoprotegerin at a dose of 1 mg/kg per day beginning at the time of the first warfarin injection. Rats were killed 7 days after the start of treatment, and the indicated tissues were extracted with acid to dissolve mineral. Each acid extract was then analyzed for calcium and phosphate as described in Methods, which can be accessed online at <http://atvb.ahajournals.org>.

* $P<0.005$ vs warfarin only; † $P<0.001$ vs warfarin only; and ‡ $P<0.01$ vs warfarin only.

tion in calcium and phosphate levels, but the levels of calcium and phosphate remained significantly above control levels. Similar results were obtained on analysis of calcium and phosphate levels in tracheal ring cartilage, with a 15-fold

increase in calcium and a 24-fold increase in phosphate in the animals treated with warfarin alone. Osteoprotegerin treatment again markedly reduced tracheal ring cartilage calcification in the warfarin-treated animals, with a 50% reduction in calcium and a 65% reduction in phosphate, but the levels of calcium and phosphate remained significantly above control levels. There was no significant difference in serum calcium and phosphate levels measured on blood obtained at the end of this experiment for the animals treated with warfarin alone, the animals treated with warfarin plus osteoprotegerin, and the age-matched control animals.

Effect of Osteoprotegerin on Soft Tissue Calcification Induced by Vitamin D Treatment

The effect of osteoprotegerin was also tested in rats treated with 500 000 IU vitamin D per kilogram for 3 days, a procedure that has been shown previously to induce extensive calcification of arteries and cartilage as well as kidneys, lungs, stomach, and small intestine. Figure 2 shows a typical example of the level of alizarin red staining for calcification seen in the arteries from the 4 rats treated with vitamin D alone and an example of the absence of alizarin red staining seen in the arteries from the 4 rats treated with vitamin D plus osteoprotegerin at a dose of 1 mg/kg per day. Note that calcification in the vitamin D-treated animals is most pro-



Figure 2. Effect of osteoprotegerin on arterial calcification induced by vitamin D treatment. Seven-week-old male rats received subcutaneous injections of 500 000 IU of vitamin D per kilogram body weight at 0, 1, and 2 days. A subset of these animals was also injected subcutaneously with osteoprotegerin at a dose of 1 mg/kg per day beginning at the time of the first vitamin D injection. 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. Top, Typical artery from an animal treated with vitamin D alone (carotid arteries on left). Middle, Typical artery from an animal treated with vitamin D plus osteoprotegerin. Bottom, Artery from an age-matched control rat.



Figure 3. Effect of osteoprotegerin on the calcification of lungs and trachea induced by vitamin D treatment. In the experiment described in the Figure 2 legend, lungs and attached trachea were dissected as a unit, fixed in formalin, and stained with alizarin red. Left, Typical lung from an animal treated with vitamin D alone. Middle, Typical lung from an animal treated with vitamin D plus osteoprotegerin. Right, Lung from an age-matched control rat.

nounced in the smaller branch arteries, such as the mesenteric, hepatic, and renal arteries. Microscopic examination of von Kossa-stained sections revealed massive calcification of the elastic lamellae in the media of arteries from the vitamin D-treated animals and absence of staining in the arteries from animals treated with vitamin D plus osteoprotegerin (figure not shown).

Figure 3 shows the typical alizarin red staining seen in the lungs of rats treated for 4 days with vitamin D alone and an example of the absence of alizarin red staining seen in lungs from the 4 rats treated with vitamin D plus osteoprotegerin at a dose of 1 mg/kg per day. Microscopic examination of von Kossa-stained sections showed that calcification is associated with the alveolar wall and pulmonary arteries (figure not shown). As can also be seen in Figure 3, vitamin D treatment caused increased calcification of tracheal ring cartilage, and osteoprotegerin treatment prevented this increase.

A repeat experiment was carried out to obtain a quantitative measure of the effects of treatment with vitamin D alone and with vitamin D plus osteoprotegerin on the accumulation of calcium phosphate mineral in arteries and other soft tissues. As shown in Table 2, treatment with vitamin D plus osteoprotegerin significantly ($P < 0.001$) decreased tissue lev-

els of calcium and phosphate in the abdominal aorta, kidney, lung, small intestine, stomach, and trachea compared with the levels seen in animals treated with vitamin D alone. In 4 of these tissues, the levels of calcium and phosphate in the animals treated with vitamin D plus osteoprotegerin were the same as those seen in age-matched control animals (Table 2), whereas in the stomach and trachea, the levels of calcium and phosphate found in the animals treated with vitamin D plus osteoprotegerin remained above control levels. Calcium levels measured on serum obtained 96 hours after the first vitamin D injection were as follows: 14.6 ± 0.5 mg/dL for rats treated with vitamin D only ($n=8$), 14.9 ± 0.8 mg/dL for rats treated with vitamin D plus osteoprotegerin ($n=8$), and 10.9 ± 0.3 mg/dL for untreated control rats ($n=4$).

Sections of arteries, kidneys, lungs, small intestine, and the stomach were also stained by hematoxylin and eosin and examined. There was no evidence of cell necrosis or degeneration in any tissue from the animals treated with vitamin D plus osteoprotegerin, and the microscopic appearance of these tissues was indistinguishable from the appearance of corresponding tissues from age-matched untreated control animals.

TABLE 2. Effect of Osteoprotegerin on Soft Tissue Calcification in Rats Treated With Vitamin D

	Vitamin D Only (n=4)	Vitamin D+ Osteoprotegerin (n=4)	Age-Matched Control (n=4)
Abdominal aorta			
Calcium, μmol	3.8 \pm 0.5	0.6 \pm 0.1*	0.7 \pm 0.1
Phosphate, μmol	2.1 \pm 0.3	0.1 \pm 0.0*	0.1 \pm 0.0
Kidney			
Calcium, μmol	55.7 \pm 16.7	5.1 \pm 0.6*	4.2 \pm 0.2
Phosphate, μmol	59.2 \pm 11.8	17.6 \pm 0.8*	19.4 \pm 0.7
Lung			
Calcium, μmol	95.1 \pm 27.8	5.7 \pm 0.4*	5.1 \pm 0.6
Phosphate, μmol	79.1 \pm 17.6	8.8 \pm 1.1*	9.1 \pm 0.5
Small intestine			
Calcium, μmol	4.2 \pm 0.4	0.8 \pm 0.1*	0.8 \pm 0.1
Phosphate, μmol	1.8 \pm 0.2	0.1 \pm 0.2*	0.1 \pm 0.2
Stomach			
Calcium, μmol	77.8 \pm 8.0	34.3 \pm 10.8*	9.1 \pm 1.0
Phosphate, μmol	60.9 \pm 7.1	28.0 \pm 8.0*	6.8 \pm 0.3
Trachea			
Calcium, μmol	2.3 \pm 0.1	1.3 \pm 0.1*	0.6 \pm 0.2
Phosphate, μmol	1.4 \pm 0.2	0.7 \pm 0.1*	0.1 \pm 0.0

Values are mean \pm SD. Male rats aged 7 weeks received subcutaneous injections of 500 000 IU vitamin D per kilogram body weight at 0, 24, and 48 hours. A subset of these rats was also injected with osteoprotegerin at a dose of 1 mg/kg per day beginning with the first vitamin D injection. The rats were killed 96 hours after the first vitamin D injection, and the indicated tissues were extracted with acid to dissolve mineral. Each acid extract was then analyzed for calcium and phosphate as described in Methods, which can be accessed online at <http://atvb.ahajournals.org>.

* $P<0.001$ vs vitamin D only.

Effect of Osteoprotegerin on Bone Resorption Activity

To confirm that osteoprotegerin did in fact inhibit bone resorption 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 Table 3, the level of cross-linked N-telopeptides was not affected by treatment with warfarin alone but was reduced by 40% in rats treated with warfarin plus osteoprotegerin. This result supports the hypothesis that warfarin induces arterial calcification by inactivating the calcification inhibitory activity of MGP rather than by accelerating bone resorption and shows that the doses of osteoprotegerin used in the present study do significantly reduce bone resorption activity in the warfarin-treated rat. As also shown in Table 3, the level of cross-linked N-telopeptides was elevated by 160% in rats treated with vitamin D alone and was reduced to control levels in rats treated with vitamin D plus osteoprotegerin. This result supports the hypothesis that vitamin D treatment induces arterial calcification by accelerating bone resorption and shows that osteoprotegerin treatment reduces bone resorption activity to control levels in the vitamin D-treated rat.

Although osteoprotegerin reduced the level of bone resorption activity in the vitamin D-treated rats to normal levels, it is intriguing to note that serum calcium levels remained 37%

TABLE 3. Effect of Osteoprotegerin on Bone Resorption Activity as Measured by Serum Cross-Linked N-Telopeptides

	Serum Cross-Linked Telopeptides, ng/mL
Warfarin-induced arterial calcification	
Control (n=6)	55.5 \pm 9.2
Warfarin only (n=6)	52.1 \pm 8.1
Warfarin+osteoprotegerin (n=6)	32.6 \pm 5.3*
Vitamin D-induced arterial calcification	
Control (n=4)	50.6 \pm 2.2
Vitamin D only (n=4)	129.6 \pm 29.8†
Vitamin D+osteoprotegerin (n=4)	50.7 \pm 3.9‡

Values are mean \pm SD. Serum samples obtained at death from the rats in the experiments described in Tables 1 and 2 were analyzed to determine the level of cross-linked N-telopeptides (OSTEOMARK NTx, Ostex), a specific marker for bone resorption activity. See Methods, which can be accessed online at <http://atvb.ahajournals.org>.

* $P<0.001$ vs control; † $P<0.001$ vs control; and ‡ $P<0.001$ vs vitamin D only.

above normal. This observation shows that the elevation in serum calcium seen in rats treated with toxic doses of vitamin D is not due to accelerated bone resorption.

Discussion

The principle conclusion of the present study is that an osteoprotegerin dose that is known to inhibit bone resorption in the rat is able to potently inhibit the calcification of arteries induced by treatment with warfarin alone and the calcification of arteries induced by treatment with toxic doses of vitamin D alone. Osteoprotegerin also inhibited the calcification of cartilage induced by warfarin and by vitamin D, and osteoprotegerin potently inhibited the calcification of kidneys, lungs, small intestine, and the stomach of animals treated with vitamin D. Previous studies have also shown that the osteoprotegerin transgene will prevent arterial calcification that is induced in mice by the targeted deletion of the osteoprotegerin gene.²⁸

One mechanism by which osteoprotegerin could inhibit arterial calcification is by inhibiting 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 that 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 previous studies, we have shown that arterial calcification induced by treatment with warfarin and by treatment with high doses of vitamin D is indeed inhibited by 2 potent inhibitors of bone resorption, the amino bisphosphonates alendronate and ibandronate, at doses of these drugs comparable to those that inhibit bone resorption in the rat.² The fact that a dose of osteoprotegerin that inhibits bone resorption (Table 3) will also inhibit arterial calcification is therefore further evidence in support of the hypothesis that arterial calcification is indeed linked to bone resorption.

Another mechanism by which osteoprotegerin could inhibit arterial calcification is by acting directly on cells in the artery to promote the expression of calcification inhibitors

such as MGP. This possibility is supported by the observation that osteoprotegerin is expressed in the media of arteries in the mouse.¹⁴ However, OPGL and RANK are not expressed in the normal adult mouse aorta²⁸; therefore, it is presently unclear whether there are cells in the artery before the onset of calcification that can be affected by osteoprotegerin via the RANK signaling pathway. Because osteoprotegerin is also a receptor for the cytotoxic ligand TNF-related apoptosis-inducing ligand (TRAIL),²⁹ which is found in the aorta and pulmonary artery of the mouse,³⁰ one possibility could be that osteoprotegerin influences arterial calcification by inhibiting TRAIL-induced apoptosis of vascular cells. However, there is presently no evidence indicating that apoptosis of vascular cells is induced by treatment with warfarin. It is also worth noting in this regard that if osteoprotegerin inhibits arterial calcification by acting directly on cells in the artery rather than by inhibiting bone resorption, then it follows that bisphosphonates would also inhibit arterial calcification by acting on cells in the artery rather than by inhibiting bone resorption. Because bisphosphonates are now known to inhibit the osteoclast by inhibiting farnesyl diphosphate synthase, an enzyme found in many cell types, the hypothesis that bisphosphonates might act on cells in the arterial wall would appear to be plausible. However, there is no evidence to indicate that doses of bisphosphonates that inhibit bone resorption in animals have any effect on the activity of farnesyl diphosphate synthase in cells other than the osteoclast.³¹ Indeed, because the mechanism by which bisphosphonates gain access to the osteoclast involves their ability to concentrate on bone mineral surfaces under the osteoclast^{32,33} and be taken up by the osteoclast during bone resorption, there is in fact little reason to suppose that cells in a normal rat artery could be equivalently sensitive to bisphosphonates. Because the only presently known physiological action of amino bisphosphonates and of osteoprotegerin in vivo is the inhibition of bone resorption, it seems more reasonable to suggest that the proven ability of these drugs to inhibit bone resorption accounts for their effectiveness as inhibitors of arterial calcification than to postulate the existence of novel and as-yet-undocumented actions of both drugs on cells in the arterial wall.

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. However, this hypothesis is not supported by the observation that doses of osteoprotegerin and of bisphosphonates that inhibit arterial calcification do not lower serum levels of calcium or phosphate in the warfarin-treated rat and do not normalize serum calcium levels in the vitamin D-treated rat. Another possibility is that soft tissue calcification is promoted by crystal nuclei generated at sites of bone resorption that travel in blood and occasionally lodge in soft tissue structures. This hypothesis is supported by the observation that under some circumstances, a complex of a calcium phosphate mineral phase and matrix Gla protein is released from bone and can be detected in blood and by the observation that the release of

this complex from bone is inhibited by inhibitors of bone resorption (authors' unpublished data, 2001).

There are clinical implications of the present findings that should be noted. Because the present studies show that doses of bisphosphonates and osteoprotegerin that inhibit bone resorption are effective inhibitors of arterial calcification in 2 entirely different animal models, it seems likely that inhibitors of bone resorption will also inhibit arterial calcification in some human patients. However, it is important to note that these animal studies show that inhibitors of bone resorption prevent the initiation of arterial calcification, not the progression of arterial calcification once it has been initiated. An important goal of future studies will be to determine whether bone resorption inhibitors such as osteoprotegerin can arrest the progression of arterial calcification once it has been established. It is also important to note that the arterial calcification induced by warfarin treatment, by vitamin D treatment, and by osteoprotegerin deficiency is primarily associated with elastic lamellae in the arterial media, a type of calcification seen in the Mönckeberg's sclerosis in human subjects. The grossly hardened regions of the atherosclerotic plaque in human patients are primarily located in the intimal region of the artery, not the media, and another important goal of future studies will be to examine intimal calcification in the rodent model and to determine the effect of bone resorption inhibitors on this calcification.

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