The Amino Bisphosphonate Ibandronate Prevents Calciphylaxis in the Rat at Doses that Inhibit Bone Resorption

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Abstract. The present experiments were carried out to test the hypothesis that there is a common underlying biochemical mechanism that accounts for the different kinds of soft tissue calcification observed in animals that are treated with toxic doses of vitamin D. In previous studies we showed that lethal doses of vitamin D cause extensive calcification of arteries, lungs, kidneys, and cartilage, and that doses of the amino bisphosphonate ibandronate that inhibit bone resorption completely inhibit each of these soft tissue calcifications and prevent death. In the present experiments we have examined the effect of ibandronate on an entirely different type of calcification, the calciphylaxis induced by administration of a challenger to rats previously treated with sub-lethal doses of vitamin D. These studies show that ibandronate doses that inhibit bone resorption completely inhibit artery calcification as well as, in the same rat, the calciphylactic responses to either subcutaneous injection of 300 µg FeCl₃ or intrascapular epilation. Since the vitamin D-treated animals had dramatically increased levels of bone resorption, and concurrent treatment with ibandronate normalized resorption, these results support the hypothesis that soft tissue calcifications in the vitamin D-treated rat may be linked to bone resorption. The ability of ibandronate to inhibit all vitamin D-associated calcifications in the rat cannot be explained by an effect of ibandronate on serum calcium, since serum calcium remained 30% above control levels in the vitamin D-treated animals that also received ibandronate.

Key words: Vitamin D — Calciphylaxis — Ectopic calcification — Artery calcification — Ibandronate — Bisphosphonates.

The present experiments were carried out to test the hypothesis that there could be a common underlying biochemical mechanism that accounts for the different kinds of soft tissue calcification observed in animals that are treated with toxic doses of vitamin D. In previous studies we showed that lethal doses of vitamin D cause extensive calcification of arteries, lungs, kidneys, and cartilage, and that doses of ibandronate that inhibit bone resorption completely inhibit each of these soft tissue calcifications and prevent death [1, 2]. In the present experiments we have examined the effect of ibandronate on an entirely different type of calcification, the calciphylaxis induced by administration of a challenger to rats previously treated with sub-lethal doses of vitamin D.

The calciphylaxis response in the rat requires two treatments, an initial treatment with a sensitizer, such as a high dose of vitamin D or PTH, followed after an interval of a day or more by treatment with a calciphylactic challenger [3]. In the present study we have employed the same 3-day vitamin D injection schedule used in the prior ibandronate studies [1, 2], but have reduced the dosage of vitamin D to nonlethal levels. Two completely different kinds of calciphylactic challengers were used, subcutaneous injection of FeCl₃ and intrascapular epilation, and both challengers were administered 24 hours after the last of the three vitamin D doses. In previous studies of these calciphylactic responses in rats sensitized by prior treatment with toxic doses of vitamin D, calcification induced by epilation (hair plucking) started in the fibrous tissue surrounding the hair follicles and subsequently spread throughout the dermis, and calcification induced by FeCl₃ was accompanied by an inflammatory response in the ventral fascia and culminated in the formation of a highly calcified tissue button [3, 4].

Materials and Methods

Materials

Vitamin D₃ (cholecalciferol) was purchased from ICN (Costa Mesa, CA) and Ibandronate (Bonderanat, Boehringer Mannheim) was purchased from Idis World Medicines (Surrey, UK). Ibandronate was diluted with 0.15 mol/l NaCl and stored at 4°C. Stock solutions of vitamin D were prepared fresh for each 3 day subcutaneous injection cycle at a concentration of 4.3 mmol/l in 7% emulphor (alkamuls EL-620, Rodia, Inc.) and then placed in foil-wrapped containers and stored at 4°C, as described previously [5]. Simonsen albino rats
Fig. 1. Effect of ibandronate on the subcutaneous calciphylaxis induced by FeCl₃ injection. Six 7-week-old male Sprague Dawley rats received subcutaneous injections of 400,000 IU of vitamin D₃/kg body weight at t = 0, 1 and 2 days. Three of these rats were also injected subcutaneously with ibandronate at a dose of 0.25 mg/kg/day beginning 4 days prior to the first vitamin D injection, and the remaining 3 rats received no ibandronate. Calciphylaxis was induced by the subcutaneous injection of the 300 µg FeCl₃ challenge at two ventral sites 3 days after the first vitamin D injection, and rats were killed 7 days after the FeCl₃ injection. The typical gross appearance of the unstained, FeCl₃-induced calciphylactic buttons in each group is shown on the top, and the appearance of the same specimen after staining for calcification with Alizarin red S is shown on the bottom. Left, treatment with vitamin D plus ibandronate; right, treatment with vitamin D alone.

(Sprague-Dawley derived) were purchased from Simonsen labs (Gilroy, CA).

Treatment of Rats

Male Sprague Dawley rats were fed ad libitum with rodent diet 5001 (Purina Mills Inc., St. Louis, MO), a diet that is 0.67% phosphorus and 0.95% calcium by weight. In all experiments, rats were killed by exsanguination while under ether anesthetic. In the calciphylaxis experiments, 7-week-old male rats were first sensitized by 3 subcutaneous injections of 400,000 IU of vitamin D₃/kg body weight made at t = 0, 24, and 48 hours. Calciphylaxis was then initiated at t = 72 hours either by subcutaneous injection with 300 µg of FeCl₃ at each of two ventral sites in the thorax (see Fig. 1) or by mechanically removing all of the hair in a 9 cm² area of skin in the infrascapular region of the anesthetized rat (epilation; see Fig. 4). To assess the effect of ibandronate on ectopic calcifications, a subset of the rats also received subcutaneous injections of ibandronate at a dose of 0.25 mg/kg/day beginning 4 days prior to the first vitamin D injection and continuing until the animals were killed. Animals were killed by exsanguination 7 days after administration of the calciphylactic challenge and the appropriate tissues were removed within 30 min of death and either immediately frozen at −20°C until chemical analysis or fixed in formalin. The effect of vitamin D dose on the level of calcification in the aorta and at the site of calciphylactic challenge was determined in rats that received subcutaneous injections of 100,000, 200,000 or 300,000 IU of vitamin D₃/kg body weight at t = 0, 24, and 48 hours and of 300 µg of FeCl₃ at t = 72 hours. The UCSD Animal Subjects Committee approved all animal experiments.

For measurement of mineral accumulation in the aorta, the abdominal aorta section beginning 1 cm above the renal branch and ending at the femoral bifurcation was demineralized in 150 mmol/L HCl overnight at room temperature. For analysis of mineral accumulation at sites of calciphylaxis, the well-defined tissue button that formed at the subcutaneous sites of FeCl₃ injection (see Fig. 1) and the region of skin that underwent epilation (see Fig. 4) were removed and demineralized in 10% formic acid. Calcium levels in serum and in the acid extract of tissues were determined colorimetrically using cresolphthalein complexone (Sigma) and phosphate levels in serum and in the acid extract of tissues were determined colorimetrically as described [6]. Serum samples were analyzed to determine the level of cross-linked N-telopeptides (OSTEO-MARK NTx) by Ostex, Inc. (Seattle, WA) using a specific ELISA assay [7].

For histological analysis of mineral accumulation, the appropriate tissues were fixed in formalin for at least 24 hours at room temperature. Sectioning and histological staining (He-