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Farnesyl Pyrophosphate Synthase Is the Molecular Target of Nitrogen-Containing Bisphosphonates

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Bisphosphonates (Bps), inhibitors of osteoclastic bone resorption, are used in the treatment of skeletal disorders. Recent evidence indicated that farnesyl pyrophosphate (FPP) synthase and/or isopentenyl pyrophosphate (IPP) isomerase is the intracellular target(s) of bisphosphonate action. To examine which enzyme is specifically affected, we determined the effect of different Bps on incorporation of [^{14}C]mevalonate (MVA), [^{14}C]IPP, and [^{14}C]dimethylallyl pyrophosphate (DMAPP) into polyisoprenyl pyrophosphates in a homogenate of bovine brain. HPLC analysis revealed that the three intermediates were incorporated into FPP and geranylgeranyl pyrophosphate (GGPP). In contrast to clodronate, the nitrogen-containing Bps (NBps), alendronate, risedronate, olpadronate, and ibandronate, completely blocked FPP and GGPP formation and induced in incubations with [^{14}C]MVA a 3- to 5-fold increase in incorporation of label into IPP and/or DMAPP. Using a method that could distinguish DMAPP from IPP on basis of their difference in stability in acid, we found that none of the NBps affected the conversion of [^{14}C]IPP into DMAPP, catalyzed by IPP isomerase, excluding this enzyme as target of NBp action. On the basis of these and our previous findings, we conclude that none of the enzymes up- or downstream of FPP synthase are affected by NBps, and FPP synthase is, therefore, the exclusive molecular target of NBp action. © 1999 Academic Press

Bisphosphonates (Bps) suppress osteoclastic bone resorption and are effective treatments of common skeletal disorders such as osteoporosis, metastatic bone disease and Paget's disease of bone. Their antiresorptive action involves inhibition of the function and survival of mature osteoclasts (1–4). Their molecular mechanism of action has been for long unknown but during the past year, studies from several laboratories documented that enzymes of the mevalonate pathway are targets of nitrogen-containing bisphosphonate (NBp) action. Luckman *et al.* (5) showed that NBps inhibit the prenylation of small GTP-binding proteins, such as Ras in J744 macrophages, a process requiring formation of the non steroidal isoprenoids FPP and GGPP. Studies with isolated osteoclasts (6) and fetal bone explants (7) showed that inhibition of osteoclastic bone resorption by NBps but not etidronate or clodronate could be totally prevented by geranylgeraniol which also rescued functioning osteoclasts, and suggested that NBps inhibit the formation of GGPP. Recently, we showed that this is due to inhibition of IPP isomerase and/or FPP synthase activity (8). The aim of the present study was to identify the specific enzyme involved in this action and to investigate whether different Bps can preferentially suppress one of these enzymes. Results showed that NBps with different structures inhibit specifically FPP synthase activity and have no effect on IPP isomerase activity.

MATERIALS AND METHODS

Bisphosphonates. Ibandronate (1-hydroxy-3-(methylpentylamino) propylidene-1,1-bisphosphonic acid) and risedronate (2-(3-pyridinyl)-1-hydroxyethylidene-1,1-bishosphonic acid) were obtained from Procter & Gamble Pharmaceuticals, Miami Valley Laboratories, Cincinnati, Ohio, USA. Olpadronate (dimethyl-amino-1-hydroxypropylidene-1,1-bisphosphonate), was from Gador SA, Buenos Aires, Argentina. Alendronate (4-amino-1-hydroxybutylidene-1,1-bisphosphonate) was obtained from Merck Sharp & Co (West Point, PA, USA). Clodronate (dichloro-methylene bisphosphonate) was from Henkel KGaA, Düsseldorf, Germany.

Reagents. RS-[2-^{14}C]mevalolactone (58 mCi/mmol), [1-^{14}C]isopentenyl pyrophosphate (IPP) (57.5 mCi/mmol), [1-^{3}H]farnesyl pyrophosphate (FPP) (20.5 Ci/mmol), and [1-^{3}H]geranylgeranyl pyrophosphate (GGPP) (22 Ci/mmol), were obtained from NEN Dupont. [1-^{14}C]dimethylallyl pyrophosphate (DMAPP) (55 Ci/mmol) was obtained from American Radiolabeled Chemicals. [^{14}C]mevalonolactone was converted to its sodium salt immediately before use. For this, mevalolactone was dissolved in 15 μl 0.2 N HCl, incubated for

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30 min at 37°C and neutralised with 15 μl 0.2 N NaOH. Tetra-n-butylammonium hydrogen sulphate (TBAS) was purchased from Alltech Associates, Inc. Deerfield, USA. The scintillation fluid used for the HPLC detector (Ultima flow) was from Hewlett-Packard, Amstelveen, The Netherlands. All other chemicals were of analytical grade.

In vitro synthesis of polyisoprenyl pyrophosphates. The *in vitro* synthesis of polyisoprenyl pyrophosphates was determined according to a modification of the procedure for the isolation and HPLC-based quantification of intermediates of the mevalonate pathway described by McCaskill *et al.* (9). In short: The reaction mixture containing 2 mg bovine brain homogenate (a dialysed 30–50% ammoniumsulphate precipitated fraction prepared according to Yokoyama *et al.* (10), Tris buffer (62.5 mM, pH 7.5), $MgCl_2$ (6.25 mM), $MnCl_2$ (10 mM)), ATP (8.75 mM) and Bovine Serum Albumin (1.25%) was incubated in the absence or presence of 1 or 100 μM Bps for 5 min at 37°C. Thereafter [^{14}C]mevalonate (80 μM), [^{14}C]IPP (25 μM) or [^{14}C]DMAPP (25 μM) was added and the mixture was incubated for another 30 min at 37°C in a total volume of 200 μl. Incubations were terminated by adding 0.5 ml of methanol. The precipitated proteins were removed by centrifugation at 13.000 rpm for 10 min and the supernatant was used for HPLC analysis and/or DMAPP/IPP quantification.

Radio-HPLC analysis. Mevalonate pathway intermediates were separated on an Adsorbosphere HS C18 column (5-mM particle size, 4,6 mm diam × 250 mm Alltech Associates) according to McCaskill *et al.* (9). In short: 50 μl of the supernatant of the reaction mixture was analysed using a ternary solvent system consisting of 10 mM TBAS in 2% (v/v) methanol (solvent A), 10 mM TBAS in 70% (v/v) methanol (solvent B), and 70% methanol (solvent C). The elution program consisted of isocratic flow of 100% solvent A for 20 min, a linear gradient to 80% solvent B:20% solvent A over the next 80 min, a linear gradient to 100% solvent C over the next 20 min, and an isocratic flow of 100% solvent C over the next 30 min, a linear gradient to 100% A over the next 5 min and an isocratic flow of 100% solvent A over the next 5 min with a flow rate of 0.75 ml/min. The total elution time was 160 min. The eluate passed through a HPLC radiodetector (Packard Flow Scintillation Analyser 500 TR) with a 500 μl liquid flow cell. Peaks were identified by spiking the ion-exchange-purified extracts with known [^{14}C] labelled intermediates, before HPLC separation.

Quantification of DMAPP and IPP by acid treatment followed by petroleum ether extraction. For distinction between DMAPP and IPP we used the method for measuring IPP isomerase activity by Slakey *et al.* (11). In short: 75 μl of the supernatant of the reaction mixture was mixed with 15 μl of 3N HCl and incubated for 10 min at 37°C. After the addition of 0.5 ml petroleum ether and 0.25 ml 2 M KCl the hydrolysed isoprenes, but not IPP (which remained in the aqueous phase), were extracted twice with petroleum ether. The combined petroleum ether phases were counted in a scintillation counter (Packard TriCarb 1900 CA) to quantify the acid labile product. Counts were corrected for background radioactivity resulting from a blank sample containing only [^{14}C]IPP label. For this, [^{14}C]IPP label was added to the reaction mixture after inactivation with methanol. As with the experimental samples, the supernatant of the mixture was treated with acid, followed by petroleum ether extraction. In 4 independent experiments we found that 4–7% of the radioactivity from [^{14}C]IPP label was petroleum ether extractable.

RESULTS AND DISCUSSION

During the last years, significant advances have been made in unravelling the intracellular target(s) and molecular mechanism of action of bisphosphonates. Previously, Amin *et al.* (12) and Luckman *et al.* (5) indicated that enzymes of the mevalonate pathway are potential molecular targets of bisphosphonates and Fischer *et al.* (6) and our group (7) showed that inhibition of bone resorption by NBps could be totally prevented by geranylgeraniol, indicating that enzymes involved in the production of GGPP are affected by NBps. Our recent studies identified FPP synthase and/or IPP isomerase as the intracellular targets of NBps, but not of clodronate and etidronate, which do not contain a nitrogen-functionality (8).

In the present study, we examined the specific involvement of these two enzymes, and also of those more upstream in the mevalonate pathway, in the mechanism of action of Bps. For this, we followed the production of mevalonate-derived intermediates of isoprenoid biosynthesis by HPLC, in response to bisphosphonates in crude homogenates of bovine brain. We specifically investigated the effects of the NBps, alendronate, risedronate, olpadronate, ibandronate and that of the non NBp clodronate on the formation of polyisoprenyl pyrophosphates using ^{14}C labelled, mevalonate, IPP and DMAPP.

Representative reversed-phase ion-pair HPLC separations of ion-exchange-purified homogenates of bovine brain incubated with [^{14}C]MVA and [^{14}C]IPP in the absence or the presence of 1 μM olpadronate and clodronate are depicted in Fig. 1. As shown in Figs. 1a and 1d, [^{14}C]MVA and [^{14}C]IPP were incorporated into FPP and GGPP, and the same pattern was seen following addition of [^{14}C]DMAPP (not shown). This confirms that the brain homogenate contains all relevant enzymes of the mevalonate pathway. As shown in Figs. 1b and 1e, 1 μM olpadronate totally inhibited the formation of FPP and GGPP. Identical responses were obtained with alendronate, ibandronate and risedronate tested at 1 and 100 μM (not shown). In contrast, clodronate (Figs. 1c and 1f) had no effect on FPP and GGPP formation. Similarly, FPP and GGPP formation from [^{14}C]DMAPP were also completely blocked by all NBps, but not by clodronate (not shown). In addition, the experiments with [^{14}C]MVA show that the enzymes phosphomevalonate kinase and pyrophosphomevalonate decarboxylase, which are responsible for the formation of IPP are not affected by NBps.

Olpadronate treatment of the homogenates, as well as the other NBps tested, increased, relative to control, the incorporation of [^{14}C]MVA into IPP/DMAPP by 3–5 fold (see Fig. 1b). These intermediates have exactly the same retention time, probably because the chemical differences between these structurally strongly related molecules were too small for individual identification by HPLC. This result does not allow any conclusion about the specific involvement of FPP synthase or IPP isomerase in the observed responses to NBps. To investigate this further, we applied a widely used assay

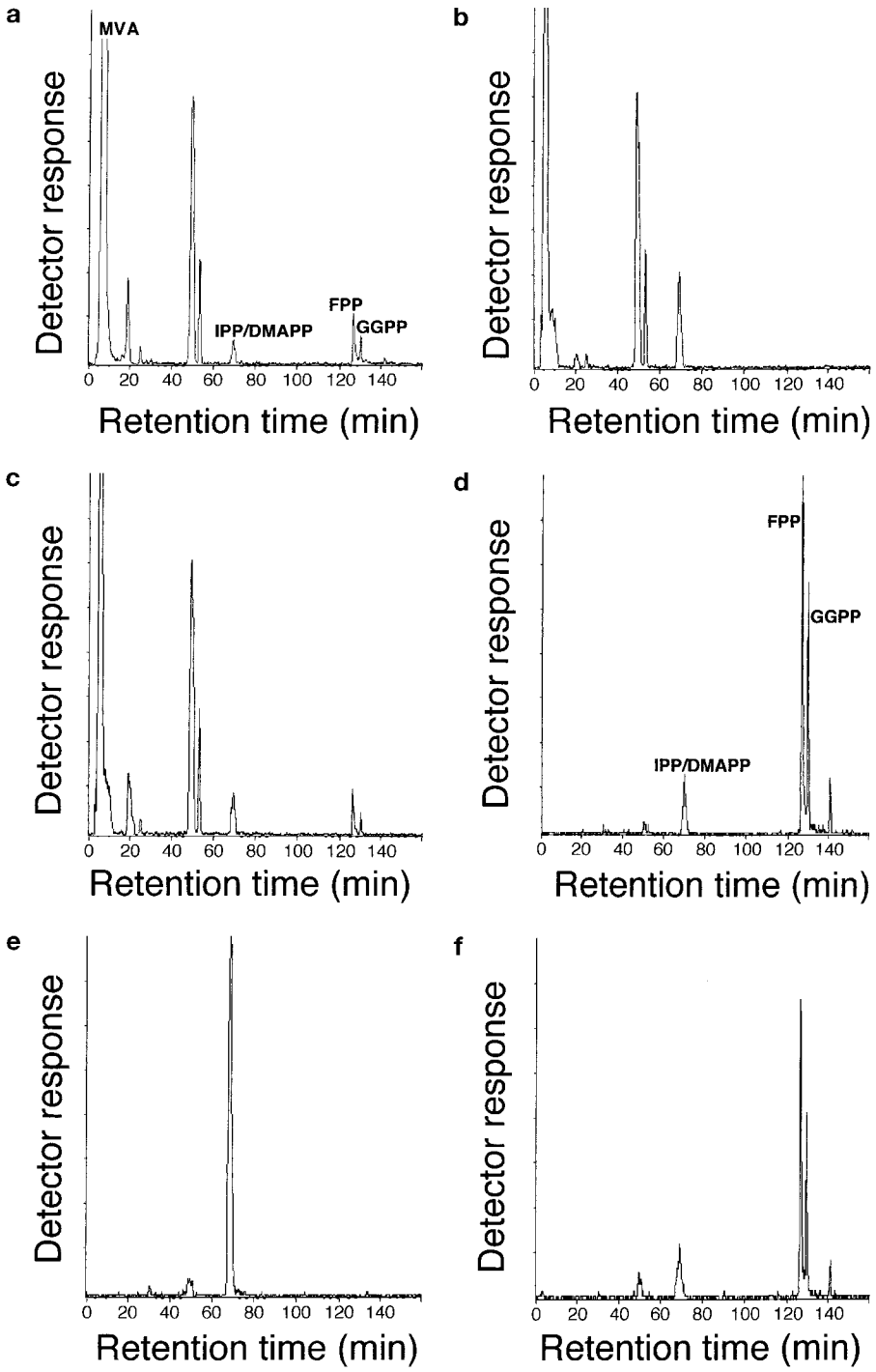

FIG. 1. Reversed-phase ion-pair HPLC separations of ion-exchange-purified homogenates of bovine brain that had been incubated in the absence or presence of bisphosphonates for 5 min at 37°C, thereafter [^{14}C]MVA (80 μM) or [^{14}C]IPP was added and incubations were continued for another 30 min at 37°C. Incubations with [^{14}C]MVA in the absence (a) or in the presence of 1 μM olpadronate (b) or 1 μM clodronate (c). Incubations with [^{14}C]IPP (25 μM), in the absence (d) or in the presence of 1 μM olpadronate (e) or 1 μM clodronate (f). See text for abbreviations.

of IPP isomerase activity, based on acid-lability of the allelic isoprenyl diphosphates (11). Allelic isoprenoids, like DMAPP, GPP, FPP and GGPP are readily converted into their corresponding alcohols under acidic conditions, whereas the homoallelic IPP is stable (13). The alcohols but not IPP can be extracted from the

aqueous phase of the reaction mixture with petroleum ether.

As shown in Fig. 2, under control conditions and in the presence of clodronate, about 75% (after correction of background radioactivity) of [^{14}C]IPP was incorporated into acid-labile products, in line with our findings of production of large quantities of FPP and GGPP. All NBps strongly inhibited the incorporation of label into acid-labile products, in line with their inhibitory effect on FPP and GGPP formation. Yet, a significant amount of label of around 25% was still incorporated into the acid-labile fraction. As HPLC analysis detected only one peak at 68 min, we conclude that the acid-labile compound formed is DMAPP, which has exactly the same retention time as IPP. So, 1 μM of NBps did not prevent the formation of DMAPP from IPP, and moreover, the same amount of DMAPP was formed even when higher concentrations (10 and 100 μM) of the NBps were used. The example for olpadronate is shown in Fig. 3. Thus, none of the NBps tested affect the conversion of [^{14}C]IPP into DMAPP and we conclude that they have no effect on IPP isomerase-activity.

The present and our previous study exclude all enzymes of the pathway from mevalonate to protein geranylgeranylation upstream or downstream of FPP synthase as targets of NBps. Therefore, for the first time we show that FPP synthase is the specific molecular target of NBps.

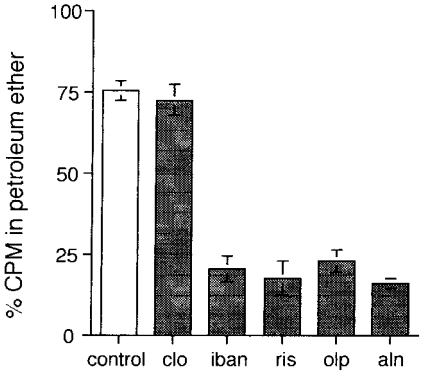

FIG. 2. Formation of acid-labile isoprenoids from [^{14}C]IPP in homogenates of bovine brain, expressed as "%CPM (radioactivity (counts per minute)) in petroleum ether." Homogenates were treated with 1 μM Bps as described in Fig. 1. After termination of the reaction with methanol, the supernatants of the reaction mixtures were treated with acid followed by another 10-min incubation at 37°C to hydrolyse acid-labile isoprenoids to their corresponding alcohols. Subsequently, the alcohols were extracted with petroleum ether and both aqueous and petroleum ether phases were counted for quantification. All values were corrected for background radioactivity resulting from petroleum ether extraction of a blank sample containing the same concentration of pure [^{14}C]IPP label. Clo = clodronate, iban = ibandronate, ris = risedronate, olp = olpadronate, aln = alendronate.

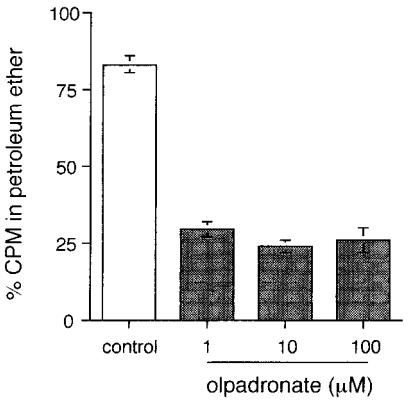

FIG. 3. Formation of acid-labile isoprenoids from [^{14}C]IPP in homogenates of bovine brain, in the absence or presence of 1, 10, and 100 μM olpadronate, as described in Fig. 2.

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