Zoledronic acid-induced IPP/Apppi production in vivo

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Abstract

Bisphosphonates are currently the most important class of anti-resorptive drugs used for the treatment of diseases involving excess bone resorption. Recently we discovered a new mechanism of action for bisphosphonates. Previously it has been shown that nitrogen-containing bisphosphonates (N-BPs) are not metabolized. However, our studies revealed that N-BPs induce formation of a novel pro-apoptotic ATP analog (Apppi), as a consequence of the inhibition of FPP synthase in the mevalonate pathway, and the subsequent accumulation of isopentenyl pyrophosphate (IPP) in vitro. The primary aim of the current study was to determine whether zoledronic acid (a N-BP) induces IPP/Apppi formation in vivo. Mass spectrometry was used to identify whether in vivo administration of zoledronic acid-induced IPP/Apppi production by mouse peritoneal macrophages or bone marrow cells. IPP/Apppi could be detected in extracts from peritoneal macrophages isolated from zoledronic acid-treated animals. Increasing IPP/Apppi accumulation was determined up to 7 days after drug injection, indicating prolonged FPP synthase inhibition by zoledronic acid. Importantly, this is the first report of in vivo production of Apppi, supporting the biological significance of this molecule.

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Introduction

Bisphosphonates (BPs) are pyrophosphate analogs that effectively inhibit osteoclastic bone resorption and are widely used in the treatment of metabolic bone diseases, such as Paget’s disease (Roux and Dougados, 1999), tumor associated osteolysis (Coleman, 2004) and postmenopausal osteoporosis (Delmas, 2002).

BPs can be divided into two distinct pharmacological classes based on their molecular mechanisms of action. Non-nitrogen-containing bisphosphonates (non-N-BPs, e.g. clodronate) are metabolized intracellularly to AppCp-type metabolites, which are cytotoxic analogs of adenosine triphosphate (ATP) (Auriola et al., 1997; Frith et al., 1997). The metabolites are formed by a back reaction catalyzed by aminoacyl-tRNA-synthetases (Rogers et al., 1996). These enzymes can probably bind bisphosphonate instead of pyrophosphate in the ATP binding site. This process leads to accumulation of cytotoxic ATP analogs in the cell cytoplasm and directly causes apoptosis by inhibiting mitochondrial adenine nucleotide translocase (ANT) (Lehenkari et al., 2002). In contrast, nitrogen-containing bisphosphonates (N-BPs, e.g. zoledronic acid) are not metabolized but appear to inhibit at least one enzyme of the mevalonate pathway (Auriola et al., 1997; Benford et al., 1999). The main target enzyme in the mevalonate pathway is currently considered to be farnesyl pyrophosphate (FPP) synthase, and its inhibition prevents the post-translational modification of important signaling proteins through addition of isoprenoid lipids. Loss of prenylated proteins causes a loss of cellular function and, consequently, indirect apoptotic cell death (Rogers, 2004).

Our recent research has revealed a new mechanism of action for N-BPs, establishing that the potent N-BPs, including zoledronic acid, induce formation of a new type of ATP analog, Apppi (triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester) (Mönkkönen et al., 2006). Apppi production...
results from the inhibition of FPP synthase in the mevalonate pathway and subsequent accumulation of the intracellular isopentenyl pyrophosphate (IPP) (Fig. 1). ApppI formation from IPP is probably catalyzed by the same enzyme family (i.e. aminoacyl-tRNA-synthetases) as the ATP analogs of non-N-BPs, but ApppI does not contain a bisphosphonate structure. Similarly to AppCp-type metabolites of non-N-BPs (Lehenkari et al., 2002), ApppI is able to induce direct apoptosis through blockade of the mitochondrial ADP/ATP translocase (Mönkkönen et al., 2006). ApppI formation introduces a new interesting metabolic concept; there are no other drugs reported to produce novel, non-endogenous molecules in the cells, without being metabolites of the drugs.

Originally, ApppI formation following N-BP treatment was detected in macrophages, osteoclasts and glioma cells in vitro (Mönkkönen et al., 2006). The purpose of the present study was to determine whether ApppI could be detected following in vivo exposure to zoledronic acid, and therefore to establish the biological significance of this molecule.

Materials and methods

Chemicals

Zoledronic acid [2-(imidazol-1-yl)-hydroxy-ethylened-1,1-bisphosphonic acid, disodium salt, 4.75 hydrate] was kindly provided by Novartis Pharma AG (Basel, Switzerland). Stock solution of zoledronic acid was prepared in phosphate-buffered saline (PBS; pH 7.4; Gibco, U.K.) and solution was filter-sterilized before use. ApppI (triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester) was synthesized as previously described (Mönkkönen et al., 2006). IPP and AppCp were purchased from Sigma (St. Louis, MO).

Animals and treatments

Eight-week-old male CD1 mice were purchased from Harlan (UK). For IPP/ApppI detection in peritoneal macrophages, animals were administered a single injection of 2.3 mg/kg zoledronic acid intraperitoneally (i.p.). The peritoneal macrophages were isolated 1, 2, 5, 7, 10 and 20 days after injection. For IPP/ApppI detection in bone marrow cells, the animals were treated 100 μg/kg zoledronic subcutaneously (s.c.) once weekly for four weeks. Bone marrow cells were isolated one week after the last dose. Samples from four animals were pooled prior to analysis. In both peritoneal macrophage and bone marrow cell experiments PBS was used as a control. Experiments were carried out with Home Office approval under project licence 40/2972.
Isolation of peritoneal macrophages and bone marrow cells

After treatment, the animals were sacrificed by cervical dislocation. For isolation of peritoneal macrophages, the skin was removed around the stomach and 5 ml of ice-cold PBS was injected into peritoneum space. The macrophages were released by gentle massage and collected using needle and syringe. For isolation of bone marrow cells, the legs were detached and bone marrow from tibia and femur were flushed with ice-cold PBS using needle and syringe. After isolation, the peritoneal macrophages and bone marrow cells were centrifuged (220 g, 5 min) and washed in ice-cold PBS. For the acetonitrile (ACN) extraction, ice-cold ACN was added to cell pellets to precipitate the macromolecules; ice-cold water was added to extract the cellular content (300 μl of ACN and 200 μl of water). The soluble and precipitated fractions were separated by centrifugation (13,000 g, 1 min). The soluble supernatant extract was transferred to a fresh Eppendorf tube. The ACN/water extract were dried down in a vacuum centrifuge and then stored at −20 °C until mass spectrometry analysis of IPP/Apppl.

Analysis of IPP and Apppl by mass spectrometry

The samples were redissolved in 150 μl of water containing sodium fluoride (0.25 mM) (Riedel-de-Haën, Germany) and sodium orthovanadate (0.25 mM) (Sigma, St. Louis, MO) as phosphatase inhibitors for preventing the degradation of Apppl. AppCp (Sigma, St. Louis, MO) was used as internal standard. The molar amounts of IPP and Apppl in cell extracts were determined by HPLC negative ion electrospray ionization mass spectrometry (HPLC-ESI-MS) as previously described (Mönkkönen et al., 2000). IPP and Apppl are very hydrophilic compounds and therefore the use of dimethylhexylamine (DMHA) as an ion-pair agent was necessary to retain these compounds into a reversed-phase column. HPLC separation was performed using a Phenomenex Gemini C18 column (2.0 × 50.0 mm, 5 μm) and an eluent system consisting 20 mM DMHA buffer (pH 7) (eluent A) and 80% methanol with 2 mM DMHA (pH 7) (eluent B). Flow-rate was 200 μl/min and injection volume 45 μl. After HPLC separation, negative ion mass spectra for IPP and Apppl were acquired using an LTQ quadrupole ion trap mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Electron).
Corporation, San Jose, CA). Selected reaction monitoring (SRM) was used for analysis of the compounds in the sample and quantitation was based on characteristic fragment ions. The standard curve was created by spiking extracts from untreated cells with synthetic IPP or ApppI. The concentrations of the samples were determined using the peak areas of the SRM chromatograms and the standard curve.

Results and discussion

This study is the first to demonstrate the formation of ApppI following a single dose of zoledronic acid in vivo. The formation of IPP and ApppI were determined by using high-performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) by monitoring the daughter fragment ions in the selected reaction monitoring (SRM) mode. The MS/MS spectra of IPP and ApppI are shown in Fig. 2, which are based on the fragment ions at m/z 79, 159, 177 and 227 for IPP (Fig. 2A), m/z 227 and 408 for ApppI (Fig. 2B). The quantitation of IPP and ApppI were based on characteristic fragment ions, m/z 245→159 for IPP and m/z 574→408 for ApppI. Transition m/z 504→406 was monitored for internal standard AppCp (data not shown). Typical SRM chromatograms of untreated macrophage extract, untreated macrophage extract spiked with IPP/ApppI and macrophages treated with zoledronic acid are shown in Fig. 3. As expected, the major components were not detected in the chromatogram cell extracts generated from untreated animals (Fig. 3A, B). The substantial peaks were present in the chromatograms of IPP and ApppI standards (Fig. 3C, D) and the cell extracts from animals treated with zoledronic acid (Fig. 3E, F). The HPLC peaks of compounds eluted with their own channels and the retention times were 6.35 min (IPP) and 6.38 min (ApppI). Potentially, N-BPs may also induce the accumulation of dimethylallyl pyrophosphate (DMAPP) in the mevalonate pathway (Fig. 1) and thus lead to production also of the ATP analog containing DMAPP. Further studies are in progress to address this question.

The kinetics of IPP/ApppI formation in peritoneal macrophages was studied after single injection of zoledronic acid (80 μg=200 nmol). ApppI production correlates well with the increased levels of IPP (Fig. 4) supporting that ApppI formation results from accumulation of IPP following inhibition of FPP synthase (Fig. 1). IPP/ApppI production reached a peak value 7 days after injection (Fig. 4). Increasing IPP/ApppI accumulation even 7 days after drug injection may be due to long-lasting FPP synthase inhibition by zoledronic acid. Our data are in agreement with those reported by Rondeau et al. (2006), who recently showed a unique mechanism of FPP synthase inhibition by N-BPs where accumulating IPP binds to and further stabilizes the inhibited FPP synthase-N-BP complex in the fully closed conformation, thereby contributing to very efficient and sustainable inhibition of enzyme. Thus, the increasing IPP/ApppI formation until 7 days (Fig. 4) supports the hypothesis of prolonged FPP synthase inhibition by N-BPs.

We were unable to detect IPP/ApppI after s.c. zoledronic acid injection in bone marrow cells (data not shown). It is possible that the treatment schedule used did not cause concentrations of zoledronic acid within the bone marrow in order to generate sufficient levels of IPP/ApppI for successful mass spectrometry analysis. It is notable that peritoneal macrophages are not clinically the target cells for bisphosphonate action and also the dose of zoledronic acid used in this study was supra-pharmacological. Following absorption, BPs are rapidly deposited into the skeleton and localize preferentially on exposed mineral at bone resorption surfaces. Bone-resorbing osteoclasts are the target cells for bisphosphonate action. Sato et al. (1991) estimated that pharmacological doses of alendronate that inhibit bone resorption in vivo could give rise to local concentrations as high as 1 mM alendronate (a N-BP) in the resorption space beneath an osteoclast. This, together with the fact that osteoclasts can internalize negatively-charged compounds via endocytosis (Salo et al., 1997; Nesbitt and Horton, 1997; Stenbeck and Horton, 2000), makes it likely that osteoclasts are also able to produce ApppI in vivo and thus to be induced to undergo mitochondria-mediated apoptosis (Mönkkönen et al., 2006). Further investigations to detect ApppI formation in osteoclasts in vivo and its relation to osteoclast apoptosis are in progress. In conclusion, this is the first study detecting ApppI formation in vivo establishing the biological significance of this molecule, and further elucidating the molecular mechanisms of action this class of anti-resorptive agents.

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