Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 307 (2003) 23–30
BBRC
www.elsevier.com/locate/ybbrc

The bisphosphonate ibandronate stimulates reverse cholesterol transport out of monocytoid cells by enhanced ABCA1 transcription ☆

Dorothea Strobach and Reinhard L. Lorenz*

Institute for Prophylaxis of Cardiovascular Diseases, University of Munich, Germany

Received 14 May 2003

Abstract

Nitrogen-containing bisphosphonates used in osteoporosis act by interference with pyrophosphorylated intermediates of the sterol pathway and are internalized by monocytes/macrophages, key players in atherogenesis. We therefore studied the effects of ibandronate on monocytic cholesterol homeostasis. In differentiated human MM6 cells and freshly prepared human PBMCs lipoprotein receptor transcription was quantified by real-time RT-PCR and receptor-mediated cellular cholesterol handling by lipoprotein-driven uptake and efflux assays. Low nanomolar concentrations of ibandronate reduced cellular cholesterol content despite reactive up-regulation of the LDL receptor. Simultaneously, the transcription of the cellular cholesterol exporter ABCA1 was severalfold stimulated, whereas the scavenger receptor CD36 was down-regulated. Thereby, ibandronate decreased the cellular uptake of modified LDL and enhanced the efflux of cholesterol to delipidated HDL. Geranylgeraniol antagonized the stimulation of ABCA1 expression by ibandronate. Ibandronate in low pharmacologic concentrations redirects monocytic cholesterol handling from favouring foam cell formation towards enhanced reverse cholesterol transport.
© 2003 Elsevier Science (USA). All rights reserved.

Keywords: Bisphosphonates; Ibandronate; ABCA1; GGPP; CD36; Macrophage; Atherosclerosis; Cholesterol

Bisphosphonates are currently used in the treatment of osteoporosis, metastatic, and Paget's disease of bone. Their chemical structure of two phosphate groups linked by a hydroxy substituted carbon atom causes a high avidity for calcium ions and a selective deposition in bone mineral. Therefore, bisphosphonates withstand degradation by phosphatases, but inhibit osteoclast-mediated bone resorption by intracellular mechanisms [1,2]. In contrast to their protective effect on bone mineral content, bisphosphonates have long been known to reduce and reverse calcifications in extraosseous tissues [3–5]. Such calcifications are commonly observed in advanced atherosclerotic lesions. Bisphosphonates are enriched in the atherosclerotic vessel wall [6–8] and indeed, bisphosphonates have been shown to attenuate and even reverse atherosclerotic lesions in several animal models without affecting plasma cholesterol levels [9–13].

Monocyte-derived macrophages and foam cells are key cellular components of atherosclerotic lesions. They accumulate lipids, secrete chemotactic, proinflammatory, and growth factors that all contribute to plaque progression. Finally, metalloproteinases secreted from monocytes may destabilize the fibrous cap of atheromas causing plaque rupture [14,15]. Monocytes/macrophages readily take up bisphosphonates by endocytosis, phagocytosis, or polyvalent transmembrane transporters [16,17]. The high polarity of bisphosphonates limits simple diffusion through the plasma membrane [2] and therefore prevents uptake in most cells.

Two intracellular modes of action depending on the molecular structure of bisphosphonates have recently

☆ *Abbreviations:* N-BP, nitrogen-containing bisphosphonates; PBMCs, peripheral blood monocytic cells; ABCA1, ATP-binding cassette transporter A1; LXR, liver x receptor; GGPP, geranylgeranyl pyrophosphate; SREBP, sterol regulatory element binding protein; dHDL, delipidated high density lipoprotein; LDL, low density lipoprotein; oxLDL, oxidized LDL; acLDL, acetylated LDL.

* Corresponding author. Present address: Kreislaufinstitut, Klinikum der Universität München, Pettenkoferstr. 9, 80336 Munich, Germany. Fax: +49-89-5160-4352.

E-mail address: rLorenz@klp.med.uni-muenchen.de (R.L. Lorenz).

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)01127-6

been elucidated. Bisphosphonates without amino substitution can be incorporated into cytotoxic ATP analogues that impair oxidative cell metabolism [1]. Osteoclasts, cells of monocytoid lineage, are exposed to high concentrations of bisphosphonates released from bone into their resorptive lacunae and are thought to be inhibited by these toxic metabolites of bisphosphonates [2].

In contrast, the most potent, nitrogen-containing bisphosphonates (N-BP) interfere with the binding of pyrophosphorylated isoprenoid intermediates of the sterol pathway to key enzymes [1]. Thereby, N-BP directly inhibit cellular cholesterol synthesis [18] but also interfere with protein prenylation, especially geranylgeranylation. Prenylation is essential for membrane anchoring of proteins and involved in signalling cascades of cell activation and cholesterol homeostasis [2,19]. The targeting of N-BP to macrophages might thus offer a new pharmacologic approach to selectively inhibit foam cell activation and stimulate reverse cholesterol transport out of the vessel wall. We therefore studied how an N-BP affects monocytoid cholesterol balance and the geranylgeranyl-dependent expression of key receptors of cellular cholesterol handling.

Materials and methods

Ibandronate was a kind gift of Dr. F. Bauss, Roche Diagnostics GmbH, Penzberg. Stock solutions (1, 10, and 100 μM) were prepared in sterile NaCl and stored at 4 °C. Geranylgeranyl pyrophosphate (GGPP) and all other chemicals were purchased from Sigma unless otherwise stated.

Cell preparation and culture. The highly differentiated monocytic cell line MM6 [20] was cultured in supplemented, filter-sterilized RPMI 1640 medium [22]. Cells were seeded at a density of 0.2×10^6 cells/ml and kept in 2 ml medium in 24-well plastic culture dishes (Falcon). Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy normolipidemic volunteer donors by Ficoll density-gradient separation [21]. PBMCs were maintained at a density of 5×10^6 cells/ml in RPMI 1640 medium with EDTA 5 mM as non-adherent cells in sterile 2 ml cups (Eppendorf).

RNA-isolation and real-time reverse-transcription PCR. Total cellular RNA was extracted from 2×10^6 MM6 cells or 5×10^6 PBMCs with the AquaPure RNA Isolation Kit (BioRad). RNA isolates were treated with RQ1-DNAse (Promega) and the RNA concentration was measured with the Gene Quant RNA/DNA Calculator (Amersham Biotech). Quantitative real-time-RT-PCR was performed in an iCycler (BioRad) using Ready-To-Go RT-PCR beads (Amersham) and the double-stranded DNA-binding fluorescent dye SYBR Green I (Molecular Probes). The reaction mixture with a final volume of 50 μl contained random hexamer primers for reverse transcription (BioRad), $MgCl_2$ 2.5 or 3 mM, specific primers 200 or 300 nM and RNA 500 ng (PBMCs) or 1000 ng (MM6). Primers were synthesized by Metabion GmbH according to sequences established previously for the LDL receptor [22], CD36 [23], ABCA1 [24], and GAPDH [24]. All Primers were checked again by a GenBank sequences BLAST search and the specificity of the amplicon was confirmed by melting curve analysis and ethidium bromide stained gel electrophoresis [25,26].

In the real-time RT-PCR protocol reverse transcription was performed at 42 °C for 30 min, denaturation at 95 °C for 5 min, followed by 40 cycles with annealing at 60 °C for 20 s, elongation at 72 °C for 20 s, melting at 95 °C for 30 s, final elongation at 72 °C for 10 min, and melting curve analysis from 55 to 98 °C. Reactions were optimized for every target and amplification efficiency was tested by serial dilution (1:10 to 1:1000) standard curves [27]. Quantification was calculated using the C_T (treshold cycle) [27] of the target signal relative to the GAPDH signal in the same RNA sample. Effects were quantified and expressed by the fold change method calculated as: Fold Change $= 2^{-\Delta(\Delta C_T)}$ with $\Delta C_T = C_{T,target} - C_{T,GAPDH}$ and $\Delta(\Delta C_T) = \Delta C_{T,stimulated} - \Delta C_{T,control}$ [28].

Flow cytometry. For assessing the surface expression of CD36 protein cells were washed with Hanks' balanced salt solution (HBSS) containing 10 mM Hepes, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.5% bovine serum albumin. Unspecific binding was blocked with 5% human serum in HBSS for 30 min at 4 °C, followed by 30 min incubation at 4 °C with mouse IgG1 isotype (MOPC21, Sigma) or mouse anti-human CD36 (Clone FA6, Immunotech) at a concentration of 2.5 μg/ml. The FITC-conjugated goat anti-mouse secondary antibody (Dako) was added at a concentration of 1 μg/ml for 30 min at 4 °C. Cells were fixed with formaldehyde 2% in FACS buffer and CD36 expression was determined as specific fluorescence relative to isotype control in a FACS scanner (Becton–Dickinson).

Isolation and modification of lipoproteins. LDL and HDL were isolated from plasma of healthy normolipidemic volunteers by 24 h ultracentrifugation on a potassium bromide gradient [29]. OxLDL was prepared by incubation of LDL with CuSO4 (5 μM) for 20 h in the presence of oxygen [30]. Acetylation of LDL was performed with acetic anhydride [31]. HDL was delipidated with methanol/ether as described [32]. Protein concentrations were determined [33] for lipoprotein quantification and samples were filter sterilized (0.22 μm), stored at 4 °C, and used within a week.

Cellular cholesterol content, loading, and efflux assay. For studying effects on cellular cholesterol handling, cells were incubated for 48 h in the presence of carrier control, ibandronate 0.8–1000 nM or dHDL 100 μg/ml as positive control. Cells were washed twice with sterile PBS (Pan Biotech) and counted to monitor growth. Total cellular cholesterol was extracted with methanol/chloroform [34], dissolved with dioxan and buffer, and quantified by a cholesterol peroxidase based enzymatic assay (Rolf Greiner Biochemica).

To investigate the effects on cellular cholesterol uptake and efflux cells were preincubated for 24 h with carrier control or ibandronate 10 nM. Then, oxLDL or acLDL 50 μg/ml was added for 48 h to allow for cellular cholesterol accumulation. After the loading phase cells were washed twice with sterile PBS and further incubated for 24 h in the presence of dHDL 20 μg/ml or carrier control to quantify HDL enhanced cholesterol efflux (modified from [34]). Throughout incubation protocols carrier or ibandronate treatment was freshly added every 24 h.

Statistical analysis. Data are presented as means ± SEM. Statistical analysis was performed by comparison to concurrent controls using Student's t test for paired samples with a P value < 0.05 considered to indicate significance.

Results

First, cells were incubated for 48 h with increasing concentrations of ibandronate added every 24 h to determine the non-toxic range. Cell morphology, viability, proliferation, and cellular protein content were not detectably affected by ibandronate in concentrations up to 1 μM. Next, effects of ibandronate on cellular sterol metabolism were monitored by measuring total cellular cholesterol content after incubation with ibandronate in increasing concentrations. Ibandronate

dose-dependently reduced cellular cholesterol content by up to 30%. A plateau effect on cellular cholesterol content was reached already at very low ibandronate concentrations between 4 and 20 nM (Fig. 1). Therefore, ibandronate 10 and 100 nM were used in subsequent experiments. For comparison, prolonged incubation of cells with a high concentration of 100 μg/ml dHDL, a massive stimulus of cellular cholesterol depletion, reduced cellular cholesterol content by about 40%.

The reduction of cellular cholesterol content induced by ibandronate was reflected by increased cellular LDL-receptor expression (Fig. 2A). Compared to control cells incubated for 24–72 h with carrier only, cells incubated with ibandronate 10 nM had a highly significant increase in LDL-receptor-specific mRNA levels. With ibandronate added only once at time 0 the LDL-receptor upregulation peaked at 48 h and was fully maintained at 72 h with ibandronate 100 nM. Therefore, increased LDL-receptor expression as the physiologic cellular counter-regulation to cholesterol depletion was not disturbed by ibandronate incubation.

In addition to the apolipoprotein B-specific LDL-receptor, cells can also take up cholesterol by scavenger receptors like CD36 binding modified lipoprotein particles. In contrast to the effects on the LDL-receptor, ibandronate reduced cellular levels of CD36-specific mRNA (Fig. 2B). This reduction in CD36 transcription by about 50% was also achieved within 48 h and fully maintained for at least 72 h after a single addition of ibandronate 10 or 100 nM at time 0. We further measured the CD36 surface expression by FACS analysis. Incubation with ibandronate 10 and 100 nM significantly reduced CD36 protein on MM6 cells after 48 and 72 h (Fig. 3).

In addition to the receptors involved in cholesterol uptake, cellular cholesterol balance is determined by the active cellular cholesterol exporter, ABCA1. The cellular level of ABCA1-specific mRNA was increased by ibandronate in a concentration-dependent manner up to

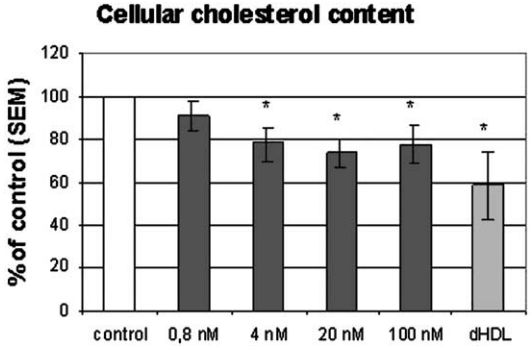

Fig. 1. Effects of ibandronate on cellular cholesterol content of MM6 cells. Cells were incubated for 48 h with ibandronate 0.8–100 nM added every 24 h or dHDL 100 μg/ml. Total cellular cholesterol in percentage of control cells incubated with carrier only (means ± SEM; $n = 5$; $^{*}p < 0.05$).

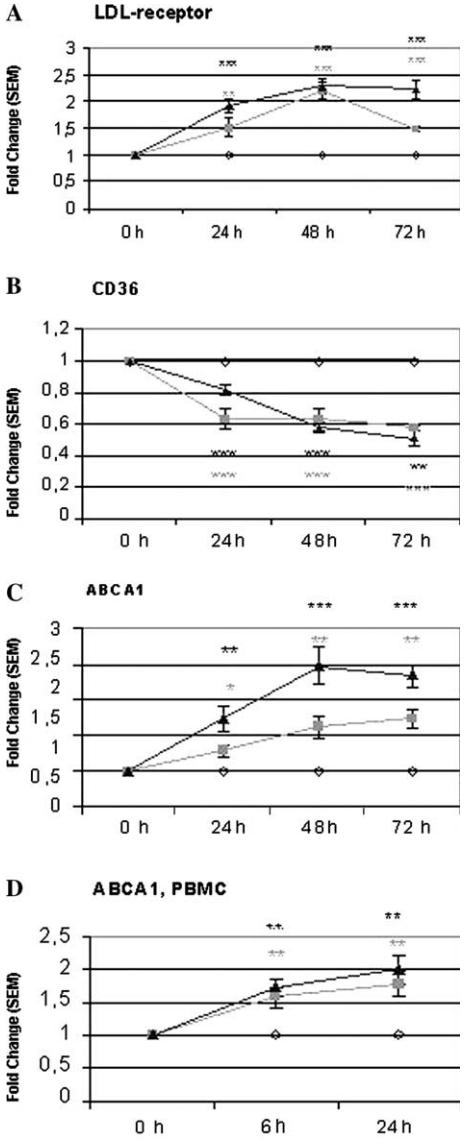

Fig. 2. Time course of effects of ibandronate 10 nM (grey symbols) and 100 nM (dark symbols) on LDL-receptor (A), CD36 (B) ABCA1 expression in MM6 cells incubated up to 72 h (C), and ABCA1 expression in freshly prepared PBMCs (D) incubated up to 24 h. Fold change of specific receptor mRNA levels measured by real-time RT-PCR, normalized to GAPDH and compared to levels in control cells (open symbols) incubated with carrier control for the same time intervals. Means ± SEM; $n = 7$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

2.5-fold and this increase was also maintained for more than 72 h (Fig. 2C). This stimulation of ABCA1 transcription by ibandronate persisted even after cholesterol depletion of the cells. In a very similar pattern, ABCA1 transcription was also very rapidly stimulated by ibandronate in freshly prepared PBMCs from healthy donors (Fig. 2D). Therefore, ibandronate is taken up by monocytes and stimulates ABCA1 even before full differentiation to macrophages.

The functional relevance of the ibandronate effects on ABCA1 and CD36 transcription was tested in a

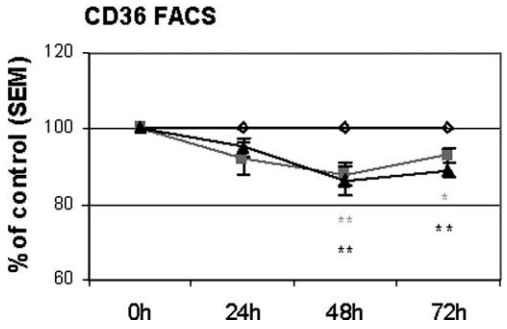

Fig. 3. Effect of ibandronate 10 nM (grey symbols) and 100 nM (dark symbols) on CD36 protein surface expression by FACS analysis relative to control cells incubated with carrier only (open circles). Means ± SEM; $n = 5$; *$p < 0.05$; **$p < 0.01$.

combined assay of ABCA1-mediated cellular cholesterol efflux to dHDL before and after cellular cholesterol loading with modified lipoproteins. Inclusion of dHDL 20 μg/ml as cholesterol acceptor in the incubation medium significantly reduced cellular cholesterol content by 25% (Fig. 4A). Ibandronate alone reduced cellular cholesterol even in the absence of HDL. The simultaneous incubation of cells with dHDL and ibandronate caused an overadditive reduction of cellular cholesterol, demonstrating the functional relevance of ibandronate stimulated ABCA1 expression.

Incubation with oxLDL or acLDL as modified LDL particles internalized by cellular scavenger receptors significantly increased cellular cholesterol content. This increase was prevented by the simultaneous incubation

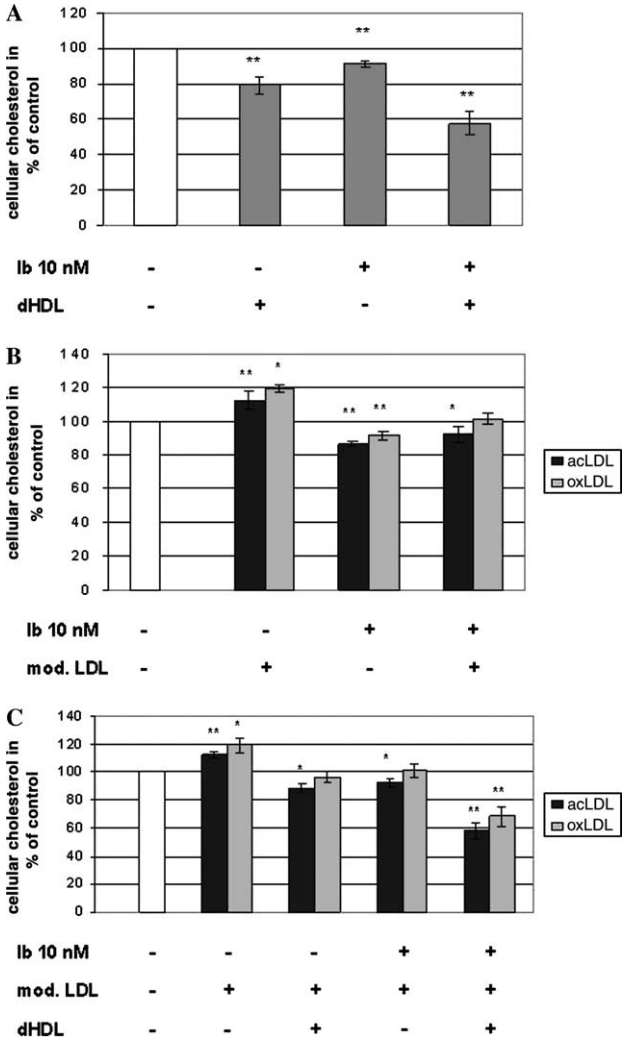

Fig. 4. Effect of incubation with ibandronate (Ib) 10 nM on cellular cholesterol content, cholesterol uptake from modified LDL particles (mod. LDL) and cholesterol efflux to delipidated HDL (dHDL) particles at baseline or after cholesterol preloading. (A) Efflux to dHDL 20 μg/ml without previous cholesterol loading. (B) Cholesterol uptake from acLDL and oxLDL 50 μg/ml. (C) Efflux to dHDL 20 μg/ml after preloading with acLDL or oxLDL 50 μg/ml. Means ± SEM; $n = 5$; *$p < 0.05$; **$p < 0.01$.

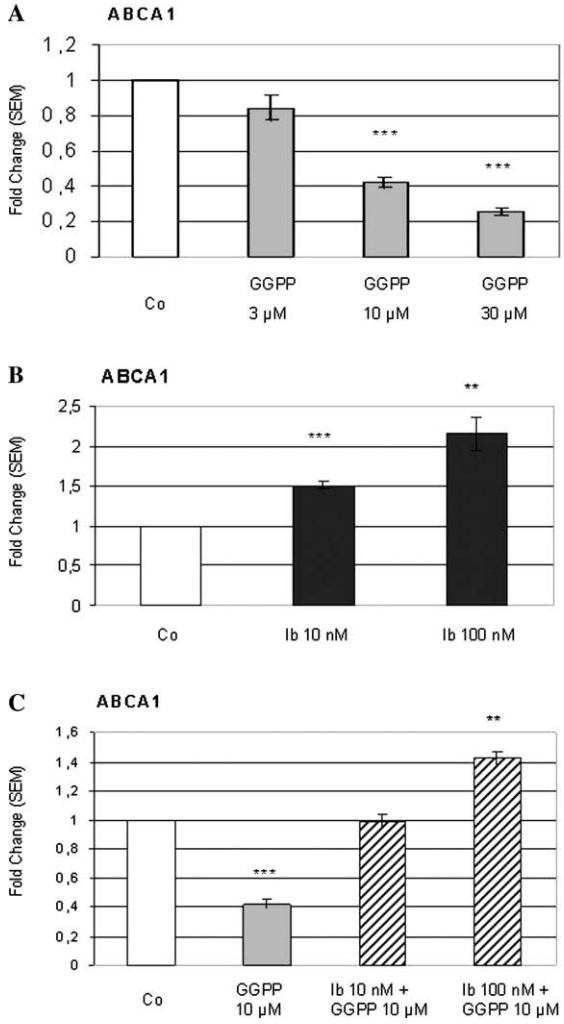

Fig. 5. Effect of GGPP and ibandronate (Ib) incubation for 48 h on ABCA1-specific mRNA expression. (A) Incubation with GGPP 3–30 μM alone inhibited ABCA1 expression in a concentration-dependent manner. (B) Incubation of cells with ibandronate 10–100 nM stimulated ABCA-expression. (C) Coincubation with GGPP 10 μM antagonized the stimulation of ABCA1 expression by ibandronate 10 and 100 nM in a competitive manner. Means ± SEM; $n = 5$; **$p < 0.01$; ***$p < 0.001$).

of cells with nanomolar concentrations of ibandronate (Fig. 3B). Moreover, ibandronate significantly reduced cellular cholesterol content compared to control conditions even in the presence of acetylated LDL particles supporting the functional relevance of the ibandronate induced reduction of CD36 and ABCA1 induction.

Finally cells were first cholesterol loaded by preincubation with oxLDL or acLDL and then exposed to dHDL to measure ABCA1-mediated cholesterol efflux (Fig. 3C). The cholesterol accumulation of cells from modified lipoproteins was both reversed by incubation with dHDL and ibandronate alone. Again, coincubation with dHDL and ibandronate caused an overadditive cholesterol loss. Cells preloaded with cholesterol from modified lipoproteins and incubated with ibandronate showed a greater cholesterol depletion after addition of dHDL compared to control cells. Therefore, ibandronate enhanced HDL-dependent cellular cholesterol efflux very effectively, especially in the presence of modified lipoprotein particles.

To support the suspected role of GGPP depletion in the ibandronate-induced ABCA1 stimulation, cells were first incubated with GGPP. GGPP 3–30 μM suppressed ABCA1 transcription in a concentration-dependent manner by up to 80% for at least 48 h (Fig. 5A). Ibandronate alone stimulated ABCA1 expression (Fig. 5B) in a concentration-dependent way. Coincubation of cells with ibandronate 10–100 nM and GGPP 10 μM reversed the inhibition of ABCA1 expression induced by GGPP alone in an obviously competitive manner (Fig. 5C).

Discussion

We studied the mechanisms involved in the effects of nitrogen-containing bisphosphonates on monocytoid cholesterol handling. The major new findings were the stimulated expression of the main cellular cholesterol exporter ABCA1 by ibandronate in very low concentrations, protecting cells from cholesterol accumulation and maintaining cholesterol loading onto HDL particles for reverse transport even after cellular cholesterol depletion.

The differentiated human monocytoid cell line MM6 used in these experiments tolerated ibandronate in concentrations up to 1 μM without signs of toxicity. In the screening test of total cellular cholesterol content first effects of ibandronate were detectable below a concentration of 1 nM and were already maximal between 4 and 20 nM. Plasma levels of ibandronate in humans after a single oral administration of 5 mg have been reported in the range of 3 nM [35]. After intravenous administration of 6 mg ibandronate, as used in the therapy of tumour hypercalcemia, peak plasma levels up to 1 μM can be reached [36]. Therefore, the concentrations having profound effects on cellular cholesterol metabolism in our experiments are well within the therapeutic range tolerated without major side effects. Previous experiments on N-BP effects in RAW 264 mouse macrophages had used much higher concentrations of ibandronate (148 μM) [37], alendronate (10 μM) [38] or liposomal preparations of ibandronate (700 nM) [37]. Species differences, the degree of monocytic differentiation, and therefore efficiency of cellular ibandronate uptake might account for this. Anyway, the accumulation mechanisms for bisphosphonates appear to be rather specific for monocytes/macrophages and higher doses of bisphosphonates have successfully been used for a selective in vivo ablation of monocytes/macrophages without major toxic effects on other tissues [39,40].

Increased LDL-receptor expression, the physiologic cellular response to cholesterol depletion mediated by the SREBP pathway [41], apparently remained intact during ibandronate incubation. This argues against an unspecific toxic effect of ibandronate at the very low concentrations used. CD36, the quantitatively most important scavenger receptor on monocytes/macrophages, was down-regulated by ibandronate. Involvement of prenylated proteins in the transcriptional regulation of CD36 has been discussed before [30]. The protein surface expression of CD36 was also significantly reduced, but to a lesser extent. This might be due to large intracellular pools of intact CD36 protein that have been demonstrated in monocytes/macrophages [42]. However, a significant decrease in uptake of modified LDL was clearly seen confirming the functional relevance of the reduction in CD36 surface expression.

Decreased uptake of acLDL by monocytes has been reported previously in the presence of suprapharmacologic concentrations of alendronate 10 μM [38] or liposomal preparations of bisphosphonates [43]. Encapsulation into liposomes enhances the penetration of bisphosphonates through the plasma membrane. Inhibition of cellular uptake of oxLDL and acLDL by low nanomolar concentrations of free ibandronate is reported here for the first time. In contrast to acLDL, oxLDL has been characterized as a pathophysiologically relevant lipoprotein detectable in humans [44] and taken up by cells via CD36. Uptake of acLDL has been shown to be mediated mainly by scavenger receptor A [45], but in MM6 cells at least one further receptor mediating uptake of acLDL was demonstrated [22,46]. AcLDL is also a ligand for scavenger receptor B, MARCO, and CD36 [47]. It is presently unknown whether other scavenger receptors are down-regulated by ibandronate in addition to CD36.

Despite increased LDL-R expression, monocytoid cells exposed to ibandronate remained cholesterol depleted for prolonged time intervals suggesting not only a suppression of cholesterol uptake and de novo synthesis

but also a continued enhancement of cellular cholesterol export. ABCA1 has been characterized as the most important cellular cholesterol exporter loading cellular cholesterol onto apoA1 containing nascent HDL particles. In fact, ibandronate stimulated transcription of ABCA1 in monocytoid cells severalfold. This finding was confirmed in freshly isolated PBMCs supporting its potential pharmacological relevance.

ABCA1 is a target gene of the nuclear transcription factor LXRα. Following activation, LXR heterodimerizes with the nuclear retinoic acid receptor RXR and associates with the nuclear steroid receptor coactivator-1 (SRC-1) [48–50]. This complex translocates to the nucleus and initiates transcription of genes like ABCA1. Recently, a dual mechanism of ABCA1 regulation by GGPP was described while other intermediate products of the sterol pathway had no effect on ABCA1 transcription. GGPP directly inhibited the interaction of LXR, RXR, and SRC-1 [51,52]. Additionally, Rho-proteins activated by transfer of a geranylgeraniol group were found to inhibit ABCA1 expression via LXR [52].

In studies with macrophages and osteoclasts, N-BPs decreased intracellular geranylgeraniol and the reduction of geranylgeranylated proteins was characterized as the molecular mechanism underlying the effects on bone metabolism [2]. In our experiments, enhanced ABCA1 expression stimulated by ibandronate could competitively be reversed by GGPP coincubation. Increased ABCA1 transcription has previously been described as a counter-regulatory response to cellular cholesterol overloading [53]. Most remarkably, ABCA1 transcription remained enhanced by ibandronate even after cellular cholesterol depletion. Inhibition of the negative GGPP signalling must have overcompensated signals of decreased sterol stores. This ability of ibandronate to stimulate and maintain enhanced ABCA1 expression in monocytes/macrophages independent of cellular cholesterol stores is of special therapeutic interest. For the regression of extracellular cholesterol accumulations in atherosclerotic plaques, cholesterol has first to be taken up by macrophages but then rapidly be transferred to apoA1-containing particles via ABCA1 to channel it into the reverse cholesterol transport and avoid foam cell formation.

Only few data relating bisphosphonates to atherosclerosis in humans are available so far. Oral etidronate for 1 year was reported to reduce intima media thickness in patients with diabetes mellitus type II [54]. Interestingly, a retrospective mortality analysis of 7900 osteoporosis patients enrolled in clinical trials with the N-BP risedronate revealed a significant reduction in stroke mortality [55]. Two trials from Italy found a small increase in serum HDL and decrease in LDL at unchanged total cholesterol levels during treatment with various N-BP [56,57]. With the rapid clearance of N-BP from the circulation, but their enrichment in the atherosclerotic vessel wall and selective uptake into macrophages, effects on peripheral lipoprotein metabolism in the vessel wall rather than in the liver may be expected.

In summary, we describe several new aspects of N-BP action on cholesterol handling in monocytoid cells. In low pharmacological concentrations ibandronate increased the expression of ABCA1 promoting the first step of reverse cholesterol transport. These changes in cellular lipoprotein receptor and transporter expression were functionally relevant and consistently decreased cellular cholesterol content. This fits well with previous reports on a reduction of the monocytic accumulation of cholesterol by N-BP [38,43]. Together with the inhibition of arterial calcification and smooth muscle cell activation [58], these actions of N-BP on lipid handling at the site of atherogenesis may provide an anti-atherosclerotic effect additive to lowering plasma lipid levels.

Acknowledgments

We thank Dr. F. Bauss from Roche Diagnostics for kindly providing ibandronate and for his helpful suggestions. B. Zimmer is thanked for excellent technical assistance in the FACS experiments. These data are part of the thesis of D. Strobach. The work was supported in part by Deutsche Forschungsgemeinschaft (Graduate Program Vascular Biology in Medicine GRK 438-Lo).

References

[1] R.G.G. Russell, P.I. Croucher, M.J. Rogers, Bisphosphonates: pharmacology, mechanisms of action and clinical uses, Osteoporos. Int. Suppl. 2 (1999) S66–S80.
[2] M.J. Rogers, S. Gordon, H.L. Benford, F.P. Coxon, S.P. Luckmann, J. Mönkkönen, J.C. Frith, Cellular and molecular mechanisms of action of bisphosphonates, Cancer 88 (2000) 2961–2978.
[3] M.D. Francis, R.G.G. Russell, H. Fleisch, Diphosphonates inhibit formation of calcium phosphate crystals in vitro and pathological calcification in vivo, Science 165 (1969) 1264–1266.
[4] C.A. Bassett, A. Donath, F. Macagno, R. Preisig, H. Fleisch, M.D. Francio, Diphosphoantes in the treatment of myositis ossificans, Lancet 2 (1969) 845.
[5] H. Fleisch, R.G.G. Russell, S. Bisaz, R.C. Mühlbauer, D.A. Williams, The inhibitory effect of phosphonates on the formation of calcium phosphate crystals in vitro and on aortic and kidney calcification in vivo, J. Clin. Invest. 1 (1970) 12–18.
[6] T. Lantto, K. Järvi, I. Toivio, S. Vahatalo, M. Vorne, The visualization of femoral vessels in delayed bone scans, a sign of atherosclerosis? A comparison of 99mTc-MDP and ppmTc-DPD, Eur. J. Nucl. Med. 15 (1989) 265–268.
[7] R. Ylitalo, J. Mönkkönen, A. Urtti, P. Ylitalo, Accumulation of bisphosphonates in the aorta and some other tissues of healthy and atherosclerotic rabbits, J. Lab. Clin. Med. 127 (1996) 200–206.
[8] R. Ylitalo, J. Kalliovalkama, R. Ylitalo, I. Pörsti, Accumulation of bisphosphonates in human artery and their effect on human and rat arterial function in vitro, Pharmacol. Toxicol. 83 (1998) 125–131.

[9] W. Hollander, J. Paddock, S. Nagraj, M. Colombo, B. Kirkpatrick, Effects of anticalcifiying and antifibrotic drugs on preestablished atherosclerosis in the rabbit, Atherosclerosis 33 (1979) 111–123.
[10] I.Y. Rosenblum, L. Flora, R. Eisenstein, The effect of disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) on a rabbit model of athero-arteriosclerosis, Atherosclerosis 22 (1975) 411–424.
[11] D.M. Kramsch, C.T. Chan, The effect of agent interfering with soft tissue calcification and cell proliferation on calcific fibrous-fatty plaques in rabbits, Circ. Res. 42 (1978) 562–571.
[12] W.D. Wagner, T.B. Clarkson, J. Foster, Contrasting effects of ethane-1-hydroxy-1,1-diphosphonate (EHDP) on the regression of two types of dietary-induced atherosclerosis, Atherosclerosis 27 (1977) 419–435.
[13] A.P. Price, A.F. Samuel, M.K. Williamson, Bisphosphonates alendronate and ibandronate inhibit artery calcification at doses comparable to those that inhibit bone resorption, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 817–824.
[14] R. Ross, The pathogenesis of atherosclerosis—a perspective for the 1990s, Nature 362 (1993) 801–809.
[15] A.J. Lusis, Atherosclerosis, Nature 407 (2000) 233–241.
[16] R. Felix, H.L. Guenther, H. Fleisch, The subcellular distribution of [14]dichloromethylenebisphosphonate and [14]1-hydroxyethylidene-1,1-bisphosphonate in cultured calvaria cells, Calcif. Tissue Int. 36 (1984) 108–113.
[17] M.J. Rogers, X. Xiong, X. Ji, J. Mönkkönen, R.G. Russell, M.P. Williamson, Inhibition of growth of *Dictyostelium discoideum* amoebae by bisphosphonate drugs is dependent on cellular uptake, Pharm. Res. 14 (1997) 625–630.
[18] D. Amin, S.A. Cornell, S.K. Gustafson, S.J. Needle, J.W. Ullrich, G.E. Bilder, M.H. Perrone, Bisphosphonates used for the treatment of bone disorders inhibit squalene synthase and cholesterol biosynthesis, J. Lipid. Res. 33 (1992) 1657–1663.
[19] S.P. Luckmann, D.E. Hughes, F.P. Coxon, R.G.G. Russell, M.J. Rogers, Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras, J. Bone Miner. Res. 13 (1998) 581–589.
[20] L. Ziegler-Heitbrock, E. Thiel, A. Fütterer, V. Herzog, A. Wirtz, G. Riethmüller, Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes, Int. J. Cancer 41 (1988) 456–461.
[21] D.F. Eiermann, C.E. Johnson, S.I. Haskill, Human monocyte inflammatory mediator gene expression is selectively regulated by adherence substrates, J. Immunol. 142 (1989) 1970–1976.
[22] R. Scheithe, A.K. Heidenthal, U. Danesch, E. Mauthner, G. Hapfelmeier, A. Becker, A. Pietsch, P.C. Weber, N. Hrboticky, Acetylated LDL endocytosis by the human monocytic mono mac 6sr cells is not mediated by the macrophage type I and II scavenger receptor, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 1024–1031.
[23] A. Pietsch, W. Erl, R.L. Lorenz, Lovastatin reduces expression of the combined adhesion and scavenger receptor CD36 in human monocytic cells, Biochem. Pharmacol. 52 (1996) 433–439.
[24] D. Kielar, W. Dietmaier, T. Langmann, C. Aslanidis, M. Probst, M. Naruszewicz, G. Schmitz, Rapid quantification of human ABCAI mRNA in various cell types and tissues by real-time reverse transcription-PCR, Clin. Chem. 47 (2001) 2089–2097.
[25] N. Steuerwald, J. Cohen, R.J. Herrera, C.A. Brenner, Analysis of gene expression in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR, Mol. Hum. Reprod. 5 (1999) 1034–1039.
[26] T.H.S. Woo, B.K.C. Patel, M. Cinco, L.-D. Smythe, M.L. Symonds, M.A. Norris, M.F. Dohnt, Real-time homogenous assay of rapid cycle polymerase chain reaction product for identification of *Leptonerma illini*, Anal. Biochem. 259 (1998) 112–117.
[27] S.A. Bustin, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assay, J. Mol. Endocrinol. 25 (2000) 169–193.
[28] ABI Prism 7700 Sequence Detection System User Bulletin #2, Relative quantitation of gene expression, Applied Biosystems (1997).
[29] T. Schulz, H. Schiffl, R. Scheithe, N. Hrboticky, L. Lorenz, Preserved antioxidative defense of lipoproteins in renal failure and during hemodialysis, Am. J. Kidney Dis. 25 (1995) 564–571.
[30] N. Hrbotitzky, G. Draude, G. Hapfelmeier, R. Lorenz, P.C. Weber, Lovastatin decreases the receptor-mediated degradation of acetylated and oxidized low density lipoproteins in human blood monocytes during the early stage of differentiation into macrophages, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 1267–1275.
[31] S.K. Basu, J.L. Goldstein, R.G. Anderson, M.S. Brown, Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts, Proc. Natl. Acad. Sci. USA 73 (1976) 3178–31821.
[32] J.C. Osborne, Delipidation of plasma lipoproteins, Methods Enzymol. 128 (1986) 213–222.
[33] M.A.K. Markwell, S.M. Haas, L.L. Bieber, M.E.A. Telbert, Modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, Anal. Biochem. 87 (1987) 206–210.
[34] G. Chinetti, S. Lestavel, V. Bocher, A.T. Remaley, B. Neve, I.P. Torra, E. Teissier, A. Minnich, M. Jaye, N. Duverger, H.B. Brewer, J.C. Fruchart, V. Clavey, B. Staels, PPAR-α and PPAR-γ activators induce cholesterol removal from human macrophage foam cells through stimulation of ABCA-1 pathway, Nat. Med. 7 (2001) 53–58.
[35] P. Ravn, G. Neugebauer, C. Christiansen, Association between pharmacokinetics of oral ibandronate and clinical response in bone mass and bone turnover in women with postmenopausal osteoporosis, Bone 30 (2002) 320–324.
[36] G. Neugebauer, C. Hagena, A. Sakalova, B. Kaufmann, R. Endele, Pharmacokinetics of ibandronate, a highly potent antiresorptive drug on bone metabolism in patients with multiple myeloma, Eur. J. Clin. Pharmacol. 54 (1998) 18.
[37] J. Mönkkönen, J. Similä, M.J. Rogers, Effects of tiludronate and ibandronate on the secretion of proinflammatory cytokines and nitric oxide from macrophages in vitro, Life Sci. 62 (1998) 95–102.
[38] O.M. Tuominen, M. Hollmen, O. Jaakkola, J. Mönkkönen, R. Ylitalo, Suppression of viability and acetyl-LDL metabolism in RAW 264 macrophage-like and smoth muscle cells by bisphosphonates in vitro, Methods Find. Exp. Clin. Pharmacol. 24 (2002) 487–492.
[39] H.D. Danenberg, I. Fishbein, J. Gao, J. Mönkkönen, R. Reich, I. Gati, E. Moerman, G. Golomb, Macrophage depletion by clodronate-containing liposomes reduces neointimal formation after balloon injury in rats and rabbits, Circulation 106 (2002) 599–605.
[40] R. Ylitalo, H. Syvälä, P. Tuohimaa, P. Ylitalo, Suppression of immunoreactive macrophages in atheromatous lesions of rabbits by clodronate, Pharmacol. Toxicol. 90 (2001) 139–143.
[41] J.L. Goldstein, M.S. Brown, Regulation of the mevalonate pathway, Nature 343 (1990) 425–430.
[42] B.H.Y. Huh, S.F. Pearce, L.M. Yesner, J.L. Schindler, R.L. Silverstein, Regulated expression of CD36 during monocyte-to-macrophage differentiation: potential role of CD36 in foam cell formation, Blood 87 (1996) 2020–2028.
[43] R. Ylitalo, J. Mönkkönen, S. Ylä-Herttuala, Effects of liposome-encapsulated bisphosphonates on acetylated LDL metabolism, lipid accumulation and viability of phagozyting cells, Life Sci. 62 (1998) 413–422.
[44] D. Steinberg, Low density lipoprotein oxidation and its pathobiological significance, J. Biol. Chem. 272 (1997) 20963–20966.

[45] J.L. Goldstein, S.K. Basu, M.S. Brown, Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition, Proc. Natl. Acad. Sci. USA 76 (1979) 333–337.
[46] R. Scheithe, N. Hrboticky, H.W.L. Ziegler-Heitbrock, P.C. Weber, Receptor-specific uptake and degradation of acetylated, oxidized and native LDL in the human monocytic mono mac 6sr cell line, Biochem. Biophys. Res. Commun. 202 (1994) 334–339.
[47] V. Terpstra, E.S. van Amersfoort, A.G. van Velzen, J. Kuiper, T.J.C. van Berkel, Hepatic and extrahepatic scavenger receptors, Arterioscleros. Thromb. Vasc. Biol. 20 (2000) 1860–1872.
[48] J.J. Repa, S.D. Turley, J.M.A. Lobaccaro, J. Medina, L. Li, K. Lustig, B. Shan, J.M. Dietschy, D.J. Mangelsdorf, Regulation of absorption and ABC-1-mediated efflux of cholesterol by RXR heterodimers, Science 289 (2000) 1524–1529.
[49] K. Schwartz, R.A. Lawn, D.P. Wade, ABC1 gene expression and ApoA-I-mediated cholesterol efflux are regulated by LXR, Biochem. Biophys. Res. Commun. 274 (2000) 794–802.
[50] P. Costet, Y. Luo, N. Wang, A.R. Tallm, Sterol-dependent transactivation of the ABC1 promoter by the liver x receptor/retinoid x receptor, J. Biol. Chem. 275 (2000) 28240–28245.
[51] B.M. Forman, B. Ruan, J. Chen, G.J. Schroepfer, R.M. Evans, The orphan nuclear receptor LXR is positively and negatively regulated by distinct products of mevalonate metabolism, Proc. Natl. Acad. Sci. USA 94 (1997) 10588–10593.
[52] X. Gan, R. Kaplan, J.G. Menke, K. MacNaul, Y. Chen, C.P. Sparrow, G. Zhou, S.D. Wright, T.Q. Cai, Dual mechanisms of ABCA-1 regulation by geranylgeranyl pyrophosphate, J. Biol. Chem. 276 (2001) 48702–48708.
[53] T. Langmann, J. Klucken, M. Reil, G. Liebisch, M.F. Luciani, G. Chimini, W.E. Kaminski, G. Schmitz, Molecular cloning of the human ATP-binding cassette transporter 1 (hABC1): evidence for sterol-dependent regulation in macrophages, Biochem. Biophys. Res. Commun. 257 (1999) 29–33.
[54] H. Koshiyama, Y. Nakamura, S. Tanaka, J. Minamikawa, Decrease in carotid intima-media thickness after 1-year therapy with etidronate for osteopenia associated with typ 2 diabetes, J. Clin. Endocrinol. Metab. 85 (2000) 2793–2796.
[55] M. Steinbuch, R.B. D'Agostino, J.S. Mandel, E. Gabrielson, M.R. McClung, A. Stemhagen, A. Hofman, Assessment of mortality in patients enrolled in a risedronate clinical trial program: a retrospective cohort study, Reg. Toxicol. Pharnacol. 35 (2002) 320–326.
[56] S. Adami, V. Braga, G. Guidi, D. Gatti, D. Gerardi, E. Fracassi, Chronic intravenous aminobisphosphonate therapy increases high-density lipoprotein cholesterol and decreases low-densitiy lipoprotein cholesterol, J. Bone Miner. Res. 15 (2000) 599–604.
[57] A. Montagnani, S. Gonnelli, C. Cepollaro, M.S. Campagna, M.B. Franci, S. Pacini, C. Gennai, Changes in serum HDL and LDL cholesterol in patients with Paget's bone disease treated with pamidronate, Bone 32 (2003) 15–19.
[58] R. Ylitalo, Bisphosphonates and atherosclerosis, Gen. Pharmacol. 35 (2002) 287–296.