Sodium nitroprusside-induced osteoblast apoptosis is mediated by long chain ceramide and is decreased by raloxifene

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Abstract

Release of high levels of nitric oxide (NO) is associated with osteoblastic cell death. The mechanisms of NO-induced cytotoxicity are not well documented and it is presently not known if estrogenic compounds prevent this effect. We studied the role of ceramides in cell death induced by the NO donor sodium nitroprusside (SNP) and we tested the possibility that 17β-estradiol, the anti-estrogen ICI 182.780 and two selective estrogen receptor modulators raloxifene and tamoxifen modify osteoblastic cell apoptosis. SNP dose-dependently decreased MC3T3-E1 osteoblast viability, increased NO production in the culture media and enhanced the release of intracellular ceramides C22 and C24. Cell death induced by SNP was partially inhibited when MC3T3-E1 cells were pretreated with raloxifene and tamoxifen but was not modified when the cells were pretreated with 17β-estradiol or ICI 182.780. Cell death induced by SNP resulted from apoptosis as demonstrated by Annexin-V and propidium iodide labeling and a reduction of SNP-induced MC3T3-E1 apoptosis was confirmed in the presence of raloxifene and tamoxifen. SNP induction of C22 and C24 production was inhibited by a pretreatment with raloxifene but not with 17β-estradiol. Moreover, the synthetic ceramide C24 (0.75 and 1 μM) decreased MC3T3-E1 cell viability and osteoblast cell death induced by C24 was partially decreased by raloxifene and to a lesser extent by 17β-estradiol. These data demonstrate that SNP-induced cell death is mediated by the long chain ceramides C22 and C24 and that raloxifene protected osteoblast from apoptosis induced by SNP, an effect that might be relevant to its pharmacological properties on bone remodeling.
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Nitric oxide (NO) is a free radical species that mediates numerous cellular effects including endothelium relaxation, neurotransmission or immune function [1]. NO is produced by bone cells and influences both osteoclastic and osteoblastic functions with a biphasic effect. Low levels of slowly released NO result in stimulation of osteoblast replication and function. On the contrary, higher amounts of rapidly released NO are associated with an inhibition of osteoblast proliferation and an induction of apoptosis [2,3]. Sex hormones, pro-inflammatory cytokines and mechanical constrain have been found to regulate NO production by bone cells [3–7]. Therefore, NO might be involved as a mediator of bone disease in postmenopausal osteoporosis or in inflammatory diseases.

Indeed, as demonstrated in vivo in mice with a targeted inactivation of the inducible NO synthase (iNOS) gene, activation of the iNOS pathway contributes to inflammation-induced osteoporosis though a decrease in bone formation caused by osteoblast apoptosis [8].

The proapoptotic effects of NO are mostly independent of cGMP but may involve the mitochondrial apoptotic pathway and activation of the caspases as well as the generation of ceramides depending on cell type [2]. Membrane-bound ceramides interact with proteins and are intracellular second messengers of apoptosis or stress signaling. The most common ceramides have acyl chain lengths of 16–24 carbon atoms (C16–C24). "Short ceramides" (C2 or C8 for example) are able to mimic the effect of proapoptotic agents such as tumor necrosis factor-α [9]. Currently however, there are little informations concerning the specific types of ceramides generated in

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NO-induced cell-death and their effect on osteoblast viability. The aim of our study was first to investigate the effects on osteoblastic cell viability of high doses of NO pharmacologically (i.e. non-enzymatically) generated by sodium nitroprusside and to determine and characterize the role of ceramides in this process. Secondly, we looked at the role of estrogens in osteoblast apoptosis as these hormones inhibit bone resorption and bone turnover but also decrease osteoblast apoptosis induced by etoposide [10]. We compared the effects of 17β-estradiol, the anti-estrogen ICI 182.780 and two selective estrogen receptor modulators (SERMs): raloxifene and tamoxifen which have estrogen agonistic properties on bone and antagonistic effects on breast [11]. Importantly, estrogens and raloxifene prevent the incidence of osteoporotic fractures in post-menopausal women [12–15]. We consequently examined whether 17β-estradiol and/or raloxifene modifies MC3T3-E1 osteoblastic cell viability in the presence of SNP. We also compared their effects on intracellular ceramide levels in response to SNP and on MC3T3-E1 osteoblastic cell death in the presence of the exogenous ceramide C24.

1. Materials and methods

1.1. Reagents

Raloxifene and ICI 182.780 were obtained from Eli Lilly (Indianapolis, IN, USA), tamoxifen and 17β-estradiol were purchased from Sigma–Aldrich (Schnelldorf, Germany). Raloxifene, tamoxifen, 17β-estradiol and ICI 182.780 were dissolved in ethanol at a concentration of 1 mM. Sodium nitroprusside (SNP) was obtained from ICN biomedicals (Asse, Belgium) and potassium hexoferrocyanate (HFC) was purchased from Sigma–Aldrich. SNP and HFC were dissolved in water at a concentration of 200 mM. C24-ceramide was synthetized by Biomol research laboratories (Plymouth, PA, USA). C24-ceramide was dissolved in ethanol/dodecan (98/2, v/v) at a concentration of 0.1 mM. Interleukin-1β (IL-1β) was obtained from R&D systems (Minneapolis, MN, USA). IL-1β was dissolved in PBS containing 1% fetal calf serum (FCS, BioWhittaker) at a concentration of 10 μg/ml.

1.2. Osteoblast cell culture

MC3T3-E1 is a non-transformed mouse osteoblastic cell line (DSMZ, ACC 210, Brawnschweig, Germany). Cells were cultured in α-minimal essential medium (α-MEM BioWhittaker, Taufkirchen, Germany) supplemented with 10% FCS, penicillin (100 IU/ml) and streptomycin (100 μg/ml) in a humidified incubator at 37 °C with 5% CO_2. Only MC3T3-E1 cells that had undergone less than 15 passages were used. For all experiments,

MC3T3-E1 cells were plated in phenol-red free α-MEM (BioWhittaker) supplemented with 1% charcoal-treated FCS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). MC3T3-E1 cells were seeded at the following densities: 10^4 cells/well in flat bottomed 96-well plates (Falcon, VWR scientific products, Leuven, Belgium) for determination of cellular viability, 2.8×10^5 cells/well in 6-well plates for trypan blue exclusion, for IL-6 measurement and for transfection experiments; 4×10^6 cells in 143 cm^2 dishes for determination of intracellular ceramide levels; and 2×10^5 cells/chamber in eight chamber slides for cytochemical staining. Cells were treated with either SNP (1–4 mM), HFC (2–3 mM) or C24-ceramide (0.5–1 μM) as indicated in the text and legends. An equal amount of solvent was added to control cultures. For establishing effect of estrogen and SERMs, cells were pretreated with either raloxifene, 17β-estradiol, tamoxifen or ICI 182.780, all at concentrations from 10^{-10} to 10^{-6} M, for 48 h before treatment with control media, SNP, C24-ceramide as indicated in the text and legends. An equal amount of solvent was added to control cultures. For raloxifene (10^{-6} M) and 17β-estradiol (10^{-6} M) effects on IL-6 protein levels and promoter activity, cells were pretreated with raloxifene or 17β-estradiol for 48 h before treatment with IL-1β (10 ng/ml) for 1–24 h as indicated in the text and legends.

1.3. MTS reduction assay

Cell viability was assessed by reduction of the yellow tetrazolium salt 3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to the blue formazan product by mitochondrial enzymes in viable cells (colorimetric proliferation assay: "Cell Titer 96 Aqueous®" Promega, Leiden, The Netherlands). At the end of the treatment period, 20 μl of MTS/PMS solution was added to each well. The reactions were incubated for 2 h at 37 °C in a humidified atmosphere of 5% CO_2. Absorbance was then measured at 492 nm with a Titertek Multiskan® Plus MK II (Flow laboratories, Brussels, Belgium) and the data were expressed as a percentage of levels of untreated control cells.

1.4. Trypan blue exclusion

After treatment, adherent cells were detached by treatment with trypsin (BioWhittaker), according to standard procedures. Floating cells were collected by centrifugation of the medium. Adherent and floating cells were pooled and resuspended in FCS-containing medium, mixed with four equal volumes of trypan blue solution and then counted and scored for dye exclusion. Results are expressed as percent viability, which was calculated as $100 \times$ (number of viable cells/number of total cells per well).

1.5. Nitrite analysis in conditioned medium

For quantitative analysis of NO_2^- in conditioned medium (CM), triplicate aliquots (100 μl) of CM or nitrite standards (NaNO$_2$) were mixed with 100 μl of Griess reagent (2% sulfanilamide in 5% phosphoric acid and 0.2% N-(1-naphtyl)ethylene-diamine) in 96-well plates. Absorbance was measured at 560 nm after 10 min with a Titertek Multiskan® Plus MK II (Flow Laboratories).

1.6. Measurement of intracellular ceramide levels

Liquid chromatography-electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) was used to measure intracellular ceramide levels as described previously [16]. Standard solutions for optimization of LC–ESI-MS/MS were prepared by dissolving ceramides in a 99.8/0.2 (v/v) mixture of ethanol/formic acid to reach a concentration of 1 μg/ml. For extraction of cellular lipids, cells were rinsed twice with ice-cold PBS, then scraped in PBS and centrifuged at $800 \times g$. The resulting pellet was homogenized in distilled water by sonication. An aliquot of the cell homogenate was reserved for determination of protein levels. Ten nanograms of C12-ceramide (as an internal standard) was added to samples of cell lysates containing 300 μg of protein. Lipids were extracted using Folch's partition with a mixture of chloroform and methanol (2/1, v/v). Samples were then centrifuged at $1500 \times g$ and washed with chloroform, methanol and water (5/48/47, v/v/v). The organic phase was evaporated to near dryness under a gentle stream of dry nitrogen. The samples were reconstituted by vortexing with 100 μl of a mixture of ethanol/formic acid (99.8/0.2) until they were completely dissolved. To avoid any loss of lipids, the entire procedure was performed in siliconized glassware. The LC–ESI-MS/MS procedure we use detects endogenous ceramides C14, C16, C18, C20, C22 and C24 [16]. C22- and C24-ceramides levels illustrated are representative of at least three distinct experiments.

1.7. Estrogen receptor mRNA levels

Estrogen receptor (ER) α and β mRNA levels were determined using reverse-transcriptase-polymerase chain reaction amplification (RT-PCR) [17]. Total RNA was extracted from MC3T3-E1 cells using RNeasy columns from Qiagen (Valencia, CA, USA), according to the manufacturer's instructions. Recovered RNA was quantified by spectrometry (Gene Quant, Pharmacia, Peapack, NJ, USA), and 1 μg of RNA was subjected to reverse transcription using the First strand cDNA synthesis kit for RT-PCR (Roche, Mannheim, Germany). The newly transcribed cDNA was amplified by PCR as previously described [17] in the presence of the following primers: ERα (accession no. M38651) sense, 5′-AATTCTGA-CAATCGACGCCAG-3′; antisense, 5′-GTGCTTCAA-CATTCTCCCTCCTC-3′; ERβ (accession no. U81451)

sense, 5′-AAAGCCAAGAGAACCAGTGGGCAC-3′; anti sense, 5′-GCCAATCATGTGCACCAGTTCCTT-3′. The expression of each transcript was normalized to β-actin (accession no. X03672). For β-actin, 32 cycles of PCR (94 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s) were performed in the presence of the sense 5′-CATGTG-CAAGGCCGGCTTCG-3′ primer and the antisense 5′-GGCCTCGGTGAGCAGCACAG-3′ primer. For negative control the RT step was omitted before the PCR. Products were separated by 3% agarose gel electrophoresis and visualized by ethidium bromide staining.

1.8. Estrogen receptor protein levels

Estrogen receptor protein levels were estimated by Western immunoblot as previously described [18]. Briefly, 10 μg of proteins were fractionated by electrophoresis on a 10% polyacrylamide gel. Proteins were transferred onto an Immobilon™ P transfer membrane (Millipore, Bedford, MA, USA). Membranes were blocked with Tris-buffered saline-Tween (20 mM Tris pH 7.5, 500 mM NaCl, 0.2% Tween) plus 5% dry milk before incubation for 3 h with an anti-ERα or an anti-ERβ antibody (sc-7207 and sc-8974 both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:1000.

Horseradish peroxidase-linked anti-rabbit antibodies (DakoCytomation, Glostrup, Denmark) were used as secondary antibodies for analysis of ERα and ERβ. The reaction was revealed with the enhanced chemiluminescence detection reagent according to the instructions of the manufacturer (ECL kit, Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK). Western blots illustrated are representative of at least two distinct experiments.

1.9. Determination of apoptosis by cytochemical staining

The presence of apoptotic cells was determined by fluorescent microscopy [19]. After treatment, cells were washed with PBS and 100 μl of HEPES buffer per slide (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl$_2$) with propidium iodide (0.5 μl per 100 μl of buffer) and Annexin-V (Annexin-V FLUOS Staining kit, Roche, Manheim, Germany, 0.5 μl per 100 μl of buffer). Cells were incubated for 15 min at room temperature and immediately visualized under fluorescent microscopy. Annexin-V (green) is a marker of apoptosis and propidium iodide (red) reflects the integrity of the cell membrane, thereby serving as a marker of necrosis [19].

1.10. IL-6 protein measurement

Conditioned media were collected from MC3T3-E1 cells treated with IL-1β. Mouse IL-6 protein levels were measured by ELISA according to the manufacturer's instructions (R&D systems, Abington, UK). The IL-6 assay has a lower limit of detection of 3,1 pg/ml. All

values were corrected for cell number. The data were expressed as a percentage of the control cultures.

1.11. Transient transfections

MC3T3-E1 cells were transfected with 1 µg of chimeric construct of the five flanking region of the rat IL-6 promoter (−2904 to +30) and the luciferase reporter gene (kindly provided by Dr. G. Fey, Friedrich-Alexander Universität, Erlangen, Germany) using Fugene reagent (Roche) with a 1:4 DNA/Fugene ratio, according to the manufacturer's instructions [20]. Cotransfection with a construct containing RSV promoter-driven β-galactosidase gene (kindly provided by Dr. R. Winkler, University of Liege, Liege, Belgium) was used to control transfection efficiency. After a 4 h recovery period, cells were transferred in phenol-red free α-MEM supplemented with 10% charcoal-treated FCS for 24 h before treatment with various agents as described in the legends. The cells were washed with phosphate buffered saline and harvested in lysis buffer (β-GAL reporter gene assay chemiluminescent, Roche). Luciferase activity was measured using a luciferase reporter gene assay kit (Roche) and β-galactosidase activity using the β-GAL reporter gene assay chemiluminescent (Roche) both in accordance with manufacturer's instructions. All data were expressed as a percentage of levels of luciferase units/β-galactosidase units normalized to control cells.

1.12. Statistical analysis

Data are presented as mean values ± S.E.M. Data were analyzed by the Mann–Whitney test.

2. Results

2.1. Cytotoxicity of SNP and HFC

Increasing concentrations of SNP, a NO-donor, dose-dependently decreased MC3T3-E1 cell viability after 24 h as determined by the MTS reduction assay (Fig. 1A) and increased NO release in the culture media of osteoblastic cells in a time-dependent manner (Fig. 1B). Unlike other NO donors, SNP contains iron cyanide in addition to nitric oxide [21]. To determine whether SNP-induced MC3T3-E1 cell death is NO-dependent, we studied the effect of potassium hexoferrocyanide (HFC), a molecule that has a similar structure as SNP but does not induce NO production [21]. HFC only slightly decreased cell viability. At a concentration of 3 mM, HFC decreased MC3T3-E1 cell viability by 10%, whereas 3 mM SNP induced a 71% decrease in MC3T3-E1 cell viability after 24 h (Fig. 1C), suggesting that NO release is required for SNP-induced cell death. This is consistent with previous reports demonstrating that high NO levels induce osteoblastic cell death [6,7].
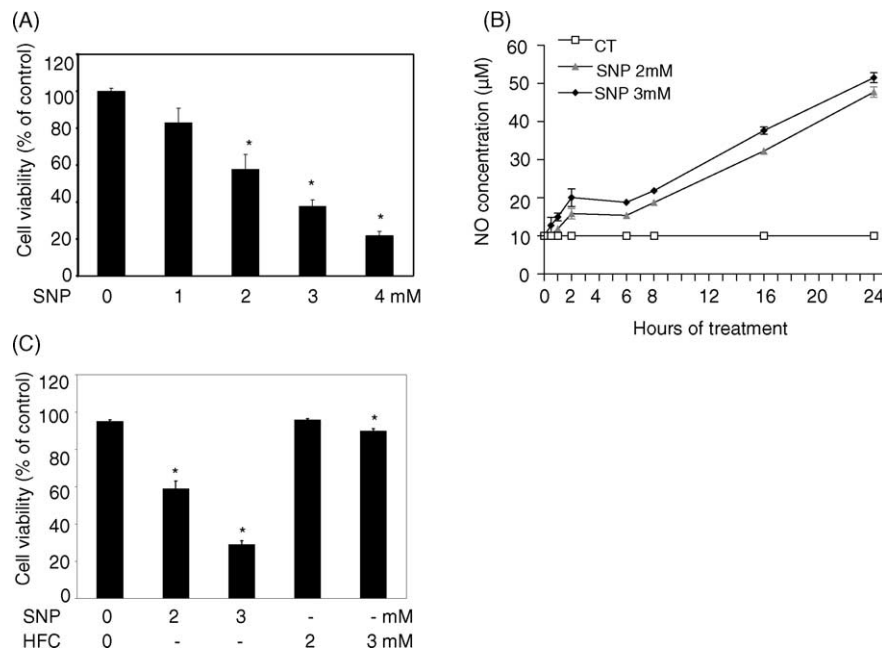

Fig. 1. Sodium nitroprusside (SNP) vs. potassium hexoferrocyanate (HFC) cytotoxicity. (A) MC3T3-E1 cells were treated with SNP for 24 h at the indicated concentrations. Cell viability was measured with the MTS reduction assay. Data are expressed as average percentage of control levels ± S.E.M. in 11 samples pooled from three independent experiments. $^*p < 0.01$ significantly different from untreated control. (B) Kinetics of NO_2^- accumulation in conditioned media of MC3T3-E1 cells. NO_2^- levels were measured by the Griess assay after MC3T3-E1 cells were treated with control media or SNP for various times from 0 to 24 h, as indicated. Data are expressed as the average NO concentration (µM) ± S.E.M. of 12 samples pooled from three independent experiments. (C) MC3T3-E1 cells were treated with SNP or HFC at 2 or 3 mM for 24 h. Cell viability was measured by the trypan blue exclusion test. Data are expressed as average percentage of control levels ± S.E.M. of 12 samples pooled from two independent experiments. $^*p < 0.05$ significantly different from untreated control.
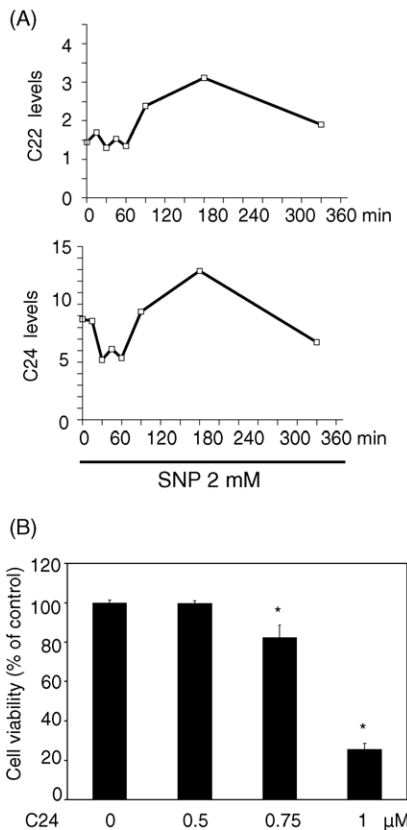
Fig. 2. SNP effect on intracellular ceramide levels and cytotoxicity of C24-ceramide. (A) Kinetics of the intracellular accumulation of C22- and C24-ceramide in MC3T3-E1 cells treated with SNP. MC3T3-E1 cells were treated with 2 mM SNP for 0 to 360 min, as indicated. Ceramide levels were then measured by LC–ESI-MS/MS. Values corresponding to the intracellular ceramide peak area were divided by the internal standard peak area. One representative experiment is shown. (B) MC3T3-E1 cells were treated with C24-ceramide for 24 h at the indicated concentrations. Cell viability was measured with the MTS reduction assay. Data are expressed as average percentage of control ± S.E.M. of 8 samples pooled from two independent experiments. $^*p < 0.05$ significantly different from untreated control.

2.2. Intracellular ceramide levels after SNP treatment

Ceramides are second messengers of apoptosis [22,23]. To determine the role of ceramides in cell death induced by NO donors, we measured the intracellular levels of ceramides C14–C24 by liquid chromatography-coupled electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). Fig. 2A shows that C22- and C24-ceramide levels increased significantly 3 h after SNP treatment. Intracellular C24 levels were higher than C22 levels at baseline, but the concentrations of both C22 and C24 were increased by up to 170% of control levels after SNP treatment (Fig. 2A). C14, C16, C18 and C20 ceramide levels were unaffected by the presence of SNP (data not shown). To further establish if C24-ceramide induces osteoblastic cell death directly, MC3T3-E1 cells were treated with synthetic C24-ceramide (Fig. 2B). Osteoblastic cell viability was decreased by 18% and 74% in the presence of 0.75 and 1 μM C24, respectively (Fig. 2B),
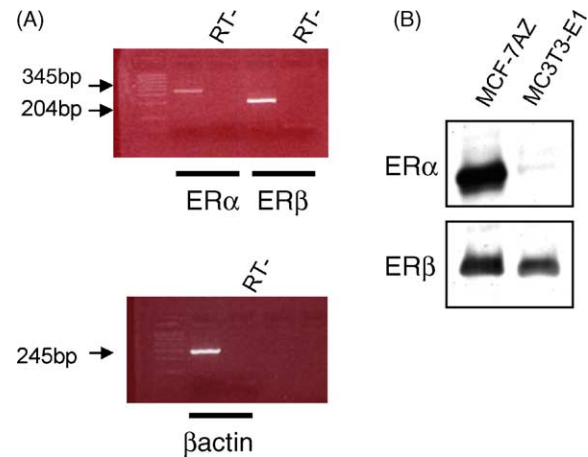

Fig. 3. Levels of ERα and ERβ in osteoblastic cells. (A) Estrogen receptors transcripts by RT-PCR in MC3T3-E1 osteoblastic cells. The 345 bp fragment of ERα and 204 bp fragment of ERβ are illustrated in the upper panel and the 245 bp fragment of β-actin from the same RT sample is shown in the lower panel. No message was identified when the RT step was omitted (RT−). (B) Estrogen receptors protein levels by Western blotting. Total cellular extracts from MC3T3-E1 and human breast cancer MCF7-AZ (positive control) cells were analyzed by Western immunoblot analysis. ERα protein was detected by an anti-ERα antibody and is illustrated in the upper panel. ERβ protein was detected by an anti-ERβ antibody and is illustrated in the lower panel.

indicating that C24 is possibly one of the mediators of SNP induced cell death.

2.3. Estrogen receptor (ERα and ERβ) mRNA and protein levels

To study the effects of estrogenic compounds on NO-induced MC3T3-E1 cell death, we first investigated whether these cells express ER. For this purpose, we evaluated by RT-PCR and Western blotting cellular levels of the two ER isoforms in MC3T3-E1 cells. Confirming previous results, MC3T3-E1 cells expressed ERα and ERβ transcripts (Fig. 3A). However at the protein level, while MC3T3-E1 osteoblastic cells expressed ERβ, the level of ERα was barely detectable in accordance to previous observations of a wide variation of ER protein expression in these cells (Fig. 3B) [17].

2.4. Effects of raloxifene, 17β-estradiol, tamoxifen and ICI 182.780 on SNP-induced cytotoxicity

Previous studies demonstrated a potent anti-apoptotic effect of estrogens on osteoblastic cells [10]. We thus compared the effect of 17β-estradiol with two SERMs, raloxifene and tamoxifen, and a pure estrogen antagonist, ICI 182.780, on osteoblastic cell death induced by SNP. Pretreatment of MC3T3-E1 cells with raloxifene at doses of 10^{-10}, 10^{-7} and 10^{-6} M for 48 h prior to the addition of SNP decreased osteoblast cell death, whereas 17β-estradiol or ICI 182.780 did not have any significant effect (Fig. 4A). The SERM tamoxifen had similar protective
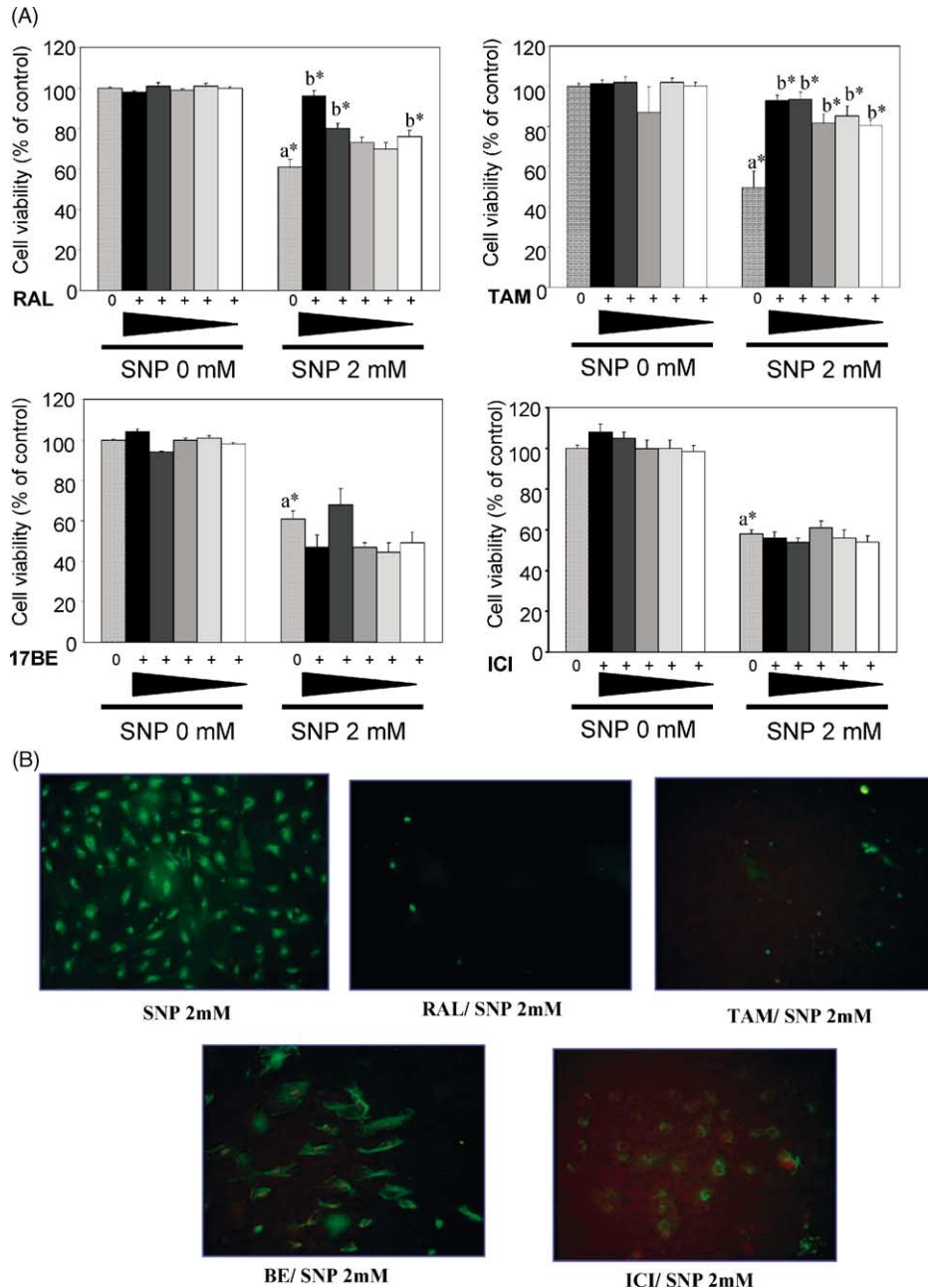
Fig. 4. Effects of raloxifene, 17β-estradiol, tamoxifen and ICI 182.780 on SNP-induced cell death. (A) Cell viability was measured with the MTS reduction assay. MC3T3-E1 cells were pretreated with control media (0) or raloxifene (RAL), 17β-estradiol (17BE), tamoxifen (TAM) or ICI 182.780 (ICI) at decreasing concentrations (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} or 10^{-10} M) for 48 h and treated in the presence or absence of 2 mM SNP for an additional 24 h. Data are expressed as average percentage of control levels ± S.E.M. of 8 samples pooled from two independent experiments. (a^*) $p < 0.01$ significantly different from untreated control and (b^*) $p < 0.05$ significantly different from SNP-treated cultures. (B) Fluorescence staining of MC3T3-E1 in the presence of SNP. Effect of raloxifene, 17β-estradiol, tamoxifen or ICI 182.780 on MC3T3-E1 apoptosis. MC3T3-E1 cells were pretreated with control media (CT) or raloxifene (RAL), 17β-estradiol (17BE), tamoxifen (TAM) or ICI 182.780 (ICI) at 10^{-6} M for 48 h prior to treatment with 2 mM SNP for an additional 16 h. After treatment, cells were labeled with Annexin-V coupled to fluorescein and propidium iodide. Cells were then examined fluorescence microscopy. The photomicrographs show apoptotic cells labeled by Annexin-V.

effects as raloxifene on the survival of MC3T3-E1 mouse osteoblastic cells at concentrations from 10^{-10} to 10^{-6} M (Fig. 4A). These results suggest that the protective effects of raloxifene and tamoxifen on osteoblast cell death are representative of this class of medication (SERMs).

To confirm that SNP induced osteoblast cell death by apoptosis, MC3T3-E1 cells were analyzed by fluorescence

staining with Annexin-V and propidium iodide (A/PI) (Fig. 4B) [19]. In the presence of SNP, MC3T3-E1 cells were labeled primarily by Annexin-V, indicating that SNP-induced cell death occurred by apoptosis. Pretreatment with raloxifene or tamoxifen but not by 17β-estradiol or ICI 182.780 induced a decrease in Annexin-V labeling after treatment with SNP (Fig. 4B), which is consistent
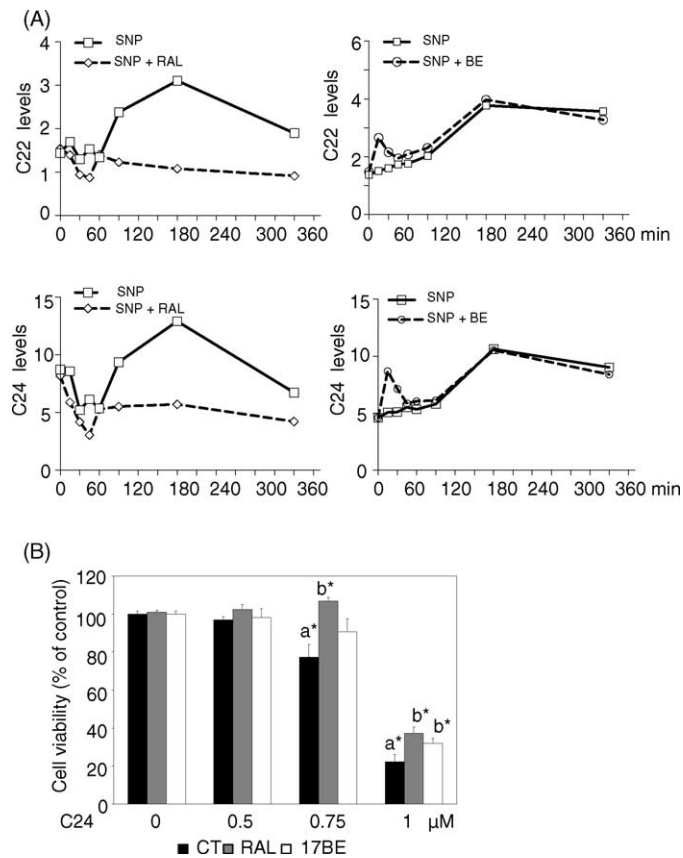
Fig. 5. Raloxifene effect on SNP-induced ceramide levels and on C24-ceramide-induced cell death. (A) Kinetics of the intracellular accumulation of C22- and C24-ceramide in MC3T3-E1 cells pretreated with raloxifene or 17β-estradiol. MC3T3-E1 cells were pretreated with control media, raloxifene or 17β-estradiol at 10^{-6} M for 48 h prior to treatment with 2 mM SNP for 0 to 360 min, as indicated. Ceramide levels were then measured by LC–ESI-MS/MS. Values corresponding to the intracellular ceramide peak area were divided by the internal standard peak area. One representative experiment is shown. (B) MC3T3-E1 cells were pretreated with control media, raloxifene or 17β-estradiol at 10^{-6} M for 48 h prior to treatment with C24-ceramide for 24 h at the indicated concentrations. Cell viability was measured with the MTS reduction assay. Data are expressed as average percentage of control ± S.E.M. of 8 samples pooled from two independent experiments. (a[*]) $p < 0.01$ significantly different from untreated control and (b[*]) $p < 0.05$ significantly different from C24-ceramide-treated cultures.

with the MTS data (Fig. 4A). We confirmed that cell cultures were at similar confluences (data not shown).

2.5. Comparative effects of raloxifene and 17β-estradiol on SNP-induced ceramide levels and C24-ceramide-induced cell death

The induction of C22- and C24-ceramide levels by SNP was decreased when the cells were pretreated with 10^{-6} M raloxifene 48 h prior to the addition of SNP at 2 mM (Fig. 5A), but treatment with 17β-estradiol at the same concentration had no effect on C22- and C24-ceramide levels (Fig. 5A). As exogenous C24-ceramide could induce osteoblastic cytotoxicity (Fig. 2B), we also tested if raloxifene protected MC3T3-E1 cells from C24-ceramide-induced cell death. Fig. 5B shows that cell viability was maintained at control levels when osteoblasts were pretreated with raloxifene at 10^{-6} M prior to treatment with 0.75 μM C24-ceramide. In the presence of C24-ceramide at 1 μM, a marked decrease in cell viability was observed but raloxifene and 17β-estra-

diol only weakly prevented cell death (Fig. 5B). All together these data suggest that the long chain ceramide C24 is a mediator of SNP-induced osteoblastic cell death and that raloxifene exerts its protective effect by decreasing ceramide production. However, a partial protection against ceramide-induced cytotoxicity could not be excluded.

2.6. Comparative effects of raloxifene and 17β-estradiol on IL-6 protein synthesis and promoter activity

As 17β-estradiol had no significant effect on SNP-induced MC3T3-E1 cell death, we next verified a relevant 17β-estradiol effect in these cells. For this purpose, we studied the effect of 17β-estradiol and raloxifene on IL-6, a cytokine known to be inhibited by these two anti-resorptive agents [24,25]. IL-6 levels were undetectable in control cultures (data not shown) but were markedly enhanced in the presence of IL-1β at 10 ng/ml for 24 h. Pretreatment of MC3T3-E1 cells with raloxifene or 17β-estradiol at 10^{-6} M for 24 h prior to the addition of IL-1β inhibited
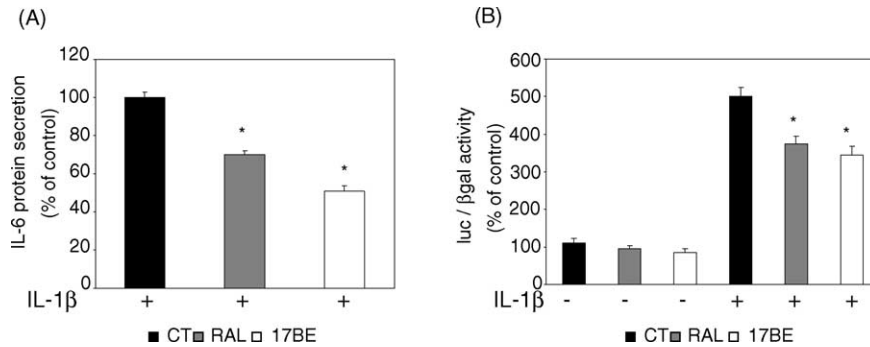
Fig. 6. Raloxifene and 17β-estradiol effects on IL-6 protein levels and promoter activity. (A) IL-6 protein levels were measured by ELISA from conditioned medium harvested from MC3T3-E1 cells pretreated with control media, raloxifene or 17β-estradiol at 10^{-6} M for 24 h prior to treatment with IL-1β at 10 ng/ml for 24 h. Data are expressed as percentage of IL-1β treated cells (control) ± S.E.M. of 12 samples pooled from four independent experiments. $^{*}p < 0.01$ significantly different from control. (B) MC3T3-E1 cells were transfected with a chimeric constructs containing the rat IL-6 promoter sequence linked to a luciferase reporter gene. To control for transfection efficiency, a RSV-β-galactosidase vector was co-transfected. Twenty-four hours post-transfection, MC3T3-E1 cells were pretreated with control media, raloxifene or 17β-estradiol at 10^{-6} M for 24 h prior to treatment with IL-1β at 10 ng/ml for 1 h. Data are expressed as percentage of control ± S.E.M. of 9 samples pooled from three independent experiments. $^{*}p < 0.01$ significantly different from IL-1β treated cultures.

IL-6 protein by 30% and 49%, respectively (Fig. 6A). We also verified that 17β-estradiol had genomic effect on IL-6 reporter gene [10] by transiently transfecting MC3T3-E1 with a chimeric constructs of the rat IL-6 promoter and the luciferase gene. Fig. 6B shows that IL-6 promoter activity was increased by IL-1β and that this effect was inhibited by pretreatment of osteoblastic cells with 17β-estradiol and raloxifene at 10^{-6} M.

3. Discussion

Nitric oxide mediates numerous biological process. NO induces a cytotoxic activity against tumor cells. In bone, stimulation of NO production in inflammatory diseases was also associated with bone loss and osteoblast apoptosis. This report confirmed that high NO levels induced apoptosis of osteoblastic cells. To study this effect, we treated MC3T3-E1 with an exogenous NO-releasing compound, SNP, and demonstrated that NO release was required for osteoblast cell death. We further determined the mechanisms by which SNP induces apoptosis in osteoblasts by evaluating intracellular ceramide levels. Ceramides are intracellular second messengers involved in stress and cell death [26–29]. Several groups have previously demonstrated that NO up-regulates intracellular ceramide levels in various cell types and suggested that this increase was involved in apoptosis [30,31]. However to date, there were little informations about the function of different ceramide species. A few reports suggested that very long chain ceramides were generated during growth arrest and apoptosis but there were no studies on the differential regulation of ceramide species by NO [32–34]. Our data show that the intracellular levels of the ceramides C22 and C24 increased after treatment of MC3T3-E1 osteoblastic cells with SNP. Moreover, we confirmed that C24-ceramide and C20-ceramide (data not shown) decreased MC3T3-E1 cell viability, demon-

strating that the long chain ceramides are able to induce osteoblastic cell death.

Previous reports revealed the role of NO in bone remodeling and demonstrated its involvement in the pathophysiology of osteoporosis [8]. Consequently, we tested whether estrogenic compounds could influence NO-induced apoptosis in osteoblasts. Estrogens decrease bone resorption but also influence osteoblast apoptosis [10]. Moreover, a decrease in circulating levels of estrogens remains the major cause of increased bone turnover in postmenopausal women and for many years, estrogen replacement therapy was considered to be the optimal prevention treatment for osteoporosis [35]. SERMs exhibit a combination of estrogen agonistic and antagonistic properties and might therefore provide a similar bone effect together with a better safety on the breast and uterus tissues. In the multiple outcomes of raloxifene evaluation (MORE) study, raloxifene was shown to significantly decrease the risk of new vertebral fractures after 3 and 4 years of treatment [6,7] and raloxifene is now used for the treatment of post-menopausal osteoporosis. In parallel to its antiresorptive effect, raloxifene increases the proliferation of osteoblasts and influence various osteoblastic functions but it is not presently known whether raloxifene affects osteoblast apoptosis [24,25,36,37]. In this study, we compared the effects of 17β-estradiol with the SERMs raloxifene and tamoxifen and the pure antagonist of estrogen ICI 182.780 on osteoblast apoptosis induced by SNP. Raloxifene inhibited osteoblast apoptosis at doses ranging from 10^{-10} to 10^{-6} M, suggesting that the anti-apoptotic effect of raloxifene might be pharmacologically relevant and related to its clinical activity. The SERM tamoxifen had similar effects as raloxifene on the survival of MC3T3-E1 mouse osteoblastic cells, suggesting that the protective effect observed is representative of this class of medication. Interestingly in our model, 17β-estradiol did not protect osteoblasts from cell death whereas both 17β-estradiol and raloxifene had similar effects on the inhibi-

tion of IL-6 protein levels and IL-6 promoter activity induced by IL-1β. IL-6 inhibition by both 17β-estradiol and raloxifene confirms that both compounds have similar ER-mediated effect in MC3T3-E1 cells in accordance to previous work [10,38]. We showed that MC3T3-E1 cells express both transcript and protein for ERβ and to lesser extent for ERα. A great variation in the cellular levels of steroid receptor seems to be a characteristic of these osteoblast-like cells [17]. Nevertheless, despite heterogeneity of ER expression in MC3T3-E1 cells and obvious difference in receptor density between single cells, we confirmed that 17β-estradiol is capable of exerting ER-mediated genomic effect in these cells as demonstrated by an inhibition of IL-6 promoter activity.

In parallel to the decrease in SNP-induced cell death, raloxifene decreased the induction of C22- and C24-ceramide levels in response to SNP. In contrast, 17β-estradiol did not modify the effects of SNP on intracellular ceramide levels. This suggests that the protective effect of raloxifene on NO-induced apoptosis in MC3T3-E1 cells could be mediated by downregulation of the ceramide production. Ceramides might be generated by an enzymatic or a non-enzymatic hydrolysis of sphingomyeline as well as by de novo synthesis by ceramide synthase. Interestingly, these pathways are linked to apoptosis as demonstrated by the defect in radiation-induced apoptosis in acid sphingomyelinase-deficient human lymphoblasts [39]. Previous studies have shown that high levels of NO lead to an increase in ceramide by a dual action of NO: on one hand, an activation of ceramide-generating sphingomyelinases and on the other hand, a degradation of the ceramidases [40]. Raloxifene might decrease SNP-induced ceramide levels by directly inhibiting sphingomyelinase activities or other enzymes involved in ceramide generation or by blocking ceramide degradation (Fig. 7). In parallel, raloxifene could exert a protective effect on exogenous ceramide-induced cell death. While 17β-estradiol had no cytoprotective effect in the presence of SNP, both 17β-estradiol and raloxifene decreased slightly C24- and C20-ceramide (data not shown) cytotoxic effect. These results suggest that while raloxifene and 17β-estradiol might moderately influence the signaling pathway activated downstream of the ceramide, the discrepancy between these two drugs on SNP-induced apoptosis should result from distinct effects on ceramide generation (i.e. ceramide synthesis or degradation) (Fig. 7).

Based on our current results, it is difficult to determine if raloxifene influences ceramide generation by a genomic or a non-genomic effect. It is tempting to postulate that the effect of raloxifene on ceramide release occurs at the membrane as membrane-bound ERs have been described in osteoblasts and as ceramide formation is taking place in the plasma membrane [28,41]. However, non-transcriptional effects are classically rapid and non-compatible with RNA and protein synthesis [41]. In our study, raloxifene pretreatment for 48 h was necessary to observe a protective
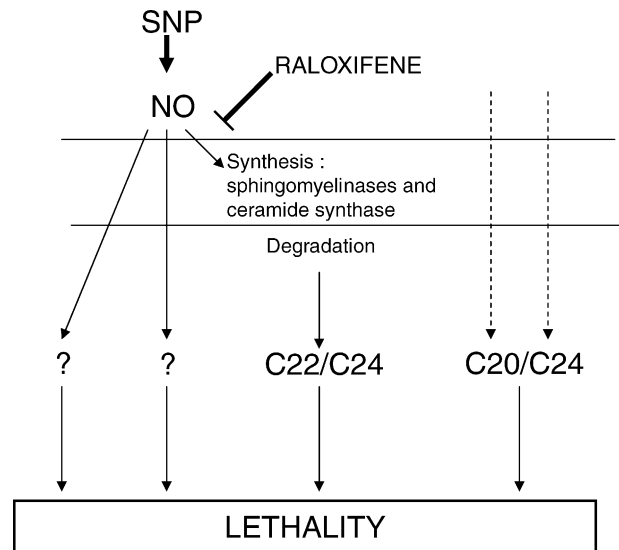

Fig. 7. Raloxifene and 17β-estradiol effects on osteoblastic cell death induced by NO and ceramides. One of the NO apoptotic pathways is mediated by ceramide generation. Raloxifene decreases the levels of intracellular C22- and C24-ceramides most likely by inhibiting ceramide synthesis or by influencing their degradation.

effect on SNP-induced cell death, indicating that de novo protein synthesis might be required and that non-genomic effects are unlikely. On the other hand, one might not exclude a non ER-mediated effect on ceramide generation as it is known that 17β-estradiol and tamoxifen might influence perturbations of membrane fluidity of human breast cancer cells [42]. These types of effects are usually observed with high doses of steroids. Concerning genomic effects, differences between estrogens and raloxifene might be explained by the involvement of distinct nuclear mechanisms such as gene promoter elements and coregulatory proteins, or simply by the nature of the gene specifically regulated by estrogen or raloxifene [43–47]. Indeed, recent data obtained by microarray technology have shown that bone-related genes regulated by 17β-estradiol or raloxifene were distinct [48]. Only 17–18% of the genes regulated by 17β-estradiol were also regulated by raloxifene or tamoxifen in U2OS stably transfected with ERα or ERβ. Moreover, genes regulated by 17β-estradiol or SERMs were different if cells were transfected with ERα or ERβ, underlying the importance of the type and the level of the ER expressed in the type of cells studied [48]. It is therefore possible that genes controlling ceramide synthesis or degradation are specifically regulated by SERMs (i.e. and not by estrogen) via ERβ and/or ERα in MC3T3-E1 cells.

In conclusion, raloxifene protects osteoblast from apoptosis induced by SNP through an inhibition of C22- and C24-ceramide generation. It is possible that the anti-apoptotic effect of raloxifene contributes to an improvement in bone quality predominantly under stressful conditions, as NO is released by bone cells in response to pro-inflammatory cytokines or mechanical strain.

Acknowledgments

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