Cyclic-AMP Inhibits Nitric Oxide-Induced Apoptosis in Human Osteoblast: The Regulation of Caspase-3, -6, -9 and the Release of Cytochrome c in Nitric Oxide-Induced Apoptosis by cAMP

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Nitric oxide (NO) induces apoptotic cell death and cAMP has a significantly protective effect on NO-induced cytotoxicity in human osteoblasts, MG-63 cells. Treatment with *S*-nitroso-*N*-acetylpenicillamine (SNAP) (0.6 mM) resulted in genomic DNA fragmentation, characteristic of apoptosis. However, concomitant incubation of the cells with either DBcAMP or forskolin markedly inhibited SNAP-induced apoptosis in a dose-dependent manner. Furthermore, pretreatment of MG-63 cells with H-89 or KT5720, which is known to inhibit cAMP-dependent protein kinase (PKA), abolished the protective effect of DBcAMP and forskolin on SNAP-induced apoptosis. In this study, we explored the involvement of caspases in the regulatory mechanism of SNAP-induced apoptosis by cAMP. Our data show that DBcAMP or forskolin blocked SNAP-induced caspase-3-like cysteine protease activation and that H-89, a PKA inhibitor, reversed the cAMP-induced regulatory effect of caspase-3 like protease. Consistent with the results, cAMP inhibited the proteolytic cleavage of caspase-3, -6, -9 and cytochrome c release to cytoplasm. The inhibition of caspase-3 activation did not block SNAP-induced cytochrome c release to cytoplasm, suggesting that caspase-3 activation may occur downstream of cytochrome c release. In summary, these findings show that the exposure of MG-63 cells to cAMP analogs renders them more resistant to NO-induced damage and suggests the presence of regulatory mechanisms of the cell death pathway by cAMP in which caspase-3, -6, and -9 and cytochrome c release serves to mediate NO-induced apoptosis.

Key words NO; cAMP; apoptosis; osteoblast; caspase; cytochrome c

Apoptosis is an active, energy-dependent mode of cell death. Apoptosis shows typical morphologic changes such as nucleoplasmic and cytoplasmic condensation and the translocation of phosphatidylserine to the cell surface and the formation of extensive membrane blebs.¹⁾ Although the events underlying the initiation of apoptosis are not well understood, the processes appear to be closely associated with the activation of one or more species of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonucleases. They introduce double-stranded breaks at internucleosomal regions of mature DNA, resulting in the appearance of “laddered” electrophoretic patterns of oligonucleosomal DNA fragments on conventional agarose gels.²⁾ Apoptosis thus appears to represent a specific and highly regulated program mediating self-destruction at the cellular level.

Nitric oxide (NO) is a labile free radical that is physiologically produced through the L-arginine/nitric oxide synthase (NOS) pathway.³⁾ Of the three known isoforms of the NOS enzyme, two are low-output, Ca^{2+} -dependent and constitutively expressed (nNOS/neuronal, eNOS/endothelial), while the third is inducible (iNOS), high-output, and Ca^{2+} -independent.⁴⁾ NO is a highly reactive molecule which interacts with multiple cellular target moieties including guanylyl cyclase, cyclooxygenase, ribonucleotide reductase, membrane phospholipids, and DNA.⁵⁾ Cytokine-induced NO synthesis causes sustained vasorelaxation in septic shock,^{6,7)} inhibits cell proliferation,⁸⁾ and mediates cytotoxicity.⁹⁾ In this study, the action of NO has been related to the induction of programmed cell death, and cAMP plays an inhibitory role in the processes of apoptosis in MG-63 cells. However, in-

creases in the intracellular cAMP level, with the use of cAMP analogs or agents which induce an endogenous cAMP increase, cause apoptotic cell death in the brain during prenatal development¹⁰⁾ and in thymocytes.¹¹⁾ In other cells, cAMP can maintain the survival of sympathetic neurons in the absence of nerve growth factor, suggesting the protective effect of cAMP on apoptosis.¹²⁾ An elevated intracellular cAMP concentration is also known to prevent various agent-induced apoptosis of T-cell hybridomas¹³⁾ and HL-60 cells.¹⁴⁾

Few insights are available regarding the signals that control NO-induced apoptosis. However, recent reports have demonstrated the role of interleukin-1 converting enzyme (ICE) or an ICE-like protease in apoptosis.^{15,16)} Indeed, the demonstration that overexpression of Ced-3 or ICE induces apoptosis has supported the involvement of the ICE/Ced-3 family of proteases in cell death signaling.¹⁷⁾ Members of the ICE/Ced-3 family include CPP32/YAMA/apopain,¹⁸⁾ Nedd2/Ich-1,¹⁹⁾ TX/Ich-2/ICE_{rel}II,²⁰⁾ ICE_{rel}III,²¹⁾ Mch2,²²⁾ Mch3/ICE-LAP3/CMH-1,²³⁾ Mch4,²³⁾ and Mch5.²⁴⁾ Among these, a recent report has indicated that NO-induced cell death involves the activation of CPP32-like protease in HL-60 cells.²⁵⁾ In addition, the finding that poly-(ADP-ribose) polymerase (PARP) is cleaved at the C-terminal to an aspartic acid residue (DEVD/G) has suggested that CPP32-like protease may be responsible for apoptosis.²⁶⁾

Currently, there is little available information regarding the existence of specific modulatory mechanisms related to NO-induced apoptosis in human osteoblasts, MG-63. Hence, in

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this study, we determined the effects of cAMP analogs or forskolin, an intracellular cAMP-elevating agent, on NO-induced apoptosis in MG-63 cells. Moreover, our findings demonstrate that signaling pathways involving caspases-3, -6, and -9 and cytochrome c release to cytoplasm are related to the cAMP-induced regulatory effect of NO-induced apoptosis in human osteoblasts, MG-63.

MATERIALS AND METHODS

Materials N^6, O^2 -dibutyl cAMP (DBcAMP), 8-bromo-cAMP (8-Br-cAMP), forskolin, dimethyl sulfoxide (DMSO), *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). H-89, KT5720, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (AC-DEVD-AMC) and *N*-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (AC-YVAD-AMC) were purchased from Calbiochem-Behring (La Jolla, CA, U.S.A.). Caspase-3, -6, -9, PARP, lamin A and cytochrome c antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK), DEVD-CHO and YVAD-CHO were purchased from Kamiya Bio Company (Seattle, WA, U.S.A.). A genomic DNA purification kit was obtained from Promega (Madison, WI, U.S.A.). Six-well tissue culture plates and 100 mm diameter culture dishes were purchased from Nunc (Naperville, IL, U.S.A.). D-MEM, PBS, and FBS, and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD, U.S.A.).

Cell Culture The MG-63, human osteoblast-like cell line (ATCC, Rockville, MD, U.S.A.) was maintained at 5×10^5 cells/ml in D-MEM supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/ml), streptomycin (100 μ g/ml), and L-glutamine (2 mM). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. Exponentially growing MG-63 cells were seeded at 1×10^6 /6-well plate, then the cells were exposed to the various agents indicated in the result section.

Determination of Cell Viability Cell viability was detected by MTT dye assay.²⁷⁾ After 4 h incubation at 37°C, the granules were lysed with dimethyl sulfoxide, and the absorbance of formazan granules was detected at 590 nm using a 96-well multiscanner autoreader. Cell viability was also detected by LDH release assay. After treatment with various agents, the maintenance medium was collected from each monolayer. Duplicate aliquots (40 μ l) of culture medium and cell lysates were placed in 96-well microtiter plates, and LDH activity was measured by monitoring the consumption of nicotinamide adenine dinucleotide (Sigma Chemical) at 340 nm using a kinetic spectrophotometric plate reader. Results are expressed as the percentage of the intracellular LDH released to the culture medium: % LDH release = [LDH activity in media/(LDH activity in cells+media)] $\times$ 100. In all experiments, the LDH activity in the culture medium was corrected for the LDH activity in FBS.

DNA Extraction and Electrophoresis The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described.²⁸⁾ Briefly, genomic DNA was purified by a Wizard Genomic DNA purification kit. After ethanol precipitation, samples of 10 μ g in each lane were subjected to elec-

trophoresis on 1.4% agarose gel at 50 V for 30 h. DNA was stained with ethidium bromide.

Detection of Phosphatidylserine with Annexin V-FITC

We used the annexin V-FITC cell membrane labeling assay to detect the translocation of phosphatidylserine from the inner face of the plasma membrane to the cell surface, where it binds an annexin V-FITC conjugate and serves as an early marker of apoptosis. Non-adherent cells, or cells grown on coverslips, were incubated with annexin V-FITC for 15 min at room temperature in the dark, and visualized by fluorescent microscopy with fluorescein filters, as described.²⁹⁾

Measurement of Nitrite Concentration Quantitation of NO synthesis was measured by a microplate assay method as described.³⁰⁾ To measure nitrite, 100 μ l aliquots were removed from a conditioned medium and incubated with an equal volume of the Griess reagent [1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H₃PO₄] at room temperature for 10 min. The absorbance at 540 nm was determined in a Titertek Multiscan (Flow Laboratories, North Ryde, Australia). NO₂⁻ was determined using sodium nitrite as standard. Cell-free medium alone contained 5 to 8 μ M of NO₂⁻; this value was determined in each experiment and subtracted from the value obtained with cells.

Fluorogenic Substrate Assay for Caspase Activity Cytosolic cell extracts were prepared by lysing the cells in a buffer containing 1% Nonidet P-40, 200 mM NaCl, 20 mM Tris/HCl, pH 7.4, 10 μ g/ml leupeptin, aprotinin (0.27 trypsin inhibitor/U/ml) and 100 μ M PMSF. Caspase-1- or caspase-3-like activity was determined by the incubation of cell lysate (containing 25 μ g of total protein) with 50 μ M of the fluorogenic substrate, AC-YVAD-AMC or AC-DEVD-AMC, respectively, in 200 μ l of a cell-free system buffer (comprising 10 mM Hepes, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH₂PO₄, 0.5 mM EGTA, 2 mM MgCl₂, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM dithiothreitol). The release of fluorescent 7-amino-4-methyl coumarin was measured for 1 h at 2 min intervals by spectrofluorometry.

Western Blot Analysis of Caspases Lysates from MG-63 cells were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Caspase isoforms were detected with isoform-specific anti-caspases (Transduction Laboratories, Lexington, KY, U.S.A.). Caspase isoforms were visualized using a peroxidase-conjugated secondary antibody and an enhanced chemiluminescence system.³¹⁾

Cytochrome c Measurements Mitochondrial fractions were prepared from 1×10^7 MG-63 cells by differential centrifugation in a buffer containing 250 mM sucrose, as described previously.³²⁾ Protein samples were loaded on sodium dodecyl sulfate-15% polyacrylamide gels, subjected to electrophoresis, then electrophoretically transferred to a nitrocellulose membrane. Western blots were probed with primary monoclonal anti-cytochrome c antibody and secondary antimouse horseradish peroxidase-conjugated antibody, then developed with enhanced chemiluminescence (Amersham Life Science).

Cyclic AMP Measurement Cyclic AMP was measured using the iodinated assay system of Yamasa (Japan). The experiment was performed by exposing 1×10^6 cells to dB-cAMP or forskolin. The reaction was terminated after 3 min by the addition of 0.1 volume of 20% ice-cold perchloric acid, followed by incubation in an ice bath. The cyclic AMP

content was determined by 100 μ l aliquots of the supernatant by radioimmunoassay.³²⁾

Data Presentation and Statistical Analysis Statistical differences were evaluated by analysis of variance (ANOVA) in dose-response experiments and two-tailed Student's *t*-tests. In each case, the statistical test used is indicated, and the number of experiments is stated individually in the legend of each figure.

RESULTS

Induction of Apoptosis by Nitric Oxide in MG-63 Cells

To address the ability of nitric oxide to induce cell death, we investigated the effect of *S*-Nitroso-*N*-acetylpenicillamine (SNAP) on cell viability using an MTT assay and a lactate dehydrogenase release assay. At 48 h, we employed two methods to investigate whether the loss of viability caused by SNAP correlates with a biochemical feature that discriminates between apoptosis and necrosis. The incubation with SNAP (0.6–1 mM), a commonly used NO generator, caused a significant decrease in cell viability in a dose-dependent manner (Fig. 1A). However, MG-63 cells, exposed to the SNAP (0.6 mM), released an amount of LDH that was similar to that released by the control cells, indicating the relative absence of lytic death as occurs in necrosis. Agarose gel electrophoresis revealed a typical ladder profile of oligonucleosomal fragments ranging from 200 to 1400 bp in the presence of SNAP (0.6, 0.8 and 1 mM) (Fig. 1B). *N*-acetyl-DL-penicillamine, which is structurally similar to SNAP except for the release of nitric oxide and oxidized-SNAP, did not induce apoptosis in MG-63 cells as determined by an *in situ* TdT-apoptosis assay (data not shown).

Inhibition of NO-Induced Apoptosis by cAMP Analogs or Forskolin in MG-63 Cells Many recent studies have described specific interactions (cross-talk) between the signal transduction pathways mediated by various protein kinases, including PKA. As shown in Fig. 2A, MG-63 cells were pretreated with 100 μ M DBcAMP or 10 μ M forskolin, and the cells were further cultured without medium replacement for 2h, then stimulated with 600 μ M SNAP. The treatment of MG-63 cells with DBcAMP markedly inhibited SNAP (0.6 mM)-induced cytotoxicity, as determined by MTT assay. The protective effect of cAMP analogs on NO-induced cytotoxicity might reflect the importance of intracellular cAMP elevation because forskolin, an intracellular cAMP-elevating agent, also markedly inhibited NO-induced DNA cleavage in MG-63 cells (Fig. 2B). In separate experiments, we evaluated the effects of these agents on the intracellular cyclic AMP level in osteoblasts. Treatment with DBcAMP or forskolin significantly increased the cyclic AMP level (data not shown). To further analyze the mechanism by which cAMP analogs protect against NO-induced apoptotic cell death, we observed the effects of two putative PKA activity modulators, H-89 and KT5720. Pretreatment of MG-63 cells with H-89 (10 μ M) or KT5720 (1 μ M) clearly blocked the protective effects of DBcAMP or forskolin on NO-induced apoptotic cell death (Fig. 2B). Although the cAMP analogue or forskolin-pretreated cells were negative for DNA fragmentation, H-89 (10 μ M) or KT5720 (1 μ M) reproduced the typical cleavage of DNA into multimers of domain-sized fragments. Neither H-89 nor KT-5720 alone induced any cell damage in

Fig. 1. Effect of Nitric Oxide (NO) on Apoptotic Cell Death of MG-63 Cells

Cells were incubated for 48 h with 0, 0.2, 0.4, 0.6, 0.8, or 1 mM SNAP. (A) Cell viability was measured according to MTT assay and LDH release. (B) Cells were then harvested and assayed for DNA fragmentation. Experiments were performed in quadruplicate a minimum of three times. Mean $\pm$ S.D. ($n=4$). *, Significantly different from control; * $p<0.05$.

the osteoblasts. These observations suggest that the cAMP-dependent activation of PKA plays a crucial role in protecting the NO-induced DNA fragmentation in MG-63 cells. Similar results were obtained with other commonly used cAMP analogs such as CPT-cAMP and 8-Cl-cAMP (data not shown). In addition, we examined MG-63 cells for an early marker, namely the translocation of phosphatidylserine from the inner surface of the plasma membrane to the cell surface, where it binds an annexin V-FITC conjugate. Indeed, after treatment of DBcAMP (100 μ M) in SNAP-stimulated cells, $9 \pm 2.5\%$ of adherent cells (mean $\pm$ S.D. from 3 separate experiments) appeared to be annexin V-stained (Table 1). Compared to the result that only 36 h of SNAP treatment led to $46 \pm 13\%$ annexin V-positive staining, the cAMP-induced effect coincided with the DNA fragmentation assay (Fig. 2B). These data showed that both 100 μ M DBcAMP and 10 μ M forskolin blocked the internucleosomal DNA fragmentation and apoptotic morphology *via* PKA. In addition, to determine whether the protective effect can be explained by the direct chemical interaction of SNAP with cAMP analogs, we measured the amount of NO release using the method of Griess (nitrite). However, there were no detectable differences in NO production (data not shown). In this study, we documented that the major effect of nitric oxide-induced cell death was attributable to apoptosis, and that cAMP had an inhibitory effect on NO-induced apoptosis *via* PKA.

Cyclic AMP Inhibits SNAP-Induced Apoptosis *via* Caspase-3 Activation in MG-63 Cells The caspase family of

Fig. 2. Effects of cAMP and PKA on NO-Induced Cell Death in MG-63 Cells

(A) The cells were incubated for 48 h with the indicated agents; SNAP (0.6 mM), DBcAMP (100 μM)+SNAP, forskolin (10 μM)+SNAP, H-89 (10 μM)+DBcAMP+SNAP, H-89+forskolin+SNAP, KT5720 (1 μM)+DBcAMP+SNAP or KT5720+forskolin+SNAP. Then, an MTT cell viability assay was performed. Mean±S.D. ($n=4$). SNAP; * $p<0.05$, DBcAMP+SNAP or forskolin+SNAP; # $p<0.05$, H-89+DBcAMP+SNAP, H-89+forskolin+SNAP, KT5720+DBcAMP+SNAP, or KT5720+forskolin+SNAP; * $p<0.05$. (B) Cells were incubated as described above, then harvested. Genomic DNA was purified and subjected to gel electrophoresis (M; molecular size marker). lane 1: control, lane 2: SNAP (0.6 mM), lane 3: DBcAMP (100 μM)+SNAP, lane 4: forskolin (10 μM)+SNAP, lane 5: H-89 (10 μM)+DBcAMP+SNAP, lane 6: H-89+forskolin+SNAP, lane 7: KT5720 (1 μM)+DBcAMP+SNAP, lane 8: KT5720+forskolin+SNAP. (C) Cells were incubated for 48 h with various concentrations of DBcAMP (25–200 μM) in the presence of SNAP (0.6 mM). Then, the MTT cell viability assay was performed as described in Materials and Methods. Experiments were performed in quadruplicate a minimum of three times. *Significantly different from control, * $p<0.05$. (D) Cells were incubated for 24 h in the presence or absence of SNAP (0.6 mM). The cells were stained with Annexin-V-FITC and Hoechst 33258 and observed with a fluorescence microscope.

Table 1. Effect of cAMP on the Number of Annexin V-positive Cells in SNAP-treated MG-63 Cells

Treatment	Annexin-positive cells (%)
Control	5.2±1.2
SNAP (0.6 mM)	46.0±13.0*
DBcAMP (100 μM)+SNAP	9.0±2.5#
Forskolin (10 μM)+SNAP	8.0±2.3#
H-89 (10 μM)+DBcAMP+SNAP	51.0±12.0*
H-89+forskolin+SNAP	50.1±13.6*

Values are mean±S.D. Cells were exposed for 24 h with the indicated agents. * $p<0.05$ vs. control or SNAP; # $p<0.05$ vs. SNAP or SNAP+DBcAMP, SNAP or SNAP+forskolin; * $p<0.05$ vs. DBcAMP+SNAP or H-89+DBcAMP+SNAP, Forskolin+SNAP or H-89+forskolin+SNAP; $n=3$ experiments.

proteases is thought to be the final execution pathway in apoptosis.^{33,34} Having established a role for caspases in cell death induced by NO, we analyzed the process of caspase activation in this system. To establish the role of the caspase pathway in NO-induced apoptosis of MG-63 cells, we treated MG-63 cells with a pan-caspase inhibitor, Z-VAD-FMK, a specific caspase-1 inhibitor, YVAD-CHO, or a specific caspase-3 inhibitor, DEVD-CHO. Figure 3A shows that pre-treatment of MG-63 cells with Z-VAD-FMK or DEVD-CHO largely prevented the cell death seen after 48 h of SNAP (0.6 mM). The formation of a DNA ladder in MG-63 cells was suppressed by simultaneous treatment with 100 μM Z-DEVD-CHO or Z-VAD-FMK (data not shown). As shown in Fig. 3B, the fluorescence intensity of the caspase-3 cleavage product AMC was monitored to obtain a linear standard

Fig. 3. Cyclic AMP or PKA Regulates NO-Induced Caspase-3 Activation in MG-63 Cells

(A) Cells were incubated for 48 h with 0.6 mM SNAP in the presence of YVAD-CHO (100 μ M), DEVD-CHO (100 μ M) or Z-VAD-CHO (100 μ M). Then, the MTT cell viability assay was performed. (B) Cells were incubated for various periods with SNAP (0.6 mM). (C) Cells were treated for 48 h with medium alone, SNAP (0.6 mM), DBcAMP (100 μ M)+SNAP, forskolin (10 μ M)+SNAP, H-89 (10 μ M)+DBcAMP+SNAP, H-89+forskolin+SNAP, KT5720 (1 μ M)+DBcAMP+SNAP or KT5720+forskolin+SNAP. Caspase activity analysis of the samples indicated was performed as described in Materials and Methods. Mean $\pm$ S.D. ($n=4$). SNAP: * $p<0.05$, DBcAMP+SNAP or forskolin+SNAP: # $p<0.05$, H-89+DBcAMP+SNAP, H-89+forskolin+SNAP, KT5720+DBcAMP+SNAP, or KT5720+forskolin+SNAP: * $p<0.05$.

curve. However, the cysteine-protease, caspase-1, was not activated in the NO-induced apoptosis signal transduction pathway in MG-63 cells. Furthermore, pretreatment of 100 μ M DBcAMP or 10 μ M forskolin completely blocked the NO-induced caspase-3-like cysteine protease activation. Consistent with Fig. 2B, pretreatment with H-89 (10 μ M) or KT5720 (1 μ M) clearly blocked the inhibitory effects of DBcAMP or forskolin on NO-induced caspase-3 cysteine protease activation (Fig. 3C). These observations suggest that the cAMP-dependent activation of PKA play a crucial role in regulating the NO-induced caspase-3 activation and subsequent apoptosis in MG-63 cells.

Cyclic AMP Inhibits SNAP-Induced Caspase-3 Cysteine Protease Proteolysis in MG-63 Cells

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Fig. 4. Cyclic AMP or PKA Regulates NO-Induced Proteolysis of Caspase-3 Pro-forms and PARP in MG-63 Cells.

(A) Cells were incubated for various periods in SNAP (0.6 mM) treatment. Decrease in pro-forms of caspase-3 (upper panel) and PARP (lower panel) in response to SNAP in MG-63 cells. (B) Cells were incubated for 48 h with medium alone (1), SNAP (0.6 mM) (2), DBcAMP (100 μ M)+SNAP (3), forskolin (10 μ M)+SNAP (4), H-89 (10 μ M)+DBcAMP+SNAP (5), H-89 (10 μ M)+forskolin+SNAP (6). Total cell lysates were prepared and then subjected to Western blot analysis using specific antibodies for caspase-3 and PARP.

3 is one of the major caspase cysteine proteases related to apoptosis in various cells, we investigated whether DBcAMP or forskolin modulates the proteolysis of caspase-3 in MG-63 cells stimulated with SNAP (0.6 mM). As shown in Fig. 4A, caspase-3 antibody could detect the 32-kDa pro-form of caspase-3 in control MG-63 cells. In SNAP (0.6 mM)-treated cells, a marked decrease in the levels of intact caspase-3 was observed. The loss of intact caspase-3 was reversed in cells pre-treated with 100 μ M DBcAMP or 10 μ M forskolin. In addition, caspase-3 is known to be the most efficient PARP-cleaving caspase, with a K_m of ca. 10 μ M. We therefore investigated whether PARP, a preferred substrate for caspase-3 cysteine protease, was cleaved in SNAP (0.6 mM)-treated MG-63 cells. Following exposure to SNAP for 30 h, PARP was cleaved extensively to its 85 kDa cleavage product. Pretreatment of cells with DBcAMP or forskolin completely abrogated cleavage of PARP in SNAP-treated cells. Furthermore, H-89 (10 μ M), a potent PKA inhibitor, clearly reversed the inhibitory effects of DBcAMP or forskolin on NO-induced caspase-3 proteolytic cleavage and PARP cleavage. These results indicate that DBcAMP or forskolin protects MG-63 cells from NO-induced apoptotic cell death, presumably nullifying CPP32-like protease activation mediated by NO. In addition, the cAMP-dependent activation of PKA plays an important role in the inhibition of NO-induced caspase-3-like cysteine protease activation and apoptosis in MG-63 cells.

Cyclic AMP Inhibits SNAP-Induced Caspase-6 Cysteine Protease Proteolysis in MG-63 Cells Next, we examined caspase-6 using caspase-6 antibody, which recognizes the full length form and active p20 subunit of caspase-6. Immunoblot analysis revealed that reduction of the band

Fig. 5. Cyclic AMP or PKA Regulates NO-Induced Proteolysis of Caspase-6 Pro-forms and Lamin-A in MG-63 Cells

(A) Cells were incubated for various periods in SNAP (0.6 mM) treatment. Decrease in pro-forms of caspase-6 (upper panel) and Lamin A (lower panel) in response to SNAP in MG-63 cells. (B) The cells were incubated for 48 h with medium alone (1), SNAP (0.6 mM) (2), DBcAMP (100 μM)+SNAP (3), forskolin (10 μM)+SNAP (4), H-89 (10 μM)+DBcAMP+SNAP (5), H-89 (10 μM)+forskolin+SNAP (6). Total cell lysates were prepared and then subjected to Western blot analysis using specific antibodies for caspase-6 and Lamin A.

corresponding to the caspase-6 pro-form occurred, and a band at approximately p20, corresponding to the carboxyl-terminal portion of caspase-6, was easily detected in cell lysates after 24 h exposure to 0.6 mM SNAP (Fig. 5A). Next, we investigated the cleavage of lamin A in SNAP-treated MG-63 cells. Lamin A, which is believed to be an endogenous substrate for caspase-6, is critical to maintaining the integrity of the nuclear envelope and cellular morphology. To ascertain whether NO-induced apoptosis is accompanied by the activation of caspase-6, we examined the cleavage of Lamin A. As shown in Fig. 5B, SNAP (0.6 mM) resulted in Lamin A cleavage in a time-dependent manner. The 46 kDa Lamin A fragment, which is indicative of proteolytic holoenzyme digestion, was visible after 24 h incubation with SNAP. Consistent with Fig. 5B, DBcAMP or forskolin completely inhibited the proteolysis of both caspase-6 and Lamin A in SNAP-treated cells. In addition, H-89 (10 μM) reversed the inhibitory effects of DBcAMP or forskolin on NO-induced cleavage of caspase-6 and Lamin A. Our data show that cAMP has regulatory effects on NO-induced caspase-6 cysteine protease activation and apoptosis *via* PKA.

Cyclic AMP Inhibits SNAP-Induced Caspase-9 Cysteine Protease Proteolysis in MG-63 Cells Recent studies have shown that caspase-9 and the following caspase-3 are activated by cytochrome c release to cytoplasm. Figure 6A demonstrates that 0.6 mM SNAP triggered caspase-9 cysteine protease proteolysis, and that both DBcAMP and forskolin have inhibitory effects on the proteolytic fragmentation in NO-induced apoptosis in MG-63 cells. This result coincided with the cAMP-induced regulatory effect on caspase-3 and -6 cysteine protease proteolysis (Figs. 4B and 5B). Our results show that DBcAMP or forskolin completely inhibited

Fig. 6. Cyclic AMP or PKA Regulates NO-Induced Proteolysis of Caspase-9 Pro-forms in MG-63 Cells

(A) Cells were incubated for various periods in SNAP (0.6 mM) treatment. Decrease in pro-forms of caspase-9 (upper panel) in response to SNAP in MG-63 cells. (B) Cells were incubated for 48 h with medium alone (1), SNAP (0.6 mM) (2), DBcAMP (100 μM)+SNAP (3), forskolin (10 μM)+SNAP (4), H-89 (10 μM)+DBcAMP+SNAP (5), H-89 (10 μM)+forskolin+SNAP (6). Total cell lysates were prepared and then subjected to Western blot analysis using specific antibodies for caspase-9.

the proteolysis of caspase-9 in SNAP-treated cells. In addition, H-89 (10 μM) reversed the inhibitory effects of DBcAMP or forskolin on the NO-induced cleavage of caspase-9. Our results show that cAMP also has regulatory effects on NO-induced caspase-9 cysteine protease activation and apoptosis *via* PKA.

Cyclic AMP Inhibits SNAP-Induced Cytochrome c Release to Cytoplasm in MG-63 Cells Many researchers have suggested that cytochrome c can induce apoptosis in carrot cytosol, and that a stereotypical DNA ladder could be observed.³⁵⁾ Cytochrome c has been used as an apoptosis inducer only recently.³⁶⁾ The steps involved in NO signaling through cytochrome c have not been previously investigated in osteoblasts. Therefore, the amounts of cytochrome c in the mitochondria and cytosol fractions were then measured by Western blot analysis. Without NO challenge, most detectable cytochrome c was found in the mitochondria fraction. Thus, the amount of cytochrome c in the cytosol fraction increased significantly after 24 h challenge with NO, and the level continued to increase for up to 36 h. The amounts of cytochrome c in the mitochondria fraction showed a corresponding decrease in a time-dependent manner (Fig. 7A). Next, we observed that pretreatment of cells with DBcAMP or forskolin was able to regulate the down regulation of cytochrome c by 0.6 mM SNAP (Fig. 7B). In addition, 10 μM H-89 reversed the inhibition of cytochrome c release into cytoplasm by DBcAMP or forskolin. The dose of H-89 had no effect on cytochrome c release in MG63 human osteoblasts. The fact that H-89 reverses the preventive effect of DBcAMP or forskolin may be because PKA is related to SNAP-induced cytochrome c release into cytoplasm. We next examined the effect of DEVD-CHO, a caspase-3 inhibitor, or Z-VAD-FMK, a pan caspase inhibitor, on the cytochrome c release after 24 h of 0.6 mM SNAP treatment. Neither of the caspase inhibitors blocked SNAP-induced cytochrome c release into cytoplasm (Fig. 7C). Thus, cytochrome c release

Fig. 7. Cyclic AMP or PKA Regulates NO-Induced Release of Cytochrome c into Cytoplasm in MG-63 Cells

(A) Cells were challenged with SNAP (0.6 mM) for various periods. (B) Cells were incubated for 24 h with medium alone (1), SNAP (0.6 mM) (2), DBcAMP (100 μ M)+SNAP (3), forskolin (10 μ M)+SNAP (4), H-89 (10 μ M)+DBcAMP+SNAP (5), or H-89+forskolin+SNAP (6). (C) The cells were treated for 24 h with medium alone (1), SNAP (0.6 mM) (2), DEVD-CHO (100 μ M)+SNAP (3), or Z-VAD-FMK (100 μ M)+SNAP (4). Mitochondrial and cytoplasmic fractions were prepared and separated on SDS-PAGE and transferred onto a nitrocellulose membrane. Cytochrome c was visualized by Western blot analysis.

appears to act upstream of caspase activation in NO-induced apoptosis in MG-63 cells.

DISCUSSION

In this study, we have shown that MG-63 cells subjected to nitric oxide (NO) exhibit chromatin condensation, nuclear fragmentation, and an apoptotic body. These generative changes are characteristic of apoptotic cell death. Recently, NO has been suggested to play an important role in bone remodeling. NO directly facilitates osteoblastic differentiation. *In vitro*, NO, acting as a paracrine factor, has been shown to inhibit osteoclast function and thereby interfere with bone resorption. Decreased nitric oxide levels stimulate osteoclastogenesis and bone resorption both *in vitro* and *in vivo*. In addition, aminoguanidine, a selective inhibitor of iNOS, induces the reduction of bone weight of growing rats and increases bone loss in ovariectomized rats. Nitroglycerine, a NO donor, blocks the bone loss induced by ovariectomy or glucocorticoid treatment. These results strongly suggest that NO is an essential mediator of bone metabolism. Our observation also suggests that NO may be an important regulator of bone remodeling. The death of NO-treated MG-63 cells can be prevented by the addition of a cAMP analog such as DBcAMP, and a cAMP level-elevating drug such as forskolin. In addition, this study, showing that cAMP inhibits apoptotic cell death by NO, is somewhat surprising, since in many existing biologic systems, the induction of apoptosis requires an elevated cAMP level. In thymocytes,³⁷⁾ B lymphocytes,³⁸⁾ and

ovarian granulosa cells,³⁹⁾ exposure to cAMP induces apoptosis. However, cAMP inhibits apoptosis due to nerve growth factor withdrawal in rat PC-12 cells⁴⁰⁾ or the exposure of primary cultures of neurons to cytokines,⁴¹⁾ macrophages to nitric oxide,⁴²⁾ and MCF-7 cells to cycloheximide.⁴³⁾ Our observations appear to be most consistent with the latter findings. However, the exact mechanism of cAMP in inhibiting NO-induced apoptosis remains to be established.

Our study shows that H-89 or KT5720, a specific inhibitor of cyclic AMP-dependent protein kinase A, completely suppressed the inhibitory effect of DBcAMP and forskolin in MG-63 cells. These findings suggest that the inhibitory effects of DBcAMP and forskolin resulted from an increase in intracellular PKA levels in MG-63 cells. Furthermore, DBcAMP also inhibited NO-induced caspase-3, -6 and -9 activation in MG-63 cells. DBcAMP did not appear to produce its inhibition by a direct action of caspase-3, -6 and -9 (data not shown). Since it has been reported that caspase-9 is upstream of caspase-3 in NO-mediated apoptosis,⁴⁴⁾ these results suggest that a possible target mechanism of cyclic AMP lies with caspase-3, following -9 activation in human osteoblasts, MG-63.

On activation, caspase-3 can cleave numerous proteins involved in cell structure, signaling and repair. Caspase-6, its activation evidenced by the disappearance of its pro-form, may participate in the cleavage of lamin A, major constituents of its nuclear envelope. Cytochrome c is an essential component of the mitochondrial respiratory chain and is also released from the mitochondria in response to apoptotic stimuli. These include UV irradiation, etoposide, staurosporine, actinomycin D, H₂O₂ and Ara-C. Zhivotovsky *et al.*⁴⁵⁾ showed that injected cytochrome c induces apoptosis in several different types of cells. More recently, it was reported that cytosolic cytochrome c activates caspase-9, and the subsequently activated caspase-9 cleaves and activates caspase-3.⁴⁶⁾ In this study, treatment with caspase inhibitory peptide (Z-VAD-FMK, a pan caspase inhibitor, or DEVD-CHO, a caspase-3 inhibitor) abrogated the exogenous NO-induced cell death in MG-63 cells (Fig. 3A). However, none of the caspase inhibitors blocked NO-induced cytochrome c release into cytoplasm (Fig. 7C). The cytochrome c molecule may be upstream from the caspase activation in NO-induced apoptosis in MG-63 cells. Moreover, we found that caspase-3, -6, -9, PARP and lamin A also degraded during the apoptotic processes induced by SNAP. The NO donor also resulted in the release of cytochrome c into cytosol (Fig. 7A). Cytochrome c is believed to be necessary for Apaf-1-mediated processing of caspase-9, which, in turn, cleaves other downstream executioner caspases and has been termed the central executioner of apoptosis. In this study, we provided evidence that cAMP completely suppressed NO-mediated apoptosis via cytochrome c release and subsequent activation of caspase-3, -6, or -9 in MG-63 osteoblasts.

Our *in vitro* results suggest that therapies targeted at the inhibition of NO-induced caspase-3-like protease activation abrogate apoptosis in osteoblasts. However, additional experiments will be required to establish which, if any, mechanisms account for the cAMP-induced regulatory effect on apoptosis in NO-exposed human osteoblasts, MG-63.

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