Cocaine-Mediated Apoptosis in Bovine Coronary Artery Endothelial Cells: Role of Nitric Oxide

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ABSTRACT

The present study examined the role of nitric oxide in cocaine-induced apoptosis in bovine coronary artery endothelial cells (BCAECs). Cocaine produced a time-dependent decrease in cell viability and an increase in apoptosis in BCAECs, which were blocked by the nitric oxide donors DETA-NONOate (DETA-NO) and S-nitroso-N-acetyl-penicillamine. In accordance, cocaine decreased nitric oxide production in BCAECs at each time point of the study. Cocaine significantly increased caspase-3 activity that was blocked by the inhibitors of cytochrome c release (cyclosporin A), caspase-3 (Ac-DEVD-CHO), and caspase-9 (Z-LEHD-FMK), respectively. In addition, cocaine activated caspase-9, which was blocked by cyclosporin A and Z-LEHD-FMK. Ac-DEVD-CHO only partially blocked cocaine-induced caspase-9 activity. DETA-NO (20 μM) blocked cocaine-mediated activation of both caspase-9 and caspase-3. Cocaine decreased Bcl-2 protein levels, which was partially blocked by Ac-DEVD-CHO and Z-LEHD-FMK, but not by DETA-NO. Furthermore, cocaine induced a translocation of Bax from the cytosol to the mitochondria in BCAECs, and increased Bax levels in mitochondria by 2.2-fold. In accordance, cytosolic Bax levels decreased about 42%. Neither Ac-DEVD-CHO nor DETA-NO affected cocaine-induced translocation of Bax. We conclude that cocaine-induced Bcl-2 protein down-regulation and Bax translocation to the mitochondria are upstream signals of caspase-9 activation that precedes caspase-3. Cocaine-induced attenuation of nitric oxide plays a key role in the activation of the caspase cascade in BCAECs.

Endothelium not only plays an important role in regulating vascular tone by releasing nitric oxide (NO) but also participates in many other cellular processes such as hemo-stasis, cellular proliferation, inflammation, and immunity. The apoptotic cell death of endothelium has been implicated in the processes of endothelial denudation, angiogenesis, thrombosis, and atherosclerosis (Lopez-Farre et al., 1998). We have recently demonstrated that cocaine induces apoptosis in coronary artery endothelial cells and fetal cardiomyocytes (He et al., 2000b; Xiao et al., 2000). Cocaine-induced apoptosis in coronary endothelium was associated with the release of cytochrome c from the mitochondria into the cytosol, and the subsequent activation of caspase-9 and caspase-3 (He et al., 2000a). It is likely that Bcl-2 family proteins play an important role in cocaine-mediated cytochrome c release. Cocaine decreased Bcl-2 protein levels but had no effect on Bax levels in coronary endothelial cells (He et al., 2000a).

Recent studies suggested that cocaine impaired endothelial NO synthesis (Mo et al., 1998; Mazzio et al., 2000). The effects of NO on apoptosis are highly tissue/cell specific (Kim et al., 1999). Studies have suggested that NO donors or stimulation of NO synthase induces apoptosis in a variety of cell types (Fehsel et al., 1995; Koglin et al., 1999; Matsuzaki et al., 1999). However, in endothelial cells, NO seems to have an anti-apoptotic effect (Tzeng et al., 1997; Ceneviva et al., 1998; Fernandez-Tome et al., 1999). It has been suggested that basal production of NO from constitutive endothelial isoform NO synthase is able to protect endothelial cells from apoptosis (Li and Billiar, 2000). NO suppressed caspase activity by its direct interaction with caspases leading to S-nitrosylation of the cysteine residue, which locates at catalytic sites of all caspases (Li et al., 1997; Mannick et al., 1999; Rossig et al., 1999). In addition, NO inhibited Bcl-2 protein cleavage and cytochrome c release (Kim et al., 1998). It is likely that multiple mechanisms may be involved in NO-mediated protection of apoptosis (for review, see Kim et al., 1999; Li and Billiar, 2000). Nevertheless, the role of NO in cocaine-induced apoptosis in coronary artery endothelial cells is unknown.

The present study was conducted to test the hypothesis that cocaine inhibits NO production, which plays a key role in...
cocaine-induced apoptosis in bovine coronary artery endothelial cells. The specific objectives of this study were to determine in bovine coronary artery endothelial cells whether 1) cocaine decreased NO production, which preceded cocaine-mediated apoptosis; 2) the NO synthase inhibitor N-nitro-l-arginine methyl ester (l-NAME) mimicked cocaine’s effects and induced apoptosis; 3) the NO donor DETA-NONOate (DETA-NO) inhibited cocaine-induced apoptosis; 4) DETA-NO inhibited cocaine-mediated activation of caspase-9 and caspase-3; and 5) DETA-NO inhibited cocaine-induced decrease in Bcl-2 protein levels and/or Bax translocation from the cytosol to the mitochondria.

**Experimental Procedures**

**Materials.** Cocaine, cyclosporin A, l-NAME, S-nitroso-N-acetylpenicillamine (SNAP), and anti-actin antibody were purchased from Sigma (St. Louis, MO). DETA-NONOate was from A.G. Scientific (San Diego, CA). Z-LEHD-FMK was from Kamiya Biomedical (Seattle, WA). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). Protein assay reagents were from Bio-Rad (San Diego, CA). Anti-Bcl-2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse IgG was from Amersham Pharmacia Biotech (Cleveland, IL). MTT cell viability assay kit, and aspase-3 and caspase-9 colorimetric assay kits were from R&D Systems (Minneapolis, MN).

**Cell Culture.** Bovine coronary artery endothelial cells (BCAECs) were obtained from Cell Applications, Inc. (San Diego, CA). Cells were grown in complete medium of Dulbecco’s modified Eagle’s medium (Mediatech Collgro Inc., VA) with glucose (4.5 g/l), 15% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% CO2, 95% air, and used for the experiments at the fifth and sixth passages at 80% confluence. Twenty-four hours before the treatment, the medium was replaced with the serum-free medium.

**Cell Viability.** MTT assay kit (R&D Systems) was used to determine cell viability. The principle of the assay was based on the reduction of MTT by metabolically active cells to insoluble purple formazan dye crystals. The experiments were performed in 96-well plates. At the end of each experiment, the cells in each well were incubated with 10 μl of MTT reagent for 2 h at 37°C. The formazan crystals were then solubilized with 100 μl of detergent solution provided in the kit in the dark for at least 2 h. The absorbance was measured at 570 nm, with 690 nm as reference using a microplate reader. The data were calculated using a standard curve and expressed as a percentage.

**Quantitative Analysis of Apoptotic Cells.** Fluorescent DNA-binding dye Hoechst 33258 was used to define nuclear chromatin morphology as a quantitative index of apoptosis as described previously (He et al., 2000a,b). Briefly, after each experiment, cells growing on cover slides were fixed by methanol/acetic acid (v/v 3:1) at 4°C for 5 min and stained with Hoechst 33258 for 10 min at room temperature in the dark. After mounting, the morphological changes of the nuclei of apoptotic cells were visualized by fluorescence microscopy. The number of apoptotic cells and total cells were counted in six randomly selected high-power fields under a fluorescence microscope (approximately 400 cells/cover slide). The percentage of apoptotic cells was calculated as the number of apoptotic cells/number of total cells x 100%.

**Measurement of Nitrate, Nitrite, and Nitrite Oxide (NOx).** NO was measured by chemiluminescence method as described previously (Yang et al., 2000). Because of the instability of NO in physiological solution, most of NO is rapidly converted to nitrite (NO2⁻) and further to nitrate (NO3⁻). Nitrite and nitrate are relatively stable in the solution, and are readily reduced back to NO in various (III/II) solution. The samples (100 μl) taken from the medium were injected into the gas purge vessel containing 5 ml of vanadium (III/HCl) and allowed to react for 1 min and reduce nitrate/nitrite in the sample back to NO. To achieve high reducing efficiency, the reduction was performed at 90°C. NO in the sample was then “stripped” into the headspace of the gas purge vessel by bubbling it with helium (12 ml/min) for 60 s. NO in the headspace was drawn into a NO Analyzer (model 270B; Sievers Instruments, Inc., Boulder, CO) and mixed with ozone (O3) in front of a cooled Hamamatsu, red-sensitive photomultiplier tube. Signals from the detector were analyzed by an on-line computer as area under the peak. The measurement reflected the combined concentrations of NOx of each sample, which was calculated from a standard curve of 10 to 1000 picomoles of nitrate run in each assay.

**Western Blot Analysis.** Bcl-2 and Bax proteins were determined with Western analysis as described previously (He et al., 2000a). Briefly, bovine coronary artery endothelial cells were harvested after treatments, and homogenized in ice-cold lysis buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml apro tinin, 10 μg/ml leupeptin) for 30 min. After centrifugation, proteins in the supernatant were quantified by a standard colorimetric assay (Bio-Rad), and were used to determine Bcl-2 protein levels. To determine Bax translocation from the cytosol to the mitochondria, the cytosolic and mitochondrial fractions were separated and the protein content of each fraction was determined as described previously (He et al., 2000a). The proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes, and incubated with primary antibodies against Bax (1:250) and Bcl-2 (1:2000), respectively, in Tris-buffered saline-Tween buffer containing 4% nonfat milk. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:2000), and visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Results were quantified by densitometric analysis using a Bio-Rad densitometer (model 670). The data were normalized by actin and presented as the percentage of the control protein levels within each group.

**Caspase Activity Assay.** Activities of caspase-3 and caspase-9 were determined using the corresponding caspase activity detection kits (R&D Systems) as described previously (He et al., 2000a). Briefly, 100 μg of total cell protein was added to 50 μl of reaction buffer and 5-μl substrates of DEVD-pNA and LEHD-pNA, respectively. Samples were incubated at 37°C for 3 h and the enzymatic-catalyzed release of pNA was quantified at 405 nm using a microtiter plate reader. The values of treated samples were normalized to corresponding untreated controls allowing determination of the fold increase in caspase activity.

**Statistical Analysis.** Data were presented as the mean ± S.E.M. Statistical analysis were performed by one-way ANOVA followed by the Newman-Keuls post hoc test. Full factorial two-way ANOVA with Bonferroni correction was used to analyze data in Figs. 2 and 4. Differences were considered significant at p < 0.05.

**Results**

**Effects of Cocaine on NO Production.** The effect of cocaine on basal NOx release in BCAECs is shown in Fig. 1. The cells were treated with control medium or medium with 100 μM cocaine for up to 72 h. NO (measured as NOx) in the medium was assayed by chemiluminescence method. Over the 72-h period of the treatment, basal NOx continued to accumulate in the medium. As shown in Fig. 1, cocaine significantly decreased NOx release from BCAECs at each time point of the study.

**Effects of NO Donors on Cocaine-Induced Apoptosis.** We have previously demonstrated in BCAECs that cocaine induces apoptosis, which is reflected by decreased cell viabil-
ity (He et al., 2000a). To study the effects of exogenous NO on cocaine-induced apoptosis, we first used 20 μM DETA-NO as the NO donor. This compound spontaneously decomposes to release two NO molecules with a half-life of \( \frac{1}{2} \) h in cell culture medium, and 20 μM DETA-NO gives 80 to 100 nM steady-state concentration of NO for at least 48 h, which resembles the physiological level of NO (1–200 nM) in the tissue (Beckman, 1999; Brown, 1999). As shown in Fig. 2, there was a spontaneous reduction in cell viability of BCAECs that occurred over the 72-h culture period in the serum-free medium. Consistent with our previous reports (He et al., 2000a,b), cocaine significantly exacerbated cell death of BCAECs in a time-dependent manner. Compared with the control, cells treated with cocaine started to show a significant decline in cell viability at 12 h (12% decline) and continued up to 72 h (35% decline) \( (p < 0.001, \) cocaine versus control). Although 20 μM DETA-NO alone had no effect on cell viability compared with the control \( (p = 0.134, \) DETA-NO versus control), it reversed the cocaine-induced decrease in cell viability (Fig. 2) \( (p = 0.074, \) DETA-NO + cocaine versus control).

To confirm apoptotic cell death, nuclear morphology was examined by the DNA-binding dye Hoechst 33258. As demonstrated previously (He et al., 2000a), apoptotic cells showed condensed, coalesced, and segmented nuclei (Fig. 3A). Cocaine significantly increased apoptosis after 24-h treatment, which was blocked by DETA-NO (Fig. 3B). To examine the specific effect of NO, NO was depleted from DETA-NO by decomposing it for six half-lives. In contrast to the parent compound, decomposed DETA-NO with depleted NO showed no effect on the cocaine-induced apoptosis (Fig. 3B), suggesting that the antiapoptotic effect of DETA-NO was due to the release of NO. This was further confirmed by another NO donor, SNAP. As shown in Fig. 3B, SNAP dose dependently inhibited the cocaine-induced apoptosis.

Fig. 1. Effect of cocaine on NO release in BCAECs. BCAECs were treated with control medium or medium with 100 μM cocaine for the indicated time periods. NO (measured as NOx) in the medium was measured by the chemiluminescence method as described under Experimental Procedures. Data are means ± S.E.M. of six experiments. *\( p < 0.05 \) versus the control.

Fig. 2. Effect of DETA-NONOate on cocaine-induced cell death in BCAECs. BCAECs were treated with control medium or medium with 100 μM cocaine (COC) in the presence or absence of 20 μM DETA-NO for the indicated time periods. Cell viability was determined by MTT method. Data are means ± S.E.M. of 10 experiments. Data were analyzed by full factorial two-way ANOVA with Bonferroni correction. Cocaine versus control, \( p < 0.001 \); DETA-NO versus control, \( p = 0.134 \); DETA-NO + cocaine versus control, \( p = 0.074 \).

Fig. 3. Effect of NO donors on cocaine-induced apoptosis in BCAECs. BCAECs were treated with control medium or medium with 100 μM cocaine (COC) in the presence or absence of DETA-NO (20 μM), decomposed DETA-NO (20 μM), and SNAP (10 and 100 μM) for 24 h. Cells were then stained with DNA binding fluorescence dye Hoechst 33258 and nuclear morphology was examined by fluorescence microscopy. A, cocaine-induced nuclear morphological changes in BCAECs. The arrows show condensed, coalesced, and segmented apoptotic nuclei. a, control; b, 100 μM cocaine; c, 100 μM cocaine + 20 μM DETA-NO; d, 100 μM cocaine + 20 μM decomposed DETA-NO. Scale bar, 20 μm. B, quantitative results. Data are means ± S.E.M. of four experiments. *\( p < 0.05 \) versus the control, \( \ast p < 0.05 \) versus cocaine alone.
To test whether the inhibition of NO production would mimic cocaine's effects, we examined the effects of endothelial NO synthase inhibitor l-NAME on cell viability of BCAECs. As shown in Fig. 4, incubation of BCAECs with 100 μM l-NAME resulted in a time-dependent decrease in cell viability compared with the control (p < 0.001, l-NAME versus control). Nevertheless, l-NAME induced decreases in cell viability to a lesser extent, compared with cocaine (p < 0.001, l-NAME versus cocaine). Apoptotic cell death induced by l-NAME was confirmed by the examination of nuclear morphology using Hoechst 33258. l-NAME (100 μM) treatment for 24 h significantly increased apoptotic cells from 6.08 ± 0.53% to 10.64 ± 0.53% (p < 0.05).

Effects of DETA-NO on Cocaine-Induced Apoptosis. Although the role of NO on cell death is still controversial, many studies have suggested that NO directly inhibits caspase family members by S-nitrosylation (Haendeler et al., 1997; Li et al., 1997). We have previously demonstrated that cocaine activates both caspase-9 and caspase-3, but not caspase-8 in BCAECs (He et al., 2000a). To test whether the inhibitory effects of DETA-NO on cocaine-induced cell death were mediated by inhibiting caspase activation induced by cocaine, we examined the effects of DETA-NO on cocaine-induced activation of caspase-9 and caspase-3 in BCAECs. As shown in Fig. 5, consistent with our previous studies (He et al., 2000a), cocaine treatment for 24 h increased caspase-3 activity 1.9-fold. Although cyclosporin A (cytochrome c release inhibitor), Ac-DEVD-CHO (caspase-3 inhibitor), and Z-LEHD-FMK (caspase-9 inhibitor) did not change the basal caspase-3 activity, they all blocked cocaine-induced increase in caspase-3 activity (Fig. 5). Similarly, cocaine increased caspase-9 activity 1.8-fold, which was blocked by cyclosporin A and Z-LEHD-FMK (Fig. 6). However, the caspase-3 inhibitor Ac-DEVD-CHO only partially blocked cocaine-induced increase in caspase-9 activity (Fig. 6). None of the inhibitors had effects on the basal caspase-9 activity. As shown in Fig. 7, DETA-NO (20 μM) itself had no effect on activities of either caspase-3 or caspase-9, but completely blocked cocaine-induced activation of both caspase-3 and caspase-9 in BCAECs.

Effects of DETA-NO on Cocaine-Induced Cleavage of Bcl-2. Our previous study has demonstrated that cocaine induces a decrease in Bcl-2 protein levels, which is partially mediated by activated caspase-3, suggesting a positive feedback amplification of the proapoptotic signaling. In an attempt to understand whether NO was involved in cocaine-mediated cleavage of Bcl-2, we determined the effects of DETA-NO on Bcl-2 protein expression in the presence of cocaine by Western blot analysis. As shown in Fig. 8, the representative Western immunoblot showed that the monoclonal antibody for the Bcl-2 protein detected a single band at expected size of 29 kDa (Fig. 8, top). Consistent with our previous findings (He et al., 2000a), cocaine decreased Bcl-2 protein levels in BCAECs. DETA-NO (20 μM) had no effect on activities of either caspase-3 or caspase-9, but completely blocked cocaine-induced activation of both caspase-3 and caspase-9 in BCAECs.

**Fig. 4.** Effect of l-NAME on cell viability of BCAECs. BCAECs were treated with control medium or medium with 100 μM l-NAME and 100 μM cocaine, respectively, for the indicated time periods. Cell viability was determined by MTT method. Data are means ± S.E.M. of 10 experiments. Data were analyzed by full factorial two-way ANOVA with Bonferroni correction. Cocaine versus control, p < 0.001; l-NAME versus control, p < 0.001; l-NAME versus cocaine, p < 0.001.

**Fig. 5.** Effects of cyclosporin A and caspase inhibitors on cocaine-induced caspase-3 activation in BCAECs. BCAECs were treated with 100 μM cocaine (COC) in the absence or presence of cyclosporin A (CSA, 1 μM), Ac-DEVE-CHO (DEVCH-CHO, 100 μM), and Z-LEHD-FMK (LEHD-FMK, 20 μM), respectively, for 24 h. The caspase-3 activity was determined using colorimetric caspase activity detection kit as described under Experimental Procedures. Data are means ± S.E.M. of six experiments. *p < 0.05 versus the control.

**Fig. 6.** Effects of cyclosporin A and caspase inhibitors on cocaine-induced caspase-9 activation in BCAECs. BCAECs were treated with 100 μM cocaine (COC) in the absence or presence of cyclosporin A (CSA, 1 μM), Ac-DEVE-CHO (DEVCH-CHO, 100 μM), and Z-LEHD-FMK (LEHD-FMK, 20 μM), respectively, for 12 h. The caspase-9 activity was determined using colorimetric caspase activity detection kit as described under Experimental Procedures. Data are means ± S.E.M. of five experiments. *p < 0.05 versus the control, **p < 0.05 versus cocaine.
on cocaine-induced decrease in the Bcl-2 protein. In contrast, both the caspase-3 inhibitor Ac-DEVD-CHO and the caspase-9 inhibitor Z-LEHD-FMK partially blocked cocaine-induced cleavage of Bcl-2.

Effects of DETA-NO on Cocaine-Induced Bax Translocation from the Cytosol to the Mitochondria. We have previously demonstrated that cocaine has no effects on Bax protein levels in BCAECs (He et al., 2000a). Other studies showed that Bax translocated from the cytosol to the mitochondrial fractions in response to certain apoptotic signals (Nomura et al., 1999; Putcha et al., 1999). To test whether Bax translocation occurred in BCAECs exposed to cocaine, we determined subcellular localization of Bax in the cytosolic and mitochondrial fractions by Western blot analysis. The representative Western immunoblot showed that the monoclonal antibody for Bax detected a single band at expected size of 21 kDa (Fig. 9, top). As shown in Fig. 9, in control cells Bax was almost exclusively located in the cytosolic fraction. After cocaine treatment, there was a 1.2-fold increase in Bax levels in the mitochondrial fraction and an accordant 42% decrease in Bax levels in the cytosolic fraction. As also shown in Fig. 9, neither the caspase-3 inhibitor Ac-DEVD-CHO nor DETA-NO had effects on cocaine-induced Bax translocation.

Discussion

The present study demonstrates for the first time that cocaine decreases NO release from endothelial cells, and suggests a role for the decreased NO in cocaine-mediated apoptotic cell death in BCAECs. This is supported by the following evidence: 1) the cocaine-mediated decrease in NO production preceded cocaine-induced decrease in cell viability, 2) the NO synthase inhibitor L-NAME mimicked cocaine's effects and decreased cell viability, and 3) the NO donor DETA-NO inhibited cocaine-induced cell death. Although the precise mechanisms underlying the protective effects of NO on cocaine-mediated apoptosis are not entirely clear at present, the inhibition of caspase activation is likely to play a key role.

The role of NO in apoptosis is controversial and complex
depending on the cell type, cell sensitivity to NO, and NO levels (Kim et al., 1999). One explanation for the paradoxical findings in different studies is likely to be the source of NO donors used in each study. Nitrosothiols have been widely used as NO donors. However, because nitrosothiols have their own chemical reactivities distinct from NO, and are far more reactive with biological thiols than NO, their actions cannot be simply equated with NO (Beckman, 1999). In the present study, we used DETA-NO as a NO donor, and 20 μM DETA-NO would maintain a steady-state concentration of 100 nM NO in cell culture medium for at least 48 h, which resembled the physiological levels of NO (1–200 nM) in the tissue (Beckman, 1999; Brown, 1999). Studies have suggested that at low concentrations NO mainly function as an antiapoptotic molecule, although excessive NO may induce cytotoxicity (Li and Billiar, 2000). The present finding that DETA-NO inhibited cocaine-induced apoptosis in BCAECs is in agreement with previous results that exogenous NO protected endothelial cells from apoptotic stimuli (Tzeng et al., 1997; Ceneviva et al., 1998; DeMeester et al., 1998; Ho et al., 1999). The finding that decomposed DETA-NO with depleted NO showed no effect on the cocaine-induced apoptosis confirms that the antiapoptotic effect of DETA-NO is due to the release of NO. This was further supported by another NO donor SNAP, which dose dependently inhibited the cocaine-induced apoptosis. Furthermore, the finding that l-NAME decreased cell viability and increased apoptosis in BCAECs suggests that endogenous NO is likely to play an important role in protecting BCAECs from apoptosis. In a previous study, DeMeester et al. (1998) demonstrated that the NO synthase inhibitor, Nω-methyl-l-arginine did not affect lipopolysaccharide-induced apoptosis in pig aortic endothelial cells, and concluded that endogenous NO did not inhibit endothelial cell apoptosis. However, the direct effect of the NO synthase inhibitor on endothelial cell apoptosis was not determined in their study (DeMeester et al., 1998). In the present study, we found that cocaine inhibited NO release, and the decreased NO preceded cocaine-induced apoptosis in BCAECs. Collectively, our data suggest that a reduction in endogenous NO is likely to play an important role in cocaine-mediated cell death in BCAECs. Nonetheless, the finding that l-NAME decreased cell viability to a lesser extent compared with cocaine suggests that decreased NO may not be the only mechanism by which cocaine mediates apoptosis in BCAECs.

It is likely that multiple mechanisms are involved in NO-mediated antiapoptotic effects. Previous studies demonstrated that NO reversibly inhibited seven members of the caspase family via S-nitrosylation in hepatocytes (Li et al., 1997). We have previously shown the participation of caspases-3 and caspase-9 but not caspase-8 in cocaine-induced apoptosis in coronary artery endothelial cells (He et al., 2000a,b). In the present study, we demonstrated that cocaine-induced activation of caspase-3 and caspase-9 was blocked by cyclosporin A, which inhibits cytochrome c release from mitochondria by stabilizing mitochondrial transmembrane potential (Green and Reed, 1998). We have previously demonstrated that cocaine induces cytochrome c release from mitochondria, and cyclosporin A inhibits cocaine-induced cytochrome c release and apoptosis in coronary artery endothelial cells (He et al., 2000a,b). Collectively, these results suggest that cocaine-mediated cytochrome c release precedes the activation of caspase-3 and caspase-9. In addition, the present finding that cocaine-induced activation of caspase-3 was completely blocked by Z-LEHD-FMK, which blocked cocaine-induced caspase-9 activation, suggests that activation of caspase-9 precedes caspase-3 in cocaine-stimulated caspase cascade in BCAECs. This is further supported by the finding that the caspase-3 inhibitor Ac-DEVD-CHO only partially blocked cocaine-induced caspase-9 activation. The fact that Ac-DEVD-CHO partially blocked caspase-9 activation suggests a positive feedback of caspase-3 on the upstream caspase(s).

The present study clearly demonstrated that DETA-NO blocked cocaine-induced activation of both caspase-3 and caspase-9 in BCAECs. It has been demonstrated that NO maintains caspase-3zymogen in an inactive form by S-nitrosylation of the catalytic-site cysteine (Mannick et al., 1999; Rossig et al., 1999). To our knowledge, the inhibition of caspase-9 activity by NO has not been previously studied. It is not clear at present whether NO inhibits caspase-9 activity directly by S-nitrosylation or indirectly by inhibiting the loss of mitochondrial inner transmembrane potential and cytochrome c release. In a recent study, Li et al., (1999) demonstrated that NO blocked TNFα/actinomycin D-reduced reduction in mitochondrial transmembrane potential and cytochrome c release in cultured hepatocytes. Nevertheless, because the caspase-3 inhibitor Ac-DEVE-CHO also abolished the mitochondrial depolarization, the authors concluded that the inhibition of cytochrome c release and loss of mitochondrial transmembrane potential by NO in hepatocytes were due to the inhibition of caspase activation (Li et al., 1999). In contrast, the present finding that cyclosporin A abolished cocaine-induced activation of caspase-9 and caspase-3 strongly suggests that loss of mitochondrial transmembrane potential is required for cocaine-mediated caspase activation in BCAECs. A recent study examined the effects of NO on the mitochondrial permeability transition pore (PTP) by exposing isolated rat liver mitochondria to NO released from NONOate NO donor, and demonstrated that NO reversibly inhibited PTP opening and cytochrome c release at physiological levels of NO, while it accelerated PTP opening at pathophysiological NO levels (Brookes et al., 2000). It is tempting to speculate that NO-inhibited caspase activation induced by cocaine in the present study may be mediated by both direct S-nitrosylation of caspases and indirect inhibition of cytochrome c release in BCAECs. Given that caspase-9 is upstream of caspase-3 in the caspase cascade in BCAECs, the possibility that DETA-NO-induced inhibition of caspase-3 was mediated in part by its inhibition of caspase-9 cannot be ruled out in the present study.

Our previous study has demonstrated that cocaine decreases Bcl-2 protein levels, which is the upstream signal for cytochrome c release from the mitochondria in BCAECs (He et al., 2000a). Bcl-2 proteins are predominantly localized to the outer mitochondrial membrane, and mediate antiapoptotic effects by stabilizing the mitochondrial membrane and inhibiting cytochrome c release (Reed, 1997; Adams and Cory, 1998). The finding that the cocaine-induced decrease in Bcl-2 proteins was partially blocked by caspases inhibitors in the present study suggests that the Bcl-2 protein is not only an upstream inhibitory signal of caspases but also a downstream substrate of caspases. The results also suggest that the activated caspases act as amplifiers in the positive feed-
back loop in cocaine-induced apoptosis. This finding is in agreement with previous studies showing that the proteolytic cleavage of Bcl-2 by activated caspases plays a key role in the amplification of proapoptotic signaling (Cheng et al., 1997; Kirsch et al., 1999). In a previous study, Kim et al. (1998) demonstrated that the NO donor SNAP inhibited Bcl-2 cleavage in hepatocytes by inhibiting caspases through a mechanism consistent with S-nitrosylation of the protease. In contrast, the present study demonstrated that DETA-NO had no effect on cocaine-induced Bcl-2 cleavage in BCAECs despite the fact that it blocked cocaine-induced activation of caspase-3 and caspase-9. The reasons for the difference between the present study and the previous one (Kim et al., 1998) are not clear at present, but may be due to the differences in the cell types (endothelial cells versus hepatocytes) and/or the NO donors (DETA-NO versus SNAP) used. The present finding suggests that exogenous NO may actually promote Bcl-2 cleavage in endothelial cells. Indeed, it has been demonstrated in neuronal cells that NO caused a decrease in Bcl-2 proteins (Tamatan et al., 1998; Matsuzaki et al., 1999). However, because NO inhibited downstream caspase activation in the present study, it blocked cocaine-induced apoptosis in BCAECs.

Unlike Bcl-2 proteins, cocaine showed no effect on Bax protein levels in BCAECs (He et al., 2000a). It has been demonstrated that Bax proteins are predominantly localized in the cytosol, and upon activation, translocate to the mitochondria and mediate cytochrome c release (Adams and Cory, 1998; Gross et al., 1998; Marzo et al., 1998; Nomura et al., 1999; Putcha et al., 1999). The present finding that cocaine-induced Bax translocation from cytosol to mitochondria suggests an important role for Bax in cocaine-mediated apoptosis in BCAECs. Neither the caspase inhibitor nor DETA-NO had an effect on cocaine-induced Bax translocation, suggesting that Bax is an upstream signal of caspase activation, and NO-mediated inhibition of caspase activation occurs downstream of Bax translocation.

Taken together, as shown in Fig. 10, we speculate that cocaine exerts its cytotoxic effects on endothelial cells through three mechanisms: 1) cleavage of Bcl-2, 2) induction of Bax translocation, and 3) attenuation of NO production. Although the mechanisms underlying cocaine-induced Bcl-2 cleavage in BCAECs are not clear at present, decreased Bcl-2 is likely to promote the translocation of Bax to the mitochondria, leading to the release of cytochrome c and subsequent activation of the caspase cascade (Nomura et al., 1999; Putcha et al., 1999; Murphy et al., 2000). Once the mitochondrial apoptotic pathway is activated, the death signal is further amplified by the cleavage of Bcl-2 by the activated caspases. This positive feedback loop ensures the cell's demise. We speculate that NO interacts with the mitochondria apoptotic pathway at two different levels: the mitochondria level and the caspase level. By inhibiting mitochondrial PTP opening and caspase activation, NO protects endothelial cells from apoptotic cell death. The finding that Fas denitrosylates endogenous procaspase-3, which was normally S-nitrosylated by endogenous NO (Mannick et al., 1999), suggests that NO-mediated S-nitrosylation of procaspases may occur in the resting state as a mechanism to inhibit caspase activation. Along with this finding, we speculate that cocaine-induced activation of caspases may be in part due to the denitrosylation of procaspases caused by the attenuation of NO. It remains unclear whether cGMP plays a role in the antiapoptotic effect of NO. Considering the multiple biological function of NO and its capacity of rapid diffuse intracellularly and intercellularly, it is likely that the cytotoxicity of cocaine is far more complicated in vivo and a variety of mechanisms may be involved.

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References


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