Cocaine Induces Apoptosis in Fetal Myocardial Cells through a Mitochondria-Dependent Pathway

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ABSTRACT
In the present study, we examined the direct cytotoxic effects of cocaine on fetal cardiac myocytes. Cocaine treatment of cultured fetal rat (21 days) myocardial cells (FRMCs) induced a time- and concentration-dependent increase in apoptotic cells in FRMCs. Cocaine induced surface exposure of phosphatidylserine in FRMCs at 12-h treatment and increased apoptotic cells up to 96 h. Corresponding DNA fragmentation induced by cocaine in these cells was demonstrated in situ by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling assay and by electrophoresis of labeled DNA fragments, showing the characteristic apoptotic ladders. The pD2 and maximum increase of cocaine-induced apoptosis in FRMCs were 4.3 and 3.2-fold, respectively. Both caspase-9 and caspase-3 inhibitors (Z-LEHD-FMK and Ac-DEVD-CHO, respectively) blocked cocaine-induced apoptosis. In addition, cyclosporin A inhibited cocaine-induced apoptosis in a concentration-dependent manner with an IC50 value of 0.1 μM. The maximum of 86% inhibition was obtained with 3 μM cyclosporin A. Cocaine induced the release of cytochrome c from the mitochondria and increased its levels in the cytosol by 3.1-fold. In accordance, the level of cytochrome c in the mitochondria fraction decreased by ~60%. Cocaine-induced translocation of cytochrome c was inhibited by cyclosporin A. The results indicate that cocaine has a direct cytotoxic effect on fetal cardiomyocytes by inducing apoptosis in the cells. Furthermore, the release of cytochrome c from the mitochondria and its subsequent activation of caspase-9 and caspase-3 play a key role in cocaine-induced apoptosis.

It is estimated that each year more than 100,000 infants who are exposed prenatally to cocaine are born in the United States. Long-term cocaine use during pregnancy has been associated with numerous adverse perinatal outcomes, such as intrauterine growth retardation, preterm delivery, abruptio placenta, and congenital anomalies (Holzman and Paneth, 1994). Cardiovascular complications related to cocaine abuse include myocardial ischemia and infarction, myocarditis, cardiomyopathy, rhythm disturbances, and sudden death. Clinical studies suggest that fetal outcome, including fetal death in utero, may be related to maternal cocaine use. The presumed cause is repetitive hypoxic insults to the fetus and the placenta as a result of cocaine-induced uterine artery vasoconstriction.

Although it has been known that cocaine crosses the placenta and may accumulate in the fetal compartment (Schenker et al., 1993), direct cocaine toxicity to the fetal heart is not well documented. Cocaine predisposes the fetus and neonate to various cardiovascular disorders. Developmental disorders observed in humans include congenital cardiac anomalies and altered cardiac function in newborns (Wiggina, 1992). Studies have demonstrated that cocaine produces changes in human fetal myocardial cell action potential configuration and contractility in vitro (Richards et al., 1990; Richards, 1997). Recently, several animal experiments have shown that cocaine can induce apoptosis in cultured neurons (Nassogne et al., 1997), thymocytes (Wu et al., 1997), and hepatocytes (Cascales et al., 1994). Apoptosis is an active physiological process that permits the removal of unwanted or damaged cells from the body through an intrinsic cell-suicide program. Compelling evidence has accumulated indicating that apoptotic cell death may also play a critical role in a variety of cardiovascular diseases, including myocardial infarction, heart failure, and atherosclerosis (MacIellan and Schneider, 1997; Haunstetter and Izumo, 1998).

The present study was undertaken to examine the cytotoxic effect of cocaine on cultured fetal rat myocardial cells (FRMCs). We characterized cocaine-induced apoptosis in the myocardial cells by determining DNA fragmentation and alterations in cell and nucleus morphology. The translocation of phosphatidylserine from the inner to the outer surface of the plasma membrane in the early phase of cocaine-induced apoptosis was detected by annexin V conjugated to fluorescein isothiocyanate (FITC). To gain a more in-depth understand-
standing of the cellular mechanisms underlying cocaine-induced apoptosis in FRMCs, in the present study, we examined the effect of cocaine on cytochrome c translocation from the mitochondria to the cytosol in these cells. Correlation of cocaine-induced cytochrome c release and apoptosis was demonstrated with the use of cyclosporin A, which blocked both apoptosis and cytochrome c release mediated by cocaine in FRMCs. We also evaluated the mitochondria pathway and determined the effects of caspase-9 and caspase-3 inhibitors on cocaine-mediated apoptosis.

**Experimental Procedures**

**Materials.** Cell culture medium 199, annexin V kit, streptavidin (Sav)-FITC, Hoechst 33258, cocaine, cyclosporin A, trypsin, gelatin, transferrin, insulin, selenium, triiodothyronine, methyl green, cytochrome c, and PBS were purchased from Sigma Chemical Co. Purified anti-cytochrome c antibody and Ac-DEVD-CHO (caspase-3 inhibitor) were obtained from PharMingen (San Diego, CA). Z-LEHD-FMK (caspase-9 inhibitor) was obtained from Kamiya Biomedical (Thousand Oaks, CA). Horseradish peroxidase-conjugated antimouse IgG and Hybrid ECL nitrocellulose membrane were purchased from Amersham Life Science (Clearbrook, IL). Prestudied protein molecular weight standards were obtained from Life Technologies (Grand Island, NY). Proteinase K, in situ cell death detection kit, DNase-free RNase, and DNase were purchased from Boehringer Mannheim (Indianapolis, IN). SYBO Gold was obtained from Molecular Probes (Eugene, OR). Type II collagenase was obtained from Worthington Biochemical (Freehold, NJ). Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT).

**Myocardial Cell Culture.** Primary culture of fetal rat cardiomyocytes was prepared from the hearts of 21-day gestational age Sprague-Dawley rats as previously described (Flink et al., 1992). Briefly, fetal heart was minced in a Ca2+-free Hanks’ balanced salt solution containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 1.0 mM NaH2PO4, 20 mM HEPES, and 5.5 mM glucose. Myocardial cells were dispersed by the addition of 0.1% trypsin and 0.5 mg/ml type II collagenase. Cells were stirred for 10 min at 37°C, and the supernatant was discarded. The pellet was incubated with fresh trypsin-collagenase for 16 min at 37°C, and the cell suspension was collected. The digestion step was repeated five times, and the cell suspension from each digestion step was combined and preplated for 1.5 h to minimize nonmyocyte contamination. After centrifugation at 500g for 6 min, cells were resuspended in culture medium 199 supplemented with 10% fetal bovine serum and 1% antibiotics (10,000 U/ml penicillin and 10,000 μg/ml streptomycin). Cells were then plated at a density of 25,000 cells/ml onto 6-well tissue culture plate or a 75-mm2 flask precoated with 1% gelatin and cultured at 37°C in 95% air/5% CO2 and 5-bromo-2′-deoxyuridine (BrDU; 0.1 mM) was added to the medium to reduce the background of nonmyocyte cells. Within 3 days, a monolayer of spontaneously beating cells was formed. As established by visual determination, >90% of the cells manifested spontaneous contractions. All experiments in the present study used 60 to 70% confluent cells.

After 3 days in the culture, cells were transferred to serum free medium 199 containing 5 μg/ml transferrin, 1 μg/ml insulin, 0.1 ng/ml selenium, and 0.5 ng/ml triiodothyronine and cultured for 24 h. Cells were then exposed to various doses of cocaine for different times. The morphological changes of the myocardial cells were examined by phase-contrast and fluorescence microscopy.

All procedures and protocols used in the present study were approved by the Animal Research Committee of Loma Linda University and followed the guidelines put forward in the National Institutes of Health “Guide for the Care and Use of Laboratory Animals.”

**Detection of Cell Surface Phosphatidylserine.** Phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane is one of the earliest apoptotic features. We used the phosphatidylserine-binding protein annexin V conjugated with Sav-FITC to identify the sites of phosphatidylserine exposure in FRMCs by fluorescence microscopy. The binding of annexin V-FITC to cell surface phosphatidylserine was detected with a commercially available annexin V kit (Sigma) according to the manufacturer’s instruction. Monolayers of cardiomyocytes grown on coverslides were washed with cold PBS and incubated with 100 μl of annexin V incubation reagent (10× 10 μl of binding buffer, 0.5 μg/10 μl propidium iodide, 0.05 μg/1 ml annexin V conjugate, 79 μl of distilled water) per sample for 15 min at room temperature in the dark. Cells were then washed with 1× binding buffer and incubated with 100 μl of 1× binding buffer containing FITC for 15 min at room temperature in the dark. After a wash with 1× binding buffer, the samples were examined immediately by fluorescence microscopy.

**DNA Fragmentation on Agarose Gels.** The characteristic formation of oligonucleosome-sized fragments of multiples of ~200 bp producing typical DNA ladders on agarose gels is the biochemical hallmark of apoptosis. After treatments, cells were harvested and lysed in a lysis buffer of 20 mM Tris (pH 8.0), 20 mM EDTA, and 1% SDS containing 300 μg of proteinase K at 55°C for 30 min. Protein was removed through the addition of sodium acetate acid and centrifugation. DNA was precipitated from the supernatant with the same volume of isopropanol and centrifuged at 14,000g for 1 min. The pellet was washed by 70% ethanol two times. DNA was dissolved in Tris-EDTA buffer containing 0.5 U of DNase-free RNase A and incubated for 2 h at 37°C. DNA (10 μg) was electrophoresed at 70 V in a 1.8% agarose gel in Tris-borate-EDTA buffer, stained with SYBO Gold, and photographed with UV illumination. DNA ladder molecular weight markers (100 bp) were added to each gel as a reference for the analysis of internucleosomal DNA fragmentation.

**In Situ Terminal Deoxynucleotidyl Transferase Biotin-DUTP Nick End Labeling (TUNEL) Assay.** To assess the occurrence of cocaine-induced apoptosis in FRMCs in situ, labeling of fragmented DNA was performed with TUNEL with a commercially available in situ cell death detection kit (Boehringer Mannheim) according to the manufacturer’s instruction. In brief, monolayers of cardiomyocytes grown on coverslides were fixed with 4% paraformaldehyde solution for 30 min at room temperature and then washed with PBS and incubated with permeabilization solution (0.1% Triton X-100, 0.01% sodium citrate) for 7 min at room temperature. Apoptotic cells were labeled with 50 μl of TUNEL reaction mixture and conjugated with alkaline phosphatase through incubation with 50 μl of converter-alkaline phosphatase for 30 min at 37°C. After substrate reaction, stained cells were analyzed under a light microscope. The nucleus was counterstained with 0.5% methyl green for 2 min at room temperature. Negative control samples for TUNEL staining lacked terminal deoxynucleotidyl transferase. Positive controls were performed through the incubation of fixed and permeabilized cells with 1 U DNase/100 μl mixture for 10 min at room temperature.

**Quantitative Analysis of Apoptotic Cells.** Fluorescent DNA-binding dyes were commonly used to define nuclear chromatin morphology as a quantitative index of apoptosis within a cell culture system (Ceneviva et al., 1998; Harada-Shiba et al., 1998). Cells grown on coverslips were washed with PBS and fixed in methanol/acetate acid (3:1) at 4°C for 5 min. After fixation, the cells were washed for 10 min with the fluorescent DNA-binding dye Hoechst 33258 at 8 μg/ml, and nuclear morphology was examined by fluorescence microscopy. Individual nuclei were visualized at 400× to distinguish the normal uniform nuclear pattern from the characteristic condensed coalesced chromatin pattern of apoptotic cells. To quantify apoptosis, 500 nuclei from random microscopic fields were analyzed, and the percentage of apoptotic cells was calculated as the number of (apoptotic cells/total number of cells) × 100%. Each experiment was conducted in triplicate and repeated at least three times.

**Determination of Cytochrome c Translocation.** To detect the release of cytochrome c from the mitochondria to the cytosol, cells were harvested after treatments, resuspended 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 aprotinin, 10 μg/ml leupeptin), and incubated for 10 min on ice. Cells were then disrupted in a microultrasonnic cell disrupter for 10 s and centrifuged at 750g for 15 min at 4°C. The supernatant (cytosolic fraction) was removed and maintained at −80°C. The pellet containing the mitochondria was resolved in lysis buffer. Protein content was determined using a standard colorimetric assay (Bio-Rad). Proteins were separated in 15% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with monoclonal antibody against cytochrome c (1:500) in Tris-buffered saline Tween 20 buffer containing 5% nonfat milk and 1% fetal bovine serum for 1 h at room temperature. After washing, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG (1:2000) and visualized using an enhanced chemiluminescence detection system. Results were quantified with a scanning densitometer (model 670; Bio-Rad). A standard of cytochrome c was added to each gel as a reference for the analysis of cytochrome c in the cytosolic and mitochondrial fractions.

Statistical Analysis. Data are presented as mean ± S.E. In all cases, n refers to the number of experiments performed. Each experiment was conducted with the cells pooled from the average of 20 fetal rat hearts obtained from two pregnant rats. Statistical analyses were performed by ANOVA followed by Newman-Keuls post hoc tests. Differences were considered significant at a value of P < .05.

Results

Cocaine-Induced Apoptosis in Cardiac Myocytes. Figure 1 shows cocaine-induced phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane detected by the phosphatidylserine-binding protein annexin V conjugated with FITC. Without membrane permeabilization, no staining of control FRMCs was observed. After treatment of the cells with cocaine (100 μM) for 12 h, binding of annexin V-FITC to the cell surface was observed (Fig. 1A), suggesting the early stage of cocaine-induced apoptosis in FRMCs. No staining of the apoptotic FRMCs with trypan blue and propidium iodide was detected at this stage. After 24-h treatment with cocaine, cells were stained by both annexin V-FITC and propidium iodide (Fig. 1B).

Figure 2 shows representative cell morphological changes induced by cocaine. Under control conditions, FRMC morphology appears normal and cell density is approaching confluence (Fig. 2A). After exposure to cocaine (100 μM for 24 h), the cells exhibited the characteristic features of cell shrinkage, rounding, and partial detachment and demonstrated the lobulated appearance of apoptotic cells (Fig. 2B). An assessment of nuclear chromatin morphology by Hoechst 33258 staining using fluorescence microscopy indicated condensed, coalesced, and fragmented nuclei induced by cocaine (Fig. 3). In accordance, cocaine induced formation of oligonucleosome-sized fragments of DNA as ladders of ~200 bp on agarose gels in a time-dependent manner (Fig. 4). Cocaine-induced DNA fragmentation in FRMCs was also detected in situ by terminal deoxynucleotidyl transferase labeling of terminal deoxynucleotidyl transferase labeling of 3’ DNA ends (Fig. 5).

Quantification of cocaine-induced apoptotic nuclei defined by the fluorescent DNA-binding dye Hoechst 33258 indicated that cocaine induced apoptosis in FRMCs in a time- and dose-dependent manner. Figure 6 shows that cocaine (100 μM) time-dependently induced apoptosis in FRMCs. At 96 h of incubation, cocaine (3–500 μM) produced concentration-dependent increases in apoptotic cells with propidium iodide and the maximal response of 4.3 ± 0.15 and 314.7 ± 15.5% of the control, respectively (Fig. 7). Cocaine-induced apoptosis (48.9 ± 5.6% versus 21.8 ± 1.6% for control, P < .01, n = 3) was blocked by the opioid receptor antagonist naltrexone (100 μM; 48.9 ± 5.6% versus 25.7 ± 2.1%, P < .01, n = 3), suggesting an involvement of opioid receptors in the cocaine-induced apoptosis in FRMCs.

Effect of Cyclosporin A on Cocaine-Induced Apoptosis. To reveal the potential cellular mechanisms, we exam-
ined the effect of cyclosporin A, which inhibits cytochrome c release from the mitochondria, on cocaine-induced apoptosis in FRMCs. As shown in Fig. 8, cyclosporin A inhibited cocaine-induced apoptosis in a concentration-dependent manner with a pD₂ value of 6.9 ± 0.3. The maximum inhibition of 86% was obtained at 3 μM cyclosporin A. Control experiments demonstrated that the solvent ethanol (0.03% in the final concentration for all treatments) did not affect basal or cocaine-induced apoptosis in FRMCs (data not shown). At the higher concentration of 10 μM, cyclosporin A itself showed cytotoxic effects and induced apoptosis in FRMCs. The inhibitory effect of cyclosporin A on cocaine-induced apoptosis was also defined on the basis of internucleosomal DNA fragmentation assessed by agarose gel electrophoresis. As shown in Fig. 9, cocaine induced DNA fragmentation in FRMCs (Fig. 9, lane 5), whereas control cells showed no detectable DNA fragmentation (Fig. 9, lane 1). The coinubcation of cyclosporin A (1 and 3 μM, respectively) with cocaine eliminated cocaine-induced DNA ladders in FRMCs (Fig. 9, lanes 2 and 3, respectively). Also shown in Fig. 9 is that the higher concentration (10 μM) of cyclosporin A itself induced DNA fragmentation in FRMCs (Fig. 9, lane 4).

**Cocaine-Induced Cytochrome c Release.** Although the inhibitory effect of cyclosporin A suggests that the release of cytochrome c from the mitochondria may play an important role in cocaine-induced apoptosis, further studies are needed to confirm this.
role in cocaine-induced apoptosis in FRMCs, we pursued further studies to examine the effect of cocaine on cytochrome c translocation in FRMCs. The representative Western immunoblot showed that the monoclonal antibody for cytochrome c detected a single band at the expected size of 15 kDa (Fig. 10, top). After cocaine (100 μM) treatment, there was an increase in cytochrome c levels in the cytosolic fraction and an accordant decrease in cytochrome c levels in the mitochondrial fraction (Fig. 10, bottom). Quantitative densitometry for three independent experiments revealed that cocaine increased cytosolic cytochrome c levels by 3.1-fold and decreased mitochondria cytochrome c levels by 60% (Fig. 10, bottom). As shown in Fig. 10, cyclosporin A (1 μM) inhibited the cocaine-induced translocation of cytochrome c in FRMCs.

Effect of Caspase Inhibitors on Cocaine-Induced Apoptosis. To further support the role of cytochrome c in cocaine-induced apoptosis in FRMCs, we determined the effect of Z-LEHD-FMK (caspase-9 inhibitor) and Ac-DEVD-CHO (caspase-3 inhibitor) on cocaine-induced apoptosis. It has been well documented that caspase-9, functioning as an initiator caspase, is involved in cell death induced by cytotoxic agents and is activated by cytochrome c and Apaf-1. As shown in Fig. 11, both caspase-3 and caspase-9 inhibitors (Ac-DEVD-CHO and Z-LEHD-FMK, respectively) blocked cocaine-induced apoptosis in FRMCs.

Discussion

The present study has demonstrated for the first time that cocaine causes a direct cytotoxic effect on fetal rat heart and induces apoptosis in FRMCs in a time- and dose-dependent manner. The release of cytochrome c from the mitochondria into the cytosol and its subsequent activation of caspase-9...
and caspase-3 play a key role in cocaine-induced apoptosis in FRMCs.

Cocaine-induced apoptosis in FRMCs was characterized by multiple morphological and biochemical features of typical apoptotic cells. It has been demonstrated that annexin V-FITC binding is a specific and sensitive method to identify early stage of apoptosis (Casciola-Rosen et al., 1996; Shounan et al., 1998; Walsh et al., 1998). The present finding of annexin V-FITC binding of FRMCs at 12-h incubation with cocaine (Fig. 1A) suggests that cocaine induces apoptosis in FRMCs as early as 12 h. At this early stage of apoptosis, cell shrinkage and DNA fragmentation induced by cocaine were still not detectable in FRMCs, confirming that phosphatidylserine externalization precedes other morphological and biochemical changes in cocaine-induced apoptotic FRMCs. In addition, as reported in many other cells (Casciola-Rosen et al., 1996; Shounan et al., 1998; Walsh et al., 1998), annexin V binding preceded the loss of membrane integrity in apoptotic cells induced by cocaine in the present study. This is supported by the finding that propidium iodide and trypan blue failed to stain apoptotic FRMCs at 12 h. The finding of propidium iodide staining after 24-h incubation of cocaine suggests that the plasma membrane becomes increasingly permeable during the later stages of apoptosis in FRMCs.

Cocaine-induced apoptosis in FRMCs was also clearly demonstrated by morphological changes such as cell shrinkage and rounding, which are characteristic features of apoptotic death. Moreover, the simultaneous assessment of nuclear chromatin morphology verified that these cells eventually manifested typical apoptotic condensed and fragmented nuclei. In addition, we have confirmed that the process of apoptosis defined on the basis of cellular and nuclear chromatin morphology correlates with apoptosis defined on the basis of internucleosomal DNA fragmentation assessed by in situ labeling and gel electrophoresis. Similar findings of cocaine-induced apoptosis have been reported in mice hepatocytes (Cascales et al., 1994) and fetal mice cortical neurons (Nassogne et al., 1997). However, in the previous studies of fetal mice cortical neurons, cocaine-induced apoptosis was observed at the very high concentration of 500 μM (Nassogne et al., 1997). In the present study, we demonstrated that a cocaine concentration as low as 3 μM is able to induce an increase in apoptosis in cultured FRMCs. The EC50 value of cocaine in inducing apoptosis in FRMCs is ~50 μM. Serum levels of cocaine in human volunteers after a dose of 0.5 mg/kg range from 0.1 to 1 μM but are often considerably higher in active abusers, reaching the 100 μM level (Nassogne et al., 1997). Cocaine crosses the placenta and accumulates in the fetal compartment with a 3-fold higher level in the fetus than in the maternal plasma (Devane et al., 1989; Schenker et al., 1993). In addition, the maternal use of cocaine results in a rapid distribution of the drug to maternal and fetal tissues with several times higher concentrations in organs than in blood (Wiggins, 1992). Cocaine concentration in fetal heart is ~3.4-fold higher than that in the blood (Poklis et al., 1985). Taken together, these studies indicate that the physiological relevance of the present finding that cocaine induces apoptosis in fetal cardiac myocytes is fully warranted.

The present finding that cyclosporin A dose dependently inhibited cocaine-induced apoptosis in FRMCs suggests that the release of cytochrome c from the mitochondria plays a key role in cocaine-induced apoptosis in fetal rat cardiomyocytes. In many, if not all, apoptosis scenarios, there is an opening of a large conductance channel known as the mitochondrial permeability transition pore and collapse of the mitochondria inner transmembrane potential, leading to the release of cytochrome c (Green and Reed, 1998). It has been reported that cocaine (10–100 μM) inhibits the activity of the terminal electron transport system of the mitochondria in fetal rat heart and decreases the heart rates (Fantel et al., 1990). More recent studies show that cocaine (10 μM to 1 mM for ≥24 h) causes a concentration- and time-dependent decrease in mitochondrial membrane potential in primary cultures of rat cardiomyocytes, and the decline in membrane potential occurs before the manifestation of cytotoxicity shown with the exposure of cocaine (Yuan and Acosta, 1996). It has been well documented that cyclosporin A prevents cytochrome c release by stabilizing the mitochondrial transmembrane potential and inhibits apoptosis (Green and Reed, 1998; Jurgensmeier et al., 1998; Marzo et al., 1998; Walter et al., 1998). In the present study, we demonstrated that cyclosporin A inhibits cocaine-induced apoptosis in FRMCs in a dose-dependent manner with an IC50 value of 0.1 μM. Similar findings were obtained in human endothelial cells, in which cyclosporin A dose dependently inhibited oxidized low-density lipoprotein-induced apoptosis (Walter et al., 1998). In contrast to the previous finding (Walter et al., 1998), present studies of both morphological changes and DNA fragmentation on agarose gel demonstrated that a high concentration of cyclosporin A (10 μM) itself induced apoptosis in FRMCs. The mechanisms underlying cyclosporin A-induced apoptosis is not clear at the present.

The further evidence supporting the role for cytochrome c in cocaine-induced apoptosis in FRMCs comes from the study of cocaine-induced cytochrome c translocation in these cells. In the present study, the mitochondria were separated from the cytosolic fraction, and the translocation of cytochrome c was detected by Western blotting. The findings of the present study demonstrate that cocaine stimulation induces cytochrome c release from the mitochondria into the cytosol. In accordance with the finding that it inhibited cocaine-induced apoptosis, cyclosporin A blocked cocaine-induced cytochrome c translocation in FRMCs. Similar findings were obtained in human endothelial cells where cyclosporin A was shown to block oxidized low-density lipoprotein-induced apoptosis and cytochrome c release (Walter et al., 1998). The notion that cocaine-induced apoptosis in FRMCs may involve the release of cytochrome c from the mitochondria has been further supported by the studies of caspase inhibitors on cocaine-induced apoptosis. It has been well documented that caspase cascade includes both initiator caspases and effector caspases (Ashkenazi and Dixit, 1998; Green and Reed, 1998; Thornberry and Lazebnik, 1998). Proapoptotic signals activate an initiator caspase that, in turn, activates effector caspases, leading to cell apoptosis. Two initiator caspases, caspase-8 and caspase-9, mediate distinct sets of death signals. Caspase 8 is associated with apoptosis involving death receptors that are activated by ligands of the tumor necrosis factor gene superfamily (Ashkenazi and Dixit, 1998). In contrast, caspase-9 is involved in death induced by cytotoxic agents and is activated by cytochrome c and Apaf-1 (Green and Reed, 1998; Thornberry and Lazebnik, 1998). In the present study, we demonstrated that cocaine-induced apoptosis in FRMCs is blocked by caspase-9 and caspase-3 inhibitors, respectively.
This finding reinforces the notions that cocaine-induced apoptosis in FRMCs is mediated by the mitochondria pathway and that the release of cytochrome c may play a key role in cocaine-induced apoptosis.

Although the mechanisms underlying cocaine-induced cytochrome c release in FRMCs are not clear at the present, recent studies have demonstrated that Bcl-2 protein associated with mitochondrial membrane prevents the loss of mitochondrial membrane potential and the efflux of cytochrome c (Adams and Cory, 1998; Green and Reed, 1998; Shimizu et al., 1998). The binding of Bax to Bel-2 proteins forms the Bax/Bel-2 complex and leads to the release of cytochrome c (Reed, 1995; Herrman et al., 1996; Adams and Cory, 1998; Green and Reed, 1998). It has been demonstrated that cardiomyocytes isolated from near-term fetal rats had a much lower content of antian apoptotic Bel-2 protein than of proapoptotic Bax (Wang et al., 1998). After the induction of apoptosis with serum withdrawal, the levels of Bax protein and the formation of Bax/Bel-2 complex were found to increase in fetal rat cardiomyocytes (Wang et al., 1998). The effects of cocaine on Bax and Bel-2 protein expression and their roles in cocaine-induced apoptosis in FRMCs remain elusive.

In summary, we have shown that cocaine induces time- and concentration-dependent increases in apoptotic cells in cultured FRMCs. Cocaine-induced apoptosis in the cardiomyocytes is associated with the release of cytochrome c from the mitochondria into the cytosol and the subsequent activation of caspase-9 and caspase-3. Increased apoptosis of cardiomyocytes in the developing heart is likely to lead to the development of cardiomyopathy and congenital heart diseases (Bromme and Holtz, 1996; Anversa et al., 1997; Maclellan and Schneider, 1997; Haustetter and Izumo, 1998). A recent study demonstrated that the induction of apoptosis in human fetal cardiac myocytes facilitated surface accessibility of SSA/Ro and SSB/La antigens to maternal autoantibodies and that the subsequent influx of leukocytes could damage surrounding healthy fetal cardiomyocytes (Miranda et al., 1998). Because cardiomyocytes are highly differentiated cells and rarely replicate after birth, the prenatal loss of cardiomyocytes through apoptosis may result in a permanent reduction of the number of functioning units in the myocardium. Indeed, the use of cocaine during pregnancy has been associated with cardiac malfunction in human newborns (Van de Bor et al., 1990; Lipschultz et al., 1991; Norris and Hill, 1992). In addition, the maternal use of cocaine during pregnancy produces an hypoxic effect on the fetus. Hypoxia enhances the expression of Fas antigen mRNA in rat cardiomyocytes (Tanaka et al., 1994), which is likely to augment the direct apoptotic effect of cocaine on the fetal heart during pregnancy.

References


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