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Cocaine induces apoptosis in fetal rat myocardial cells through the p38 MAPK and mitochondrial/cytochrome c pathways

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FRMCs:	fetal rat myocardial cells
MAPK:	mitogen-activated protein kinase
ERK:	extracellular signal-regulated kinase
MEK:	mitogen-activated protein kinase kinase
JNK:	c-Jun N-terminal kinase
Ac-DEVD-CHO:	N-acetyl-Asp-Glu-Val-Asp-CHO

Abstract

Cocaine induces apoptosis in fetal rat myocardial cells (FRMCs). However, the mechanisms are not clear. The present study examined the role of p38 MAPK and cytochrome c release in the cocaine-induced apoptosis in primary culture of FRMCs prepared from the fetal heart of 21-day gestational age. Cocaine induced time-dependent, concurrent increases in cytochrome c release and activities of caspase-9 and caspase-3, which preceded apoptosis. Caspase-8 was not activated. In accordance, cyclosporin A and the inhibitors of caspase-9 and caspase-3 inhibited cocaine-induced caspase activation and apoptosis. Cocaine stimulated a transient increase in the p38 MAPK activity at time point of 15 min, but reduced the ERK activity at 5 and 15 min in FRMCs. The p38α MAPK inhibitor SB203580 inhibited cocaine-induced activation of caspases and apoptosis. In contrast, the p38β MAPK and MEK/ERK inhibitors SB202190 and PD98059, respectively, increased apoptosis in the absence of cocaine, and potentiated cocaine-induced apoptosis. Consistent with its inhibition of apoptosis, SB203580 inhibited cocaine-induced cytochrome c release and activation of caspase-9 and caspase-3. In addition, cocaine induced a decrease in Bcl-2 protein levels, with no effect on Bax levels. The cocaine-mediated reduction of Bcl-2 levels was not affected with SB203580 and the caspase inhibitors. The results suggest that in FRMCs, p38α MAPK plays an important role in the cocaine-induced apoptosis by promoting cytochrome c release, downstream or independent of Bcl-2 protein-mediated regulation. In contrast, p38β MAPK and ERK protect fetal myocardial cells against apoptosis.

Cocaine abuse among women of childbearing age is prevalent in the United States. A number of developmental disorders in human infants have been attributed to cocaine exposure *in utero*. These include decreased birth weight and head circumference, sudden infant death syndrome, systemic hypertension and tachycardia (Handler et al., 1991; Silvestri et al., 1991). Cocaine can cross the placenta and accumulate in the fetus (Schenker et al., 1993). Our recent studies have demonstrated that maternal cocaine administration during pregnancy induces apoptosis in the fetal heart (Xiao et al., 2001). Cocaine also directly induces an increase in apoptotic cell death in fetal rat myocardial cells (FRMCs) (Xiao et al., 2000). However, the cellular and molecular mechanisms responsible for cocaine-induced cardiac myocyte apoptosis are presently not clear.

Apoptotic cell death is characterized by the activation of a unique class of aspartate-specific proteases, *i.e*. caspases. Sequential activation of caspases results in cleavage of substrate proteins and breakdown of DNA molecules. It has been well documented that the caspase cascade involved in apoptosis includes both initiator caspases and effector caspases. Proapoptotic signals activate an initiator caspase that, in turn, activates effector caspase, e.g. caspase-3, leading to cell apoptosis. Two initiator caspases, caspase-8 and caspase-9, mediate distinct sets of death signals. Caspase-8 is activated by the death signals that bind to death receptors on cell surface (Ashkenazi and Dixit, 1998). The ligands that bind to death receptors belong to the tumor necrosis factor gene superfamily. In contrast, caspase-9 is activated by cytochrome c released from mitochondria (Green and Reed, 1998). The mitochondria/cytochrome c death pathway-mediated myocyte apoptosis has been demonstrated in neonatal rat cardiomyocytes (Cook et al., 1999; Remondino et al., 2003; Yamanaka et al., 2003). It has been reported that cocaine inhibits the activity of the terminal electron transport system of mitochondria in fetal rat heart, and

decreases heart rate (Fantel et al., 1990). Other studies showed that cocaine caused a concentration- and time-dependent decrease in the mitochondrial membrane potential in primary cultures of rat cardiomyocytes, and the decline in the membrane potential occurred prior to manifestation of cytotoxicity shown with the exposure to cocaine (Yuan and Acosta, 1996). We have found that treatment of fetal rat cardiomyocytes with cocaine for 48 h increases cytochrome c release and apoptosis (Xiao et al., 2000). Although these studies suggest a likelihood role of the mitochondria/cytochrome c pathway in cocaine-induced apoptosis in myocardial cells, it remains unknown whether cocaine-induced cytochrome c release trails or precedes the activation of caspases and apoptosis.

It has been shown that cocaine induces oxidative stress in the heart and liver (Devi and Chan, 1996, 1999; Moritz et al., 2003, 2003). Stress-responsive mitogen-activated protein kinases (JNK and p38 MAPKs) have been implicated in cell death/apoptosis in the myocardium (Sugden and Clerk, 1998). Studies of pharmacological inhibitors and the dominant negative mutants suggest that activation of p38 MAPKs is involved in the mitochondrial activation-mediated cell death pathway (Assefa et al., 2000; Zhuang et al., 2000; Cheng et al., 2001; Park et al., 2003). Nevertheless, the role of p38 MAPKs in cardiac myocyte apoptosis remains controversial (Wang et al., 1998; Zechner et al., 1998, Ma et al., 1999; Mackay and Mochly-Rosen, 1999). Studies in neonatal rat cardiomyocytes demonstrated that among the 3 major MAPKs only p38 MAPKs were activated by ischemia, and that inhibition of p38α MAPK by SB203580 reduced myocyte apoptosis (Mackay and Mochly-Rosen, 1999). In contrast, other studies showed that overexpression of MAPK kinase 6, an upstream activator of p38 MAPKs, resulted in a protection of cardiac myocyte from apoptosis (Zechner et al., 1998). Activation of different p38 MAPK isoforms may explain

these findings, and it has been suggested that p38α is pro-apoptotic, whereas p38β is anti-apoptotic in neonatal rat cardiomyocytes (Sugden and Clerk, 1998; Wang et al., 1998).

In the present study, we determined the time courses of cocaine-induced cytochrome c release, caspase activities, and apoptosis in FRMCs. We also determined the time courses of cocaine in stimulating activities of p38 MAPKs and extracellular signal-regulated kinases (ERK). To test the hypothesis that p38α MAPK is an upstream signal of the cocaine-induced apoptosis, we determined the effects of SB203580, a relatively selective chemical inhibitor of p38α MAPK (Sugden and Clerk, 1998), on cocaine-induced cytochrome c release and caspase activation. Given that Bcl-2 family proteins are key regulators of mitochondrial integrity and cytochrome c release, and are important modulators of cardiac myocyte apoptosis, we also determined the effect of cocaine on Bcl-2 and Bax protein levels, and the role of p38α MAPK.

Methods

Materials. Cell culture medium DMEM was purchased from Mediatech Cellgro (Herndon, VA). Monoclonal anti-α-cardiac sarcomeric actin antibody, Hoechst 33258, cocaine, trypsin, cyclosporin A and PBS were from Sigma Chemical Co (St. Louis, MO). Anti-cytochrome c antibody and Ac-DEVD-CHO were from PharMingen (San Diego, CA). Z-LEHD-FMK was from Kamiya Biomedical (Seattle, WA). SB203580, SB202190 and PD98059 were from CalBioChem. ERK and p38 MAPKs nonradioactive IP-kinase assay kits were from New England Biolabs. Caspase-3, -8, -9 colorimetric assay kits were from R&D Systems (Minneapolis, MN). Bcl-2 and Bax antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Primary cardiomyocyte culture. A primary culture of fetal rat myocardial cells was prepared from the hearts of 21-day gestational age Sprague-Dawley rats, as previously described (Xiao et al., 2000). Cells were plated at a density of 25,000 cells/ml in 6-well tissue culture plate in DMEM supplemented with 15% fetal bovine serum (FBS) and 1% antibiotics (10,000 U/ml penicillin and 10,000 μg/ml streptomycin), and were cultured at 37 °C in 95% air/5% CO_2. BrdU (0.1 mM) was added in the medium to reduce the growth of non-myocyte cells. Within 3 days, a monolayer of spontaneously beating cells was formed. As established by visual determination of immunostaining, >95% of the cells manifested spontaneous contractions and were α-cardiac sarcomeric actin positive. All experiments in the present study used 70% to 80% confluent cells. After three days in the culture, cells were transferred to 0.1% FBS medium and cultured for 24 h. Cells were then exposed to various doses of cocaine and other chemicals for the indicated times.

All procedures and protocols used in the present study were approved by the Animal Research Committee of Loma Linda University and followed the guidelines in the NIH Guide for the Care and Use of Laboratory Animals.

Quantitative analysis of apoptosis. Cells grown on coverslips were examined for apoptosis by examining nuclear chromatin morphology stained with fluorescent DNA-binding dye Hoechst 33258, as described previously (Xiao et al., 2000). Cells with condensed or fragmented nuclei were considered to be apoptotic. 500 nuclei were examined for each sample, and the number of apoptotic cells was expressed as the percentage of total cell population.

p38 MAPK and ERK activity assay. p38 MAPK and ERK activities were determined using the p38 MAPK and ERK activity assay kits from New England Biolabs. Cells were washed twice with ice-cold PBS, and lysed in 500 μl lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM PMSF) for 5 min. Cells were then sonicated for 4 times at 5 s each. After centrifugation, the supernatant was collected. 200 μl cell lysate (~ 200 μg total protein) was transferred to a microcentrifuge tube containing 20 μl of re-suspended immobilized phospho-p38 MAPK (Thr^{180}/Tyr^{182}) monoclonal antibody or 15 μl of re-suspended immobilized phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) monoclonal antibody, and incubated with gentle rocking overnight at 4 °C. The samples were then centrifuged at 200 ×g for 30 s at 4 °C. The pellet was washed twice with 500 μl of lysis buffer and with 500 μl of kinase buffer, provided in the kits. The pellet was then re-suspended in 50 μl kinase buffer supplemented with 200 μM ATP and 2 μg ATF-2 or Elk-1 fusion proteins (provided in the kits), respectively, and incubated for 30 min at 30 °C. The reaction mixture was separated by SDS-PAGE. Western blots were probed with the phospho-ATF-2 antibody (specific for phospho-Thr^{71}) and phospho-Elk-1antibody

(specific for phospho-Ser^{383}), respectively, provided in the kits. Immunoreactivity was detected by enhanced chemiluminescence.

Western blot analysis. Cells were harvested after treatments, and incubated in ice-cold lysis buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 µg/ml aprotinin, 10 µg/ml leupeptin) for 30 min. To detect cytochrome c, proteins in cytosolic and mitochondrial fractions were separated as previously described (Xiao et al., 2000). Total cellular protein was used to determine Bax and Bcl-2 protein levels. Protein content was determined using a standard colorimetric protein assay (Bio-Rad). Proteins were separated with 15% (cytochrome c) and 12% (Bax and Bcl-2) SDS-polyacrylamide gels, respectively. They were then transferred to nitrocellulose membranes, and incubated with primary antibodies against Bax, Bcl-2, and cytochrome c, respectively. After washing, the membranes were incubated with HRP-conjugated secondary antibodies, and visualized using an enhanced chemiluminescence detection system (Amersham). Results were quantified with KODAK Electrophoresis Documentation and Analysis System and KODAK 1D Image Analysis Software.

Caspase activity assay. Activities of caspase-3, caspase-8, caspase-9 were determined using the corresponding caspase activity detection kits from R&D Systems, as previously described (He et al., 2000, 2001). Briefly, 100 µg total cell protein was added to 50 µl reaction buffer and 5 µl substrates of DEVD-*p*NA, IETD-*p*NA, and LEHD-*p*NA, respectively. Samples were incubated at 37 °C for 2 h and the enzyme-catalyzed release of *p*NA was quantified at 405 nm using a microtiter plate reader. At each time point of study, the values of cocaine treated samples were normalized to corresponding untreated controls allowing determination of the fold increase in caspase activity.

Statistical analysis. Data are presented as the means ± SEM. Statistical analyses were performed by ANOVA followed by Newman-Keuls post hoc test. Differences were considered significant at $P < 0.05$.

Results

Time courses of cocaine-induced cytochrome c release and apoptosis. As shown in Fig. 1, 100 μM cocaine produced a time-dependent increase in cytochrome c levels in the cytosolic fraction in FRMCs. Cytochrome c release was first seen at 4.5 h and peaked at 24 h. Cocaine-induced time-dependent increases in apoptosis trailed the release of cytochrome c (Fig. 1). Our previous study showed that 100 μM cocaine produced a sub-maximal increase in apoptosis in FRMCs (Xiao et al., 2000). The serum levels of cocaine in active drug abusers are often >100 μM and repeated uses of cocaine produce a dose-related accumulation in serum cocaine concentrations (Benowitz, 1993; Nassogne et al., 1997; Jufer et al., 1998). Furthermore, cocaine crosses the placental and accumulates in the fetal compartment with 3-fold higher in the fetus than in maternal plasma (DeVane et al., 1989; Schenker et al., 1993). Therefore, the pathophysiological relevance of 100 μM cocaine used in the present study is warranted.

Cocaine-induced caspase activity. To further support the role of cytochrome c and its subsequent activation of the caspase cascade in cocaine-induced apoptosis in FRMCs, we determined the time courses of cocaine-induced activation of the protease activities of caspase-9, caspase-8, and caspase-3. As shown in Fig. 2, cocaine produced concurrent time courses in activating caspase-9 and caspase-3. The activities of caspase-9 and caspase-3 were increased at 4.5 h. Caspase-9 reached the peak at 6 h, and caspase-3 reached the peak at 12 h. In contrast, caspase-8 activity did not change significantly during the time period of study (Fig. 2). To determine whether cytochrome c played a role in cocaine-induced increases in caspase-9 and caspase-3 activities, we examined the effect of cocaine treatment for 12 h on the caspase activities in the presence of cyclosporin A (cytochrome c release inhibitor), Ac-DEVD-CHO (caspase-3 inhibitor), and Z-LEHD-FMK (caspase-9 inhibitor). As shown in Fig. 3, cyclosporin

A, Ac-DEVD-CHO, and Z-LEHD-FMK did not change the basal caspase-3 activity, but blocked cocaine-induced increase in the caspase-3 activity. Similarly, cocaine-induced caspase-9 activity was blocked by cyclosporin A and Z-LEHD-FMK (Fig. 3). However, the caspase-3 inhibitor Ac-DEVD-CHO only partially blocked cocaine-induced increase in the caspase-9 activity (Fig. 3). None of the inhibitors had effects on the basal caspase-9 activity. In accordance with the inhibition of caspase-9 and caspase-3 activities, cyclosporin A, Ac-DEVD-CHO, and Z-LEHD-FMK blocked cocaine-induced apoptosis in FRMCs (data not shown). Our previous study showed that cyclosporin A and the caspase inhibitors blocked cocaine-induced apoptosis at 24 h and 48 h treatments in FRMCs (Xiao et al., 2000). Taken together, these studies suggest that the effects of these inhibitors are not simply a delay of the onset of cell death, but rather a protection against cocaine-induced apoptosis in FRMCs.

Effects of p38 MAPKs and ERK on cocaine-induced caspase activation and apoptosis. To determine the potential role of p38 MAPKs and ERK in cocaine-induced apoptosis, we first examined the effect of cocaine on the activities of p38 MAPKs and ERK in FRMCs. As shown in Fig. 4, cocaine induced a rapid and transient increase in the activity of p38 MAPKs at 15 min. In contrast, cocaine produced a transient decrease in the activity of ERK at 5 and 15 min (Fig. 5). We then determined the effects of selective inhibitors of p38 MAPKs and ERK on cocaine-induced apoptosis. These inhibitors were PD98059 for MEK/ERK, and two relatively specific inhibitors of p38α MAPK and p38β MAPK, SB203580 and SB202190 (Sugden and Clerk, 1998). As shown in Fig. 6, SB203580 did not affect myocyte apoptosis in the absence of cocaine, but blocked cocaine-induced apoptosis. In contrast, both SB202190 and PD98059 significantly increased myocyte apoptosis in the absence of cocaine, and potentiated cocaine-induced apoptosis in FRMCs (Fig. 6). Consistent with its inhibition of apoptosis,

SB203580 blocked cocaine-induced increases in the activities of caspase-9 and caspase-3 (Fig. 7). We further determined the effect of SB203580 on cocaine-induced cytochrome c release in FRMCs. As shown in Fig. 8, SB203580 had no effect on the basal cytosolic cytochrome c levels, but blocked cocaine-induced increase in cytochrome c levels in the cytosol.

Effect of cocaine on Bax and Bcl-2 protein levels. Given that Bcl-2 family proteins are key regulators of mitochondrial integrity and cytochrome c release, and are important modulators of cardiac myocyte apoptosis, we determined the effect of cocaine on Bcl-2 and Bax protein levels, and the role of p38α MAPK. Fig. 9 shows that cocaine treatment (100 μM for 12 h) decreases Bcl-2 protein levels, but does not affect Bax levels in FRMCs. The cocaine-mediated decrease of Bcl-2 levels was not affected with SB203580 and the inhibitors of caspase-9 and caspase-3 (Fig. 10).

Discussion

The present study has demonstrated that cocaine induces a time-dependent cytochrome c release, which precedes cocaine-induced apoptosis in FRMCs. In addition, cocaine activated caspase-9 and caspase-3, but not caspase 8, with concurrent time courses as that of cytochrome c release. Furthermore, cocaine-induced activation of caspase-9 and caspase-3 was blocked by cyclosporin A, an inhibitor of mitochondrial cytochrome c release (Green and Reed, 1998; Xiao et al., 2000). Collectively, these results suggest that cytochrome c release is an upstream signal and a cause of the cocaine-induced apoptosis in FRMCs. 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. This is further supported with 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).

In the present study, we demonstrated that cocaine induced a rapid and transient increase in p38 MAPK activity in FRMCs. The time course of cocaine-induced activation of p38 MAPK was comparable to those found in the previous studies in which ischemia/reperfusion activated p38 MAPK in the heart (Ma et al., 1999; Yue et al., 2000). Activation of p38 MAPK has been implicated in induction of apoptosis in response to various stimuli (Yue et al., 2000; Ma et al., 1999; Zhuang et al., 2000; Martin et al., 2002; Kim et al., 2002). The present finding of rapid activation of p38 MAPK after cocaine treatment suggests that activation of p38 MAPK plays a key role in the early events of cocaine-induced apoptosis. This is supported by the observation

that inhibition of p38 MAPK by SB203580 blocked apoptotic cell death induced by cocaine. The finding that SB202190 induced apoptosis in the absence of cocaine and potentiated cocaine-induced apoptosis is intriguing, and suggests potential differences in p38 MAPK isoforms in the regulation of apoptosis in FRMCs. The p38 subfamily of MAPKs consists of p38α, p38β, p38γ, and p38δ. It has been demonstrated that the pyridinyl imidazole compounds are selective inhibitors of p38α and p38β, but not p38γ and p38δ (Lee et al., 1994; Jiang et al., 1996; Lechner et al., 1996; Cuenda et al., 1997; Goedert et al., 1997; Wang et al., 1997). SB203580 and SB 202190 have been suggested to be relatively specific inhibitors of p38α MAPK and p38β MAPK, respectively (Sugden and Clerk, 1998). It has been shown that p38α MAPK is pro-apoptotic, whereas p38β MAPK is anti-apoptotic in neonatal rat cardiomyocytes (Sugden and Clerk, 1998; Wang et al., 1998; Mackay and Mochly-Rosen, 1999; Kaiser et al., 2004). Consistent with the present finding, many studies demonstrated that inhibition of p38 MAPK by SB203580 inhibited apoptosis induced by various stimuli (Ma et al., 1999; Mackay and Mochly-Rosen, 1999; Yue et al., 2000; Junn and Mouradian, 2001; Park et al., 2003). On the other hand, results of inhibition of p38 MAPK by SB202190 were controversial, and both increased and decreased apoptosis were reported (Karahashi et al., 2000; Park et al., 2002; Cicconi et al., 2003; Deacon et al., 2003; Caughlan et al., 2004). It has been shown that SB202190 induces apoptosis through inhibition of p38β MAPK (Nemoto et al., 1998). Taken together, the results suggest that p38α MAPK plays a key role in cocaine-induced apoptosis in FRMCs, whereas p38β MAPK may be part of the cellular survival response that is designed to protect FRMCs from apoptotic cell death.

The finding that the cocaine-induced increase in p38 MAPK activity preceded increased cytosolic cytochrome c levels suggests that activation of p38 MAPK is an upstream signal of cytochrome c release. This is supported by the results that SB203580 blocked cocaine-induced

cytochrome c release and activation of caspase-9 and caspase-3. Consistent with the present finding, it has been shown that dopamine induces oxidative stress and activation of p38 MAPK within 10 min of dopamine treatment, followed by cytochrome c release, caspase activation and apoptosis, which are blocked by SB203580 (Junn and Mouradian, 2001). In addition, previous studies showed that inhibition of p38 MAPK by SB203580 counteracted both cytochrome c release and apoptosis without affecting the processing of pro-caspase-8 (Zhuang et al., 2000; Assefa et al., 2000). This is in agreement with the present finding that cocaine had no effect of caspase-8 activation. These results suggest that p38 MAPK mediates apoptosis through caspase-8 independent, mitochondria/cytochrome c pathway death pathway in FRMCs.

The mechanisms for p38 MAPK-mediated cytochrome c release in FRMCs are not clear at present. It has been demonstrated that inhibition of p38 MAPK promotes a dramatic up-regulation of Bcl-2 protein levels in the hearts of transgenic mice (Kaiser et al., 2004). In the same study, it was shown that in primary neonatal cardiomyocyte cultures, adenoviral-mediated gene transfer of a p38 MAPK inhibitory mutant up-regulated Bcl-2, whereas expression of an activated p38 MAPK mutant down-regulated Bcl-2 protein levels (Kaiser et al., 2004). In the present study, we found that cocaine induced a down-regulation of Bcl-2 levels, without affecting Bax levels in FRMCs. The decreased Bcl-2 was not affected with the inhibitors of caspase-9 and caspase-3, suggesting it is an upstream signal of cocaine-induced caspase activation. The finding that SB203580 inhibited cocaine-induced cytochrome c release but did not affect the cocaine-mediated decrease in Bcl-2 levels suggests that the effect of p38 MAPK on mitochondria/cytochrome c release is downstream or independent of Bcl-2-mediated responses in FRMCs. In agreement with the present studies, it has been shown that inhibition of p38 MAPK with SB203580 decreased H. pylori-induced apoptosis without affecting Bcl-2 levels

(Choi et al., 2003). Furthermore, recombinant p38 MAPK caused a direct release of cytochrome c from mitochondria isolated from human memory B cells (Torcia et al., 2001). However, our results do not exclude the possibility that activation of p38 MAPK may lead to phosphorylation of Bcl-2 and translocation of Bax from the cytosol to mitochondria, as reported in other cell types (Yoshino et al., 2001; Deacon et al., 2003; Ishikawa et al., 2003; Park et al., 2003; Pastorino et al., 2003; Miyoshi et al., 2004).

In contrast to activation of p38 MAPK, cocaine induced an inhibition of ERK activity in FRMCs. Existing evidence suggests that ERK exerts a cytoprotective effect and counteracts pro-apoptotic effects associated with JNK and p38 MAPK activation (Xia et al., 1995, Kyriakis and Avruch, 1996; Robinson and Cobb, 1997; Chang and Karin, 2001). In the present study, we demonstrated that inhibition of ERK activity with the MEK/ERK inhibitor PD98059 induced apoptosis and potentiated cocaine-induced apoptosis. This is consistent with the previous studies showing that PD98059 increased apoptosis in the heart and other cell types (Aikawa et al., 1997; Yue et al., 2000; Yu et al., 2001; Park et al., 2003; Carvalho et al., 2004). In cardiac myocytes, PD98059 significantly increased myocyte apoptosis, and potentiated daunomycin-induced apoptosis (Zhu et al., 1999). The present finding that cocaine caused a rapid decrease in ERK activity and an increase in p38 MAPK activity suggests that dynamic balance of their activities is critical in determining cell survival and apoptosis in cardiomyocytes. Similar finding was observed in human cancer cells in which phytosphingosine induced a rapid decrease in ERK activity and a marked increase of p38 MAPK activity within 10 min of treatment, resulting in apoptosis (Park et al., 2003). In the present study, we found that cocaine decreased the ERK activity after 5 min of treatment, which preceded the increase of p38 MAPK activity observed at 15 min of treatment. The finding is intriguing and suggests a possibility that decreased ERK

activity may be an upstream signal of increased activity of p38 MAPK caused by cocaine. It has been shown that inhibition of ERK activity by PD98059 significantly increases p38 MAPK activity and apoptosis in myocytes and leukemia cells (Yue et al., 2000; Yu et al., 2001). Consistent with the central role of the mitochondria/cytochrome c death pathway demonstrated in cocaine-induced apoptosis in FRMCs, it has been shown that the proximal cause of enhanced apoptosis by inhibition of ERK is cytochrome c release and subsequent caspase activation (Yu et al., 2001).

In conclusion, the present study has clearly demonstrated that cocaine-induced apoptosis is mediated by the mitochondria/cytochrome c death pathway in FRMCs. Activation of p38α MAPK plays a key role in cocaine-induced cytochrome c release and subsequent caspase activation and apoptosis. In contrast, p38β MAPK and ERK present part of the cellular survival response that is designed to protect the myocytes from apoptotic cell death. The effect of p38α MAPK on cocaine-induced mitochondria/cytochrome c release in FRMCs may be downstream or independent of Bcl-2-mediated responses. However, the potential mechanisms of p38α MAPK activation in phosphorylation of Bcl-2 and translocation of Bax from the cytosol to mitochondria in FRMCs remain an intriguing area for further investigation.

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Footnotes

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Figure Legends

Figure 1. Time courses of cocaine-induced cytochrome c release and apoptosis in FRMCs. Fetal rat myocardial cells (FRMCs) were incubated with 100 μM cocaine for the time periods indicated. Apoptotic nuclei in control and cocaine-treated cells, respectively, at each time point were determined using Hoechst 33258 staining as described in *Experimental Procedures*. Cytosolic cytochrome c levels in control and cocaine-treated cells, respectively, at each time point were determined by Western blots using monoclonal cytochrome c antibody. Top panel shows the representative Western immunoblot of cytochrome c obtained at the time point of 4.5 h. Bottom panel shows the quantitative results. Results are expressed as percentage of apoptosis or cytochrome c levels of cocaine-treated samples versus the corresponding controls at each time point. Data are means ± SEM of five to six experiments. * $P < 0.05$ *vs*. control.

Figure 2. Time courses of cocaine-induced caspase activation in FRMCs. Fetal rat myocardial cells (FRMCs) were incubated with 100 μM cocaine for the time periods indicated. Caspase activities were determined in control and cocaine-treated cells, respectively, at each time point, using corresponding caspase colorimetric assay kits. Results are expressed as percentage of caspase activities of cocaine-treated samples versus the corresponding controls at each time point. Data are means ± SEM of six experiments. * $P < 0.05$ *vs*. control.

Figure 3. Effects of cyclosporin A and caspase inhibitors on cocaine-induced caspase activation in FRMCs. Fetal rat myocardial cells (FRMCs) were incubated with 100 μM cocaine (COC) for 12 h in the absence or presence of cyclosporin A (CSA, 1 μM), Ac-DEVE-CHO (100

μM), and Z-LEHD-FMK (20 μM), respectively. The caspase-3 activity (upper panel) and caspase-9 activity (lower panel) were determined using caspase-3 and caspase-9 colorimetric assay kits. Data are means ± SEM of six experiments. [a] $P < 0.05$ *vs*. control, [b] $P < 0.05$ *vs*. cocaine alone.

Figure 4. **Cocaine-induced p38 MAPK activation in FRMCs.** Fetal rat myocardial cells (FRMCs) were incubated with 30 μM cocaine for the time periods indicated. The activity of p38 MAPK was determined using a p38 MAPK activity assay kit from New England Biolabs as described in *Experimental Procedures*. Top panel shows the representative Western immunoblot of p38 MAPK-induced phospho-ATF-2 for control (C) and cocaine treated (T) samples at 5, 15, 30 and 60 min, respectively. Bottom panel shows the quantitative results. Results are expressed as percentage of p38 MAPK activity of cocaine-treated samples versus the corresponding controls at each time point. Data are means ± SEM of three experiments. * $P < 0.05$ *vs*. control.

Figure 5. **Cocaine-induced ERK activity in FRMCs.** Fetal rat myocardial cells (FRMCs) were incubated with 30 μM cocaine for the time periods indicated. The activity of ERK was determined using a ERK activity assay kit from New England Biolabs as described in *Experimental Procedures*. Top panel shows the representative Western immunoblot of ERK-induced phospho-Elk-1 for control (C) and cocaine treated (T) samples at 5, 15, 30 and 60 min, respectively. Bottom panel shows the quantitative results. Results are expressed as percentage of ERK activity of cocaine-treated samples versus the corresponding controls at each time point. Data are means ± SEM of three experiments. * $P < 0.05$ *vs*. control.

Figure 6. Effects of ERK and p38 MAPK inhibitors on cocaine-induced apoptosis in FRMCs. Fetal rat myocardial cells (FRMCs) were incubated with 100 μM cocaine (COC) for 12 h in the absence or presence of SB202190 (10 μM), SB203580 (10 μM), and PD98059 (30 μM), respectively. Apoptotic nuclei were determined using Hoechst 33258 staining. Data are means ± SEM of six experiments. [a] $P < 0.05$ *vs*. control, [b] $P < 0.05$ *vs*. the inhibitors alone.

Figure 7. Effects of SB203580 on cocaine-induced caspase activation in FRMCs. Fetal rat myocardial cells (FRMCs) were incubated with 100 μM cocaine for 12 h in the absence or presence of SB203580 (10 μM). The activities of caspase-9 and caspase-3 were determined using corresponding colorimetric caspase assay kits. Data are means ± SEM of six experiments. * $P < 0.05$ *vs*. control.

Figure 8. **Effects of SB203580 on cocaine-induced cytochrome c release in FRMCs.** Fetal rat myocardial cells (FRMCs) were incubated with 100 μM cocaine (COC) for 12 h in the absence or presence of SB203580 (SB, 10 μM). Cytosolic cytochrome c (Cyto c) levels were determined by Western blots using monoclonal cytochrome c antibody. Top panel shows the representative Western immunoblot of cytochrome c. PC, positive control of cytochrome c. Bottom panel shows the quantitative results. Data are means ± SEM of four experiments. * $P < 0.05$ *vs*. control (C).

Figure 9. Effects of cocaine on Bcl-2 and Bax protein levels in FRMCs. Fetal rat myocardial cells (FRMCs) were incubated with 100 μM cocaine for 12 h. Bcl-2 and Bax levels were determined by Western blots using Bcl-2 and Bax antibodies. Top panel shows the representative

Western immunoblot of Bcl-2 and Bax. Bottom panel shows the quantitative results. Data are means ± SEM of four experiments. * $P < 0.05$ *vs*. control.

Figure 10. **Effects of SB203580 and caspase inhibitors on cocaine-induced changes of Bcl-2 levels in FRMCs.** Fetal rat myocardial cells (FRMCs) were incubated with 100 μM cocaine for 12 h in the absence or presence of SB203580 (10 μM), Ac-DEVE-CHO (100 μM), and Z-LEHD-FMK (20 μM), respectively. Bcl-2 levels were determined by Western blots using Bcl-2 antibody. Data are means ± SEM of five experiments. * $P < 0.05$ *vs*. control.

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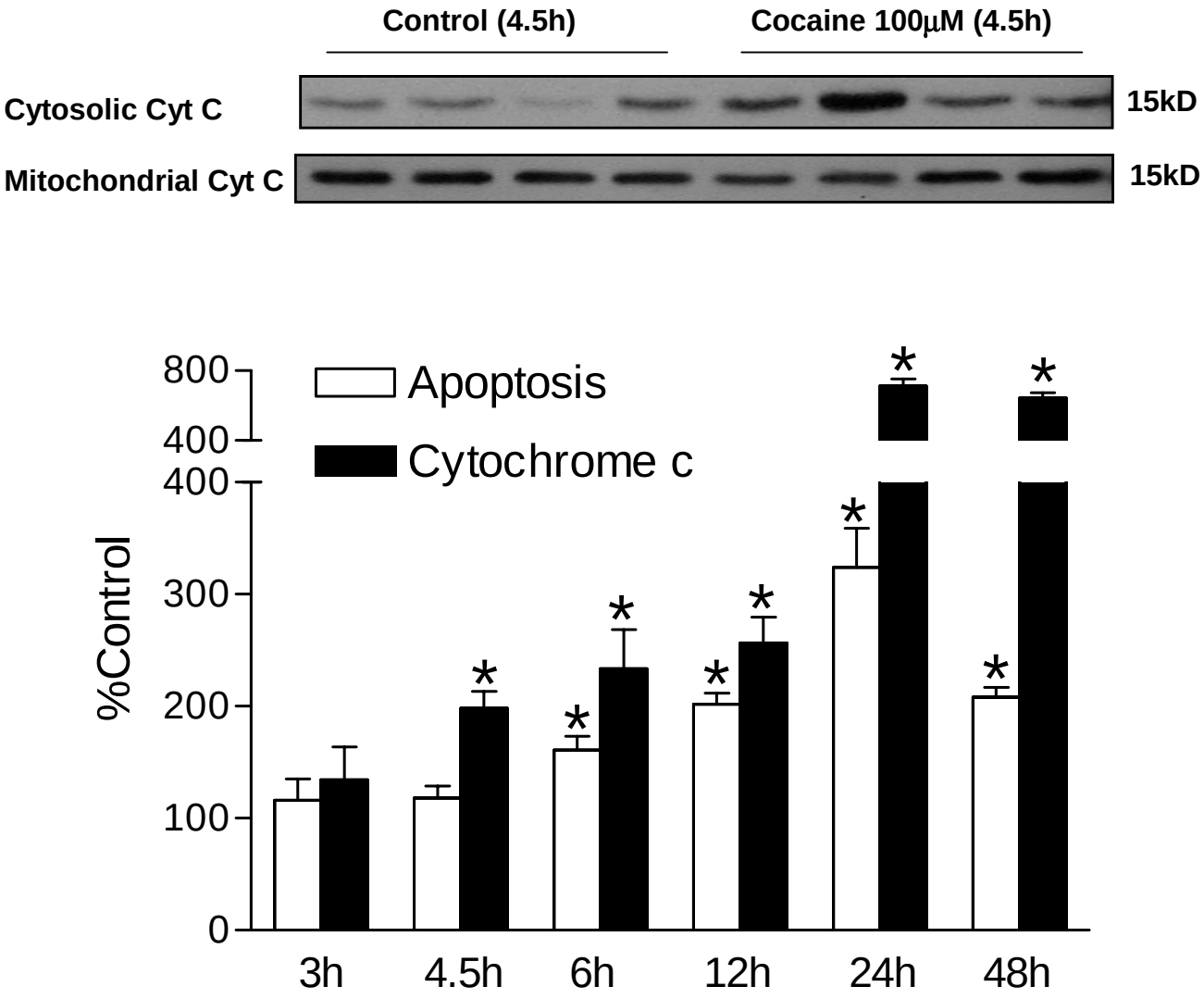

Figure 1

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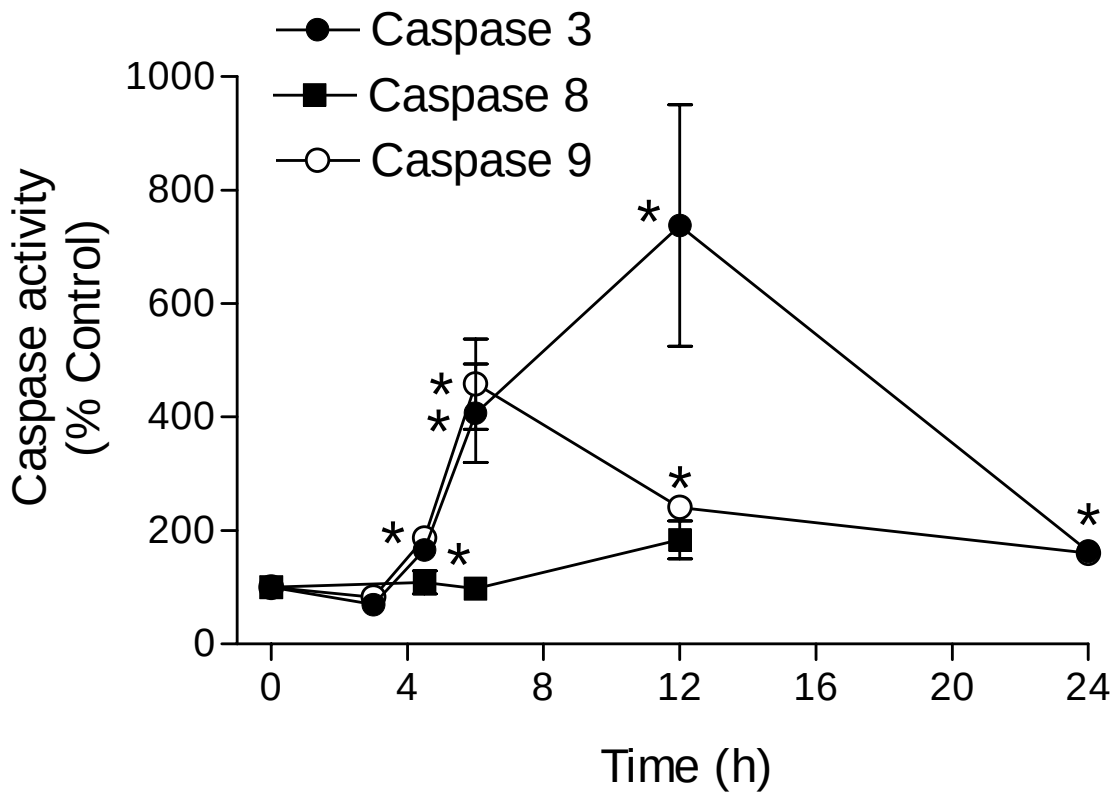

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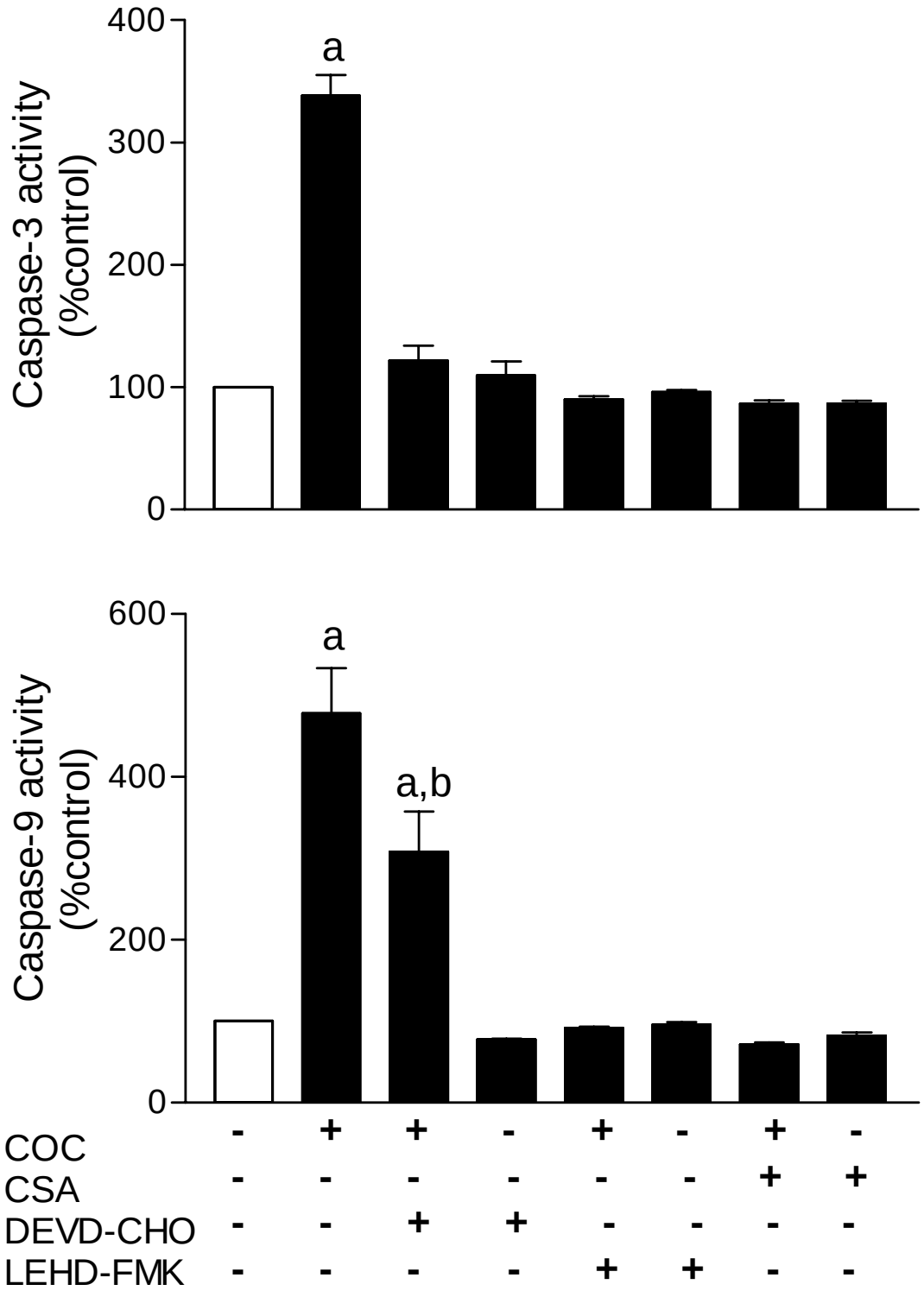

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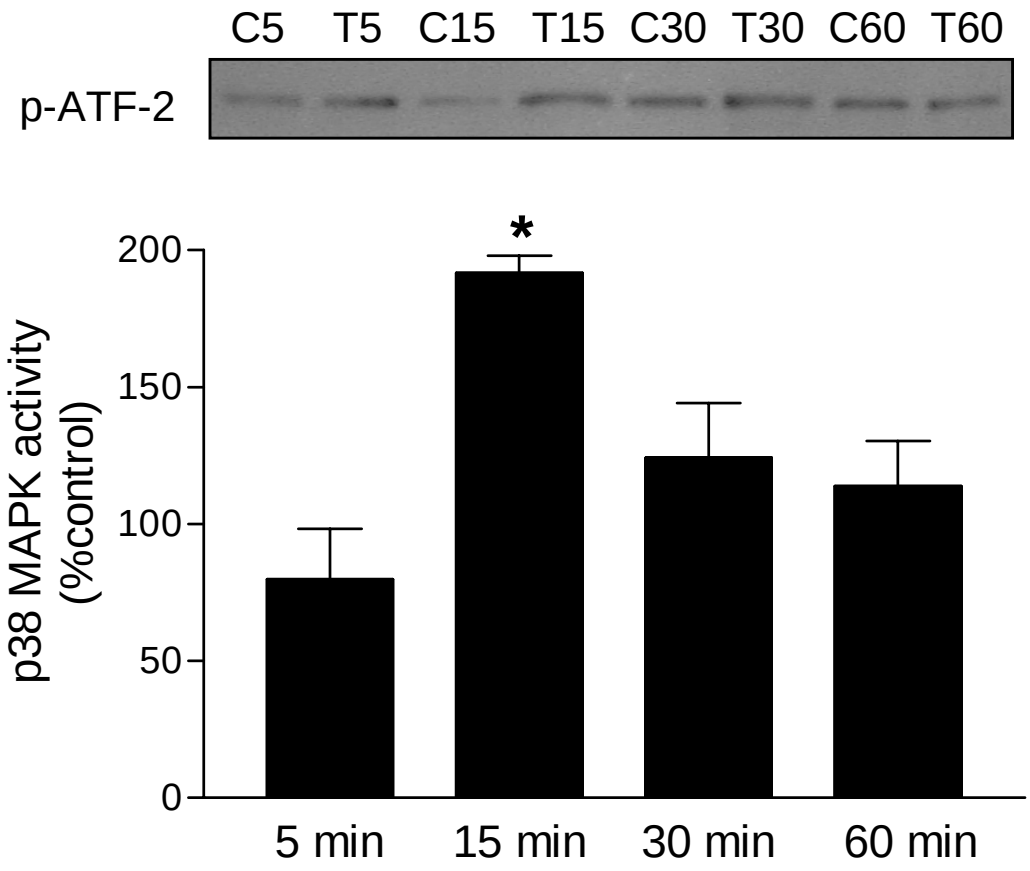

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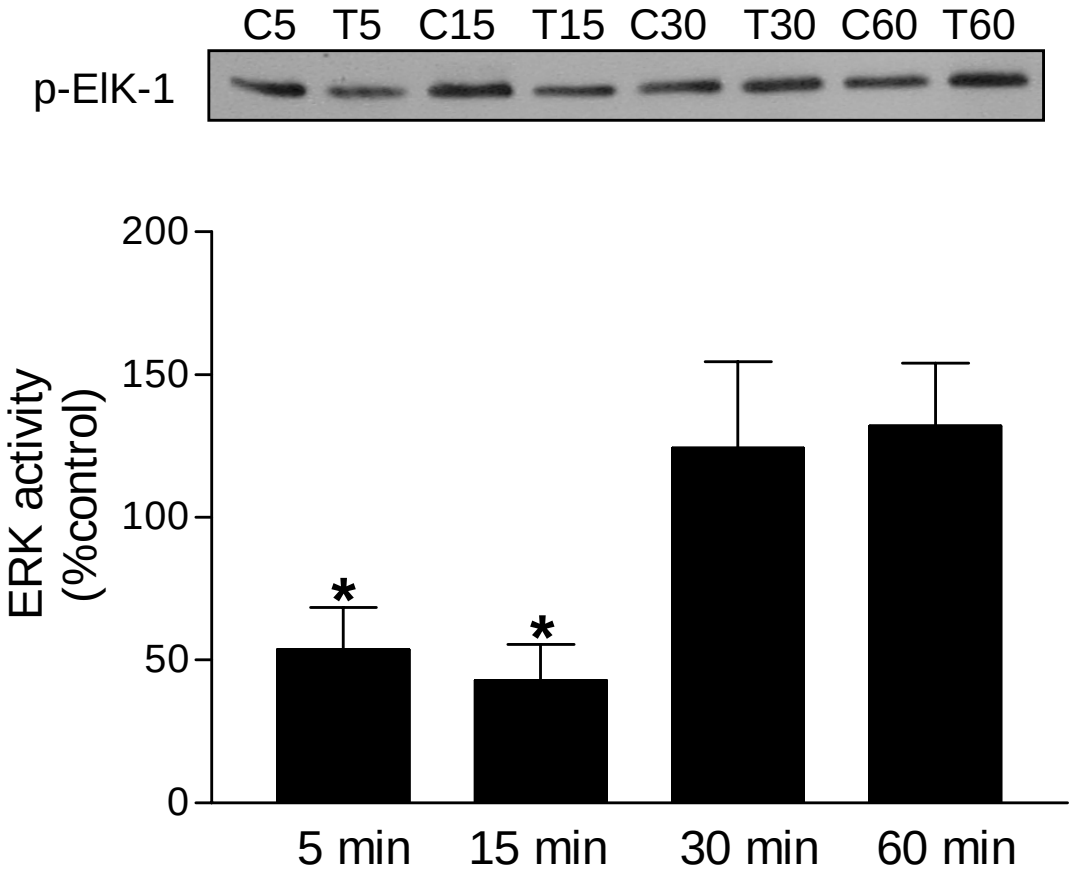

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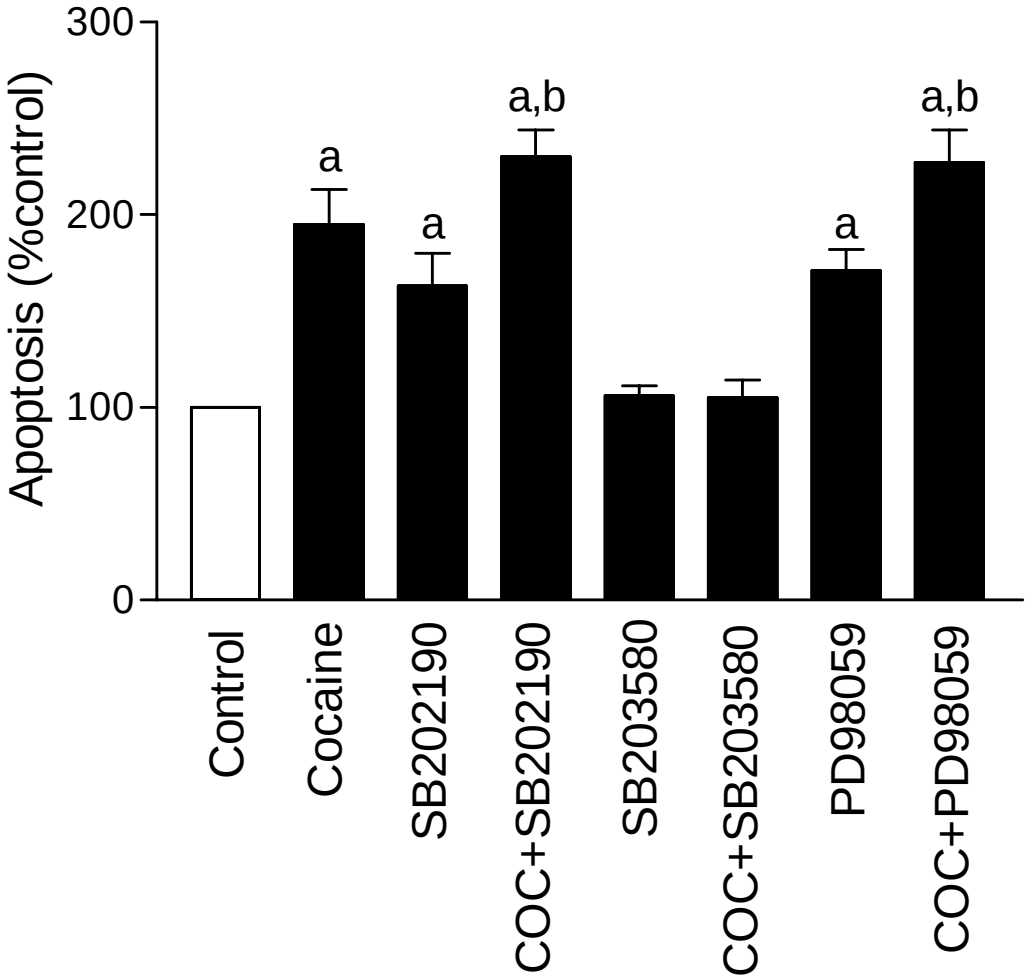

Figure 6

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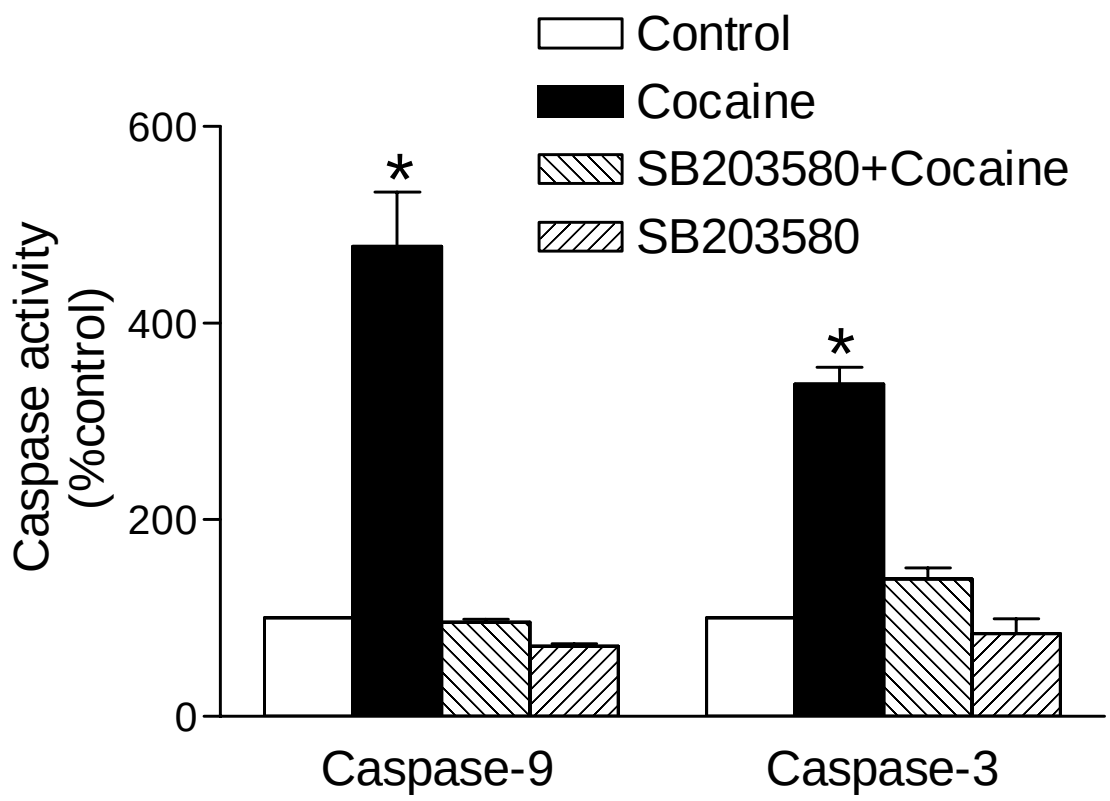

Figure 7

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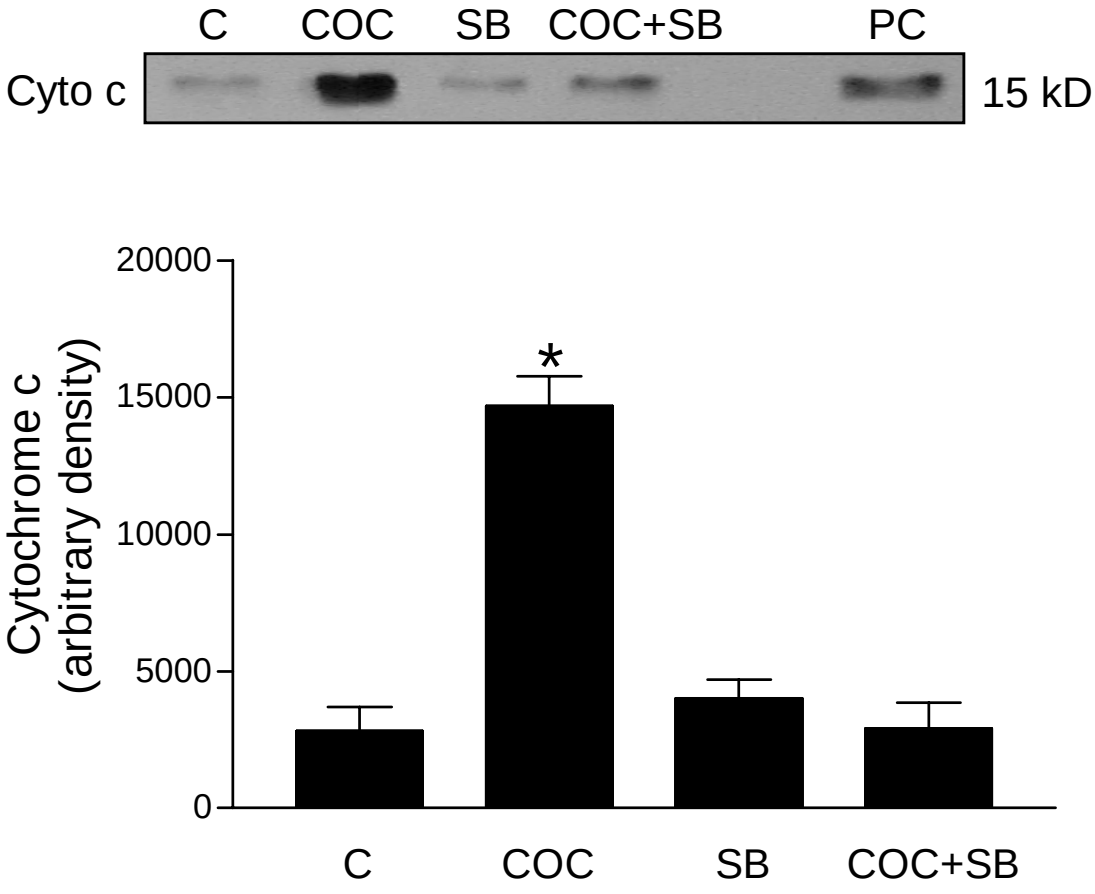

Figure 8

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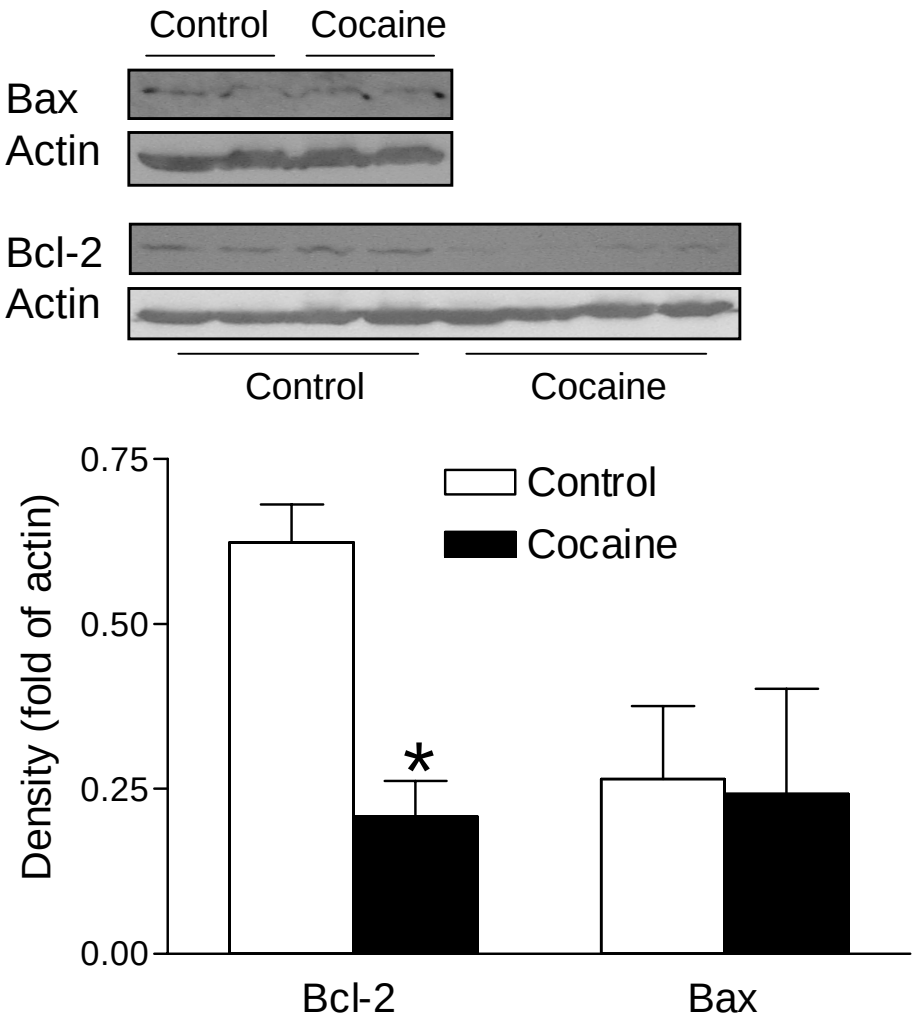

Figure 9

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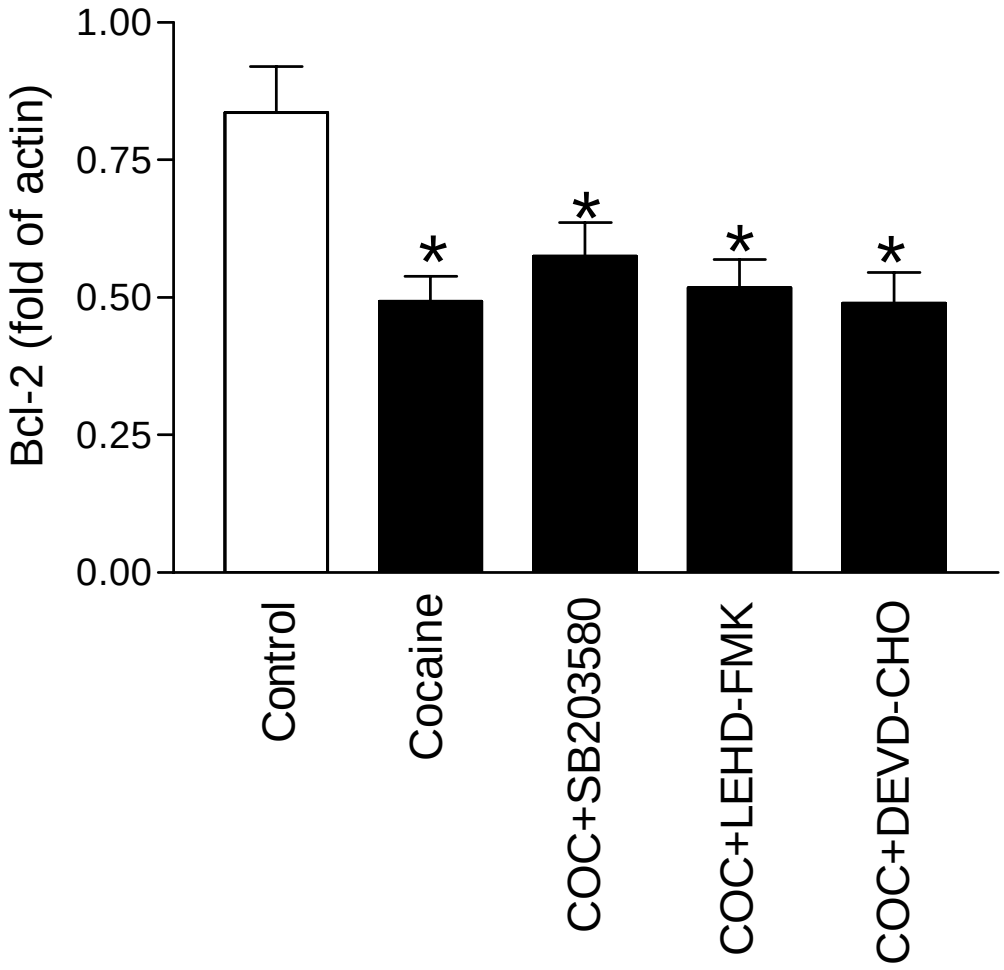

Figure 10