Induction of Cardiac Cytochrome P450 in Cocaine-Treated Mice

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Cytochrome P450 (P450) is a ubiquitous family of enzymes responsible for the metabolism of a wide variety of drugs and their metabolites, including cocaine. To investigate the effects of cocaine on myocardial injuries and cardiac P450 expression, BALB/c mice were injected daily intraperitoneally with cocaine (30 mg/kg) or cocaine plus pretreatment of P450 inhibitors for 14 days. Tumor necrosis factor-α (TNF-α) content and creatine phosphokinase (CPK) activity in mice hearts and serums were significantly increased after long-term treatment with cocaine. Pretreatment with the P450 inhibitor, cimetidine (Cime, 50 mg/kg) or metyrapone (Mety, 40 mg/kg) abolished or significantly attenuated the effects of cocaine on TNF-α and CPK activity. Western blot analysis shows that mouse cardiac tissues express the P450 isoforms CYP1A1, CYP1A2, and CYP2J2. The protein levels normalized with cyclophilin A were 1.20 ± 0.07, 0.67 ± 0.03, and 1.48 ± 0.01 for CYP1A1, CYP1A2, and CYP 2J2, respectively. After cocaine administration, CYP2J2 increased by 43.6% and CYP1A1 increased by 108.5%, but CYP1A2 was not significantly altered. However, the cytochrome P450 inhibitors Cime and Mety suppressed the cocaine-induced increase in CYP1A1 and CYP2J2 expression. Moreover, application of Cime or Mety alone did not alter the level of cardiac TNF-α or the expression of P450. Our results demonstrate that long-term exposure to cocaine causes an increase in cardiac CYP1A1 and CYP2J2 concentration. We speculate that induction of P450 isoforms may cause cardiac injury due to cocaine metabolites locally catalyzed by P450 or the increase in P450 expression itself. [Exp Biol Med Vol. 227(3):182–188, 2002]

Key words: cardiac P450; cocaine; TNF-α; mouse

The cytochrome P450-dependent mixed-function oxidases are one of the most important enzyme systems that oxidize numerous exogenous compounds, including many drugs, carcinogens, and environmental pollutants, as well as endogenous compounds. The products formed by these reactions can have equal, greater, or less biological activity or toxicity than their parent compounds (1, 2). Some studies reported that P450-derived arachidonic acid (AA) metabolites might be involved in the pathophysiology of hypertension (1, 3). Also, some metabolites cause renal artery vasoconstriction, inhibit cardiac myocyte shortening, and exacerbate the response of the heart to ischemia and reperfusion (2, 4, 5). Therefore, the P450 system may play an important role in modulation of cardiovascular physiology and pathophysiology.

Numerous clinical and laboratory studies have demonstrated that cocaine causes myocardial ischemia and infarction as well as cardiac arrhythmias (6, 7). Also, cocaine induces myocardial ultrastructural alterations and infiltration by inflammatory cells (6, 8). Furthermore, cocaine administration has also been reported to cause hepatotoxicity in man (9–11). These studies described morphological changes in patients that were similar to those reported in mice with cocaine exposure. In animals, cocaine administration produces severe hepatotoxicity, including fat infiltration, midzonal and periportal necrosis, and apoptosis. The hepatotoxicity induced by cocaine is related directly to its biotransformation by cytochrome P450 (12). The cytochrome P450 metabolites of cocaine such as norcocaine and *N*-hydroxynorcocaine were demonstrated to be hepatoxic, but benzoylecgonine and ecogonine methyl ester, which are produced by the esterase pathway, were not. Furthermore, Jeong and co-workers (13) reported that metabolism of cocaine by P450 is a critical pathway for the generation of cocaine metabolites. These P450-catalized metabolites of cocaine are capable of immunosuppression. In addition, inhibition of the esterase pathway of cocaine shunts the metabolism of cocaine into an immunotoxic pathway (13).

Recently, cardiac isoforms of cytochrome P450 in mammalian hearts have been cloned and expressed (14).

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The role of these isoforms in cardiac function is largely unknown. Cocaine-induced hepatotoxicity and immunosuppression is dependent on the cytochrome P450 system. In the present study, we treated mice with cocaine and evaluated cocaine's cardiotoxicity and expression of cytochrome P450. In addition, we assessed the effects of cytochrome P450 inhibitors on induction of P450 and cardiotoxicity caused by cocaine in the murine model. Our results indicate that long-term administration of cocaine causes myocardial injuries and P450 induction.

Materials and Methods

Animals. BALB/c mice (Taconic Farms, Germantown, NY) of either sex and with body weights from 20–25 g were divided into six groups: saline (n = 20), cocaine (Coc, 30 mg/kg, n = 20), cimetidine (Cime, 50 mg/kg, n = 20), metyropane (Mety, 40 mg/kg, n = 20), Coc + Cime (30 + 50 mg/kg, n = 20), and Coc + Mety (30 + 40 mg/kg, n = 20). Mice were injected daily with cocaine and/or P450 inhibitors i.p. for 14 days. Mety or Cime were administrated 30 min before cocaine treatment in P450 inhibitors plus cocaine groups. Four hours after the last injection, mice were anesthetized with ketamine and xylazine (40 + 5 mg/kg). Blood was collected through the inferior vena cava to prepare serum. Mouse hearts were quickly excised and dropped into liquid nitrogen and were kept at −80°C until Western blot assay of protein levels of P450 isoforms and ELISA measurement of TNF-α. Animal experiments were performed in accordance with the guidelines of the National Institutes of Health. Protocols were approved by the Animal Care and Use Committee of our institution.

Creatine Phosphokinase (CPK) Assay. Serum CPK activity was measured with commercially available diagnostics kits (Sigma Assay kit; Sigma, St. Louis, MO). Detection of CPK activity was performed on an ultraviolet/visible scanning spectrophotometer (Beckman DU-640, Beckman Instruments, Fullerton, CA). The activity of CPK is expressed as Sigma units/ml.

TNF-α Measurement. Mice hearts (n = 10) were homogenized with PBS buffer. The homogenate was centrifuged at 13,000g for 30 min at 4°C. The supernatant was taken to measure cardiac TNF-α concentrations using a TNF-α immunoassay kit (R&D Systems, Minneapolis, MN). TNF-α serum concentrations were also measured with the same method.

Western Blot Analysis. Mice hearts were homogenized in buffer containing 145 mM NaCl, 0.1 mM $MgCl_2$, 15 mM HEPES, 10 mM EGTA, 0.5% Triton X-100, 1 μg/ml Aprotinin, 1 μg/ml leupeptin, 1μg/ml pepstatin, and 1 mM Na_3VO_4. Proteins were quantified using the Lowry Assay kit (Sigma). The immunoprecipitation method was used to purify and condense the P450 proteins. Fifty micrograms of protein was loaded onto a 10% SDS-PAGE gel. The blots were blocked with 0.05% Tween-20 and 5% nonfat milk for 1 hr and were then incubated with different isoforms of P450 primary antibodies at 4°C overnight. The antibody of CYP2J2 was supplied by Dr. Darryl C. Zeldin (NIEHS). The antibodies of CYP1A1 and CYP1A2 were purchased from GENTEST Corporation (Woburn, MA). After incubation with a second antibody, the blots were subjected to enhanced chemiluminescent (ECL) solution and were exposed to Kodak X-OMAT film for 5–60 sec. The protein levels were normalized with cyclophilin A (Upstate Biotechnology, Lake Placid, NY).

Data Analysis. All data are presented as mean ± SEM. The overall statistical significance for the differences among groups was tested using analysis of variance (ANOVA). A P value of less then 0.05 was considered to be significant.

Results

Effects of Cocaine on TNF-α and CPK. Clinical and laboratory evidence indicates that TNF is an important mediator of cardiovascular pathology. Also, recent evidence indicates that myocardial TNF is an autocrine contributor to myocardial dysfunction and cardiomyocyte death. Our previous study demonstrated that cocaine stimulates myocardial TNF-α production and that the elevation of TNF-α resulted in cocaine-induced cardiotoxicity. Figure 1 shows that after long-term treatment with cocaine, TNF-α content in mice hearts was significantly increased from 2.3 ± 0.2 ng g^{-1} protein for saline control (n = 10) to 7.6 ± 0.8 ng g^{-1} protein ($P < 0.01$) for cocaine treatment (n = 10), respectively. Pretreatment of the P450 inhibitors, Mety or Cime, significantly suppressed the increase in cardiac TNF-α induced by cocaine ($P < 0.05$ vs cocaine group). However, cardiac TNF-α content in mice treated with cocaine plus the P450 inhibitors was still higher than the value of the saline control ($P < 0.05$). In contrast, Mety or Cime alone had no effects on the production of cardiac TNF-α.

We also examined the production of TNF-α in mouse serum. Figure 2A shows that serum TNF-α content was also significantly elevated in mice after long-term cocaine treatment ($P < 0.01$). Administration of either P450 inhibitor (Mety or Cime) only slightly inhibited the cocaine-induced increase in serum TNF-α production ($P > 0.05$ vs cocaine group) in cocaine-treated mice. This result is different from that in cardiac tissues where P450 inhibitors significantly attenuated the cocaine-induced increase of cardiac TNF-α production. CPK activity in serum was also significantly increased in cocaine-treated mice compared with the saline group ($P < 0.05$; Fig. 2B). Pretreatment with Mety or Cime suppressed the effects caused by cocaine ($P < 0.05$). In contrast, Mety or Cime alone did not alter these biochemical parameters. These results indicate that application of cocaine increased TNF-α production in cardiac tissues and in serum. Pretreatment of P450 inhibitors Mety or Cime abolished or significantly attenuated the cocaine's effects.

Cytochrome P450 Expression in Mouse Hearts. P450 and its associated monooxygenase activity have been

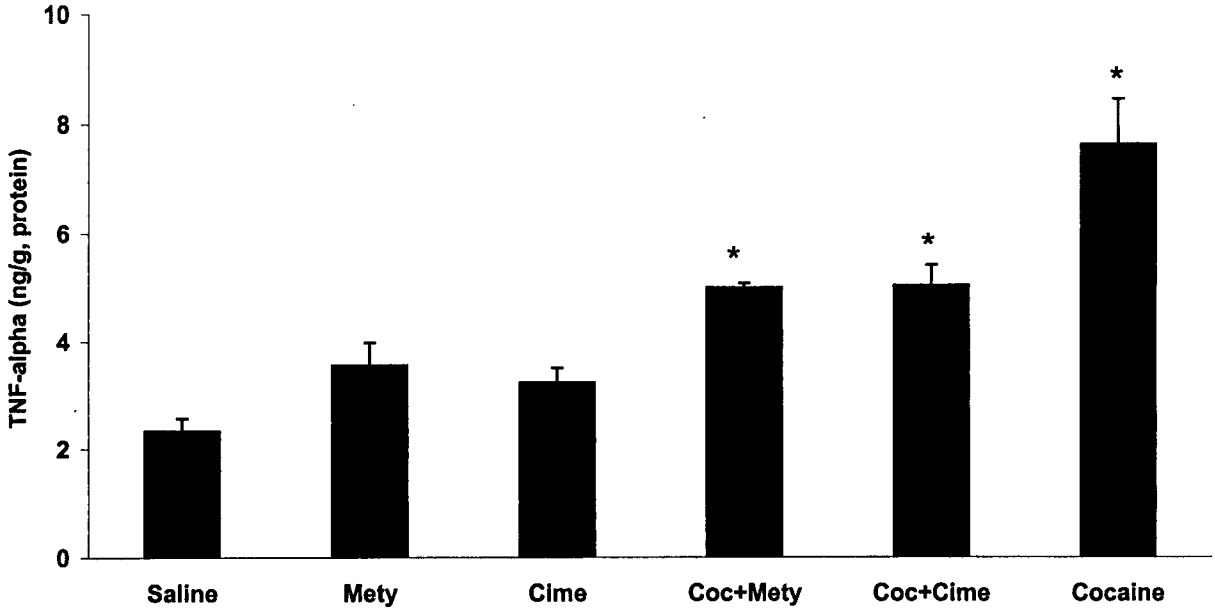

Figure 1. Effects of cocaine on cardiac TNF-α production in long-term cocaine-treated mice. Animals were treated i.p. with cocaine or cocaine plus P450 inhibitors daily for 14 days. Mety, 40 mg/kg; Cime, 50 mg/kg; Coc + Mety, 30 mg/kg + 40 mg/kg; Coc + Cime, 30 mg/kg + 50 mg/kg. Each experimental group consisted of 10 mice. $^{*}P < 0.05$; $^{**}P < 0.01$ vs saline control. $\#P < 0.05$ vs cocaine alone.

identified in hearts from several mammalian species, including rat, guinea pig, rabbit, and pig. Recently, a new cardiac isoform of P450, CYP2J2, has been cloned and is reported to be highly expressed in the human heart. Due to a lack of information about P450 in mouse hearts, we analyzed the protein expression of P450 isoforms in our experiments. Three different antibodies of the P450 isoforms CYP1A1, CYP1A2, and CYP2J2 were used in Western blot analysis experiments. Figure 3 shows that the three isoforms were detected in mouse hearts, but the level of CYP1A2 was the lowest. The protein levels of CYP1A1 and CYP2J2 were 1.8- and 2.2-fold higher than the value of CYP1A2, respectively. CYP2J2 concentration was highest among the three P450 isoforms examined in the present experiments.

Induction of Cardiac P450 by Cocaine. The above results demonstrated that cocaine increases cardiac TNF-α production in long-term cocaine-treated mice and that this increase was significantly attenuated by addition of the P450 inhibitors (Fig. 1). It is well known that the P450 system participates in cocaine metabolism. To test whether the cocaine-induced increase in cardiac TNF-α was accompanied by an induction of P450 expression, we examined the changes in protein levels of three P450 isoforms in cardiac tissues of cocaine-treated mice. Figure 4 shows that cocaine increased the expression of CYP1A1 and CYP2J2. The protein level of CYP1A1 was increased by 108.5% ($P < 0.01$, $n = 7$ vs control) and the increase of CYP2J2 was 43.6% ($P < 0.05$, $n = 7$ vs control). In contrast, the protein level of cardiac CYP1A2 was not altered in cocaine-treated mice.

To test whether the P450 inhibitors Cime and Mety altered the cocaine-induced increase of the P450 isoforms, mice were treated with cocaine plus Cime or Mety. Figure 5 shows that both Cime and Mety significantly suppressed the cocaine-caused induction of CYP2J2 (Fig. 5A, $P < 0.01$) and CYP1A1 (Fig. 5B, $n = 7$, $P < 0.01$). Cime reduced the cocaine-caused P450 induction by 52.4% and 50.9% for CYP1A1 and CYP2J2, respectively. In addition, Mety inhibited the cocaine-induced increase by 57.2% for CYP1A1 and by 52.8% for CYP2J2. In contrast, cocaine and the P450 inhibitors had no effect on the expression of the CYP1A2 isoform. Furthermore, Figure 5 shows that Cime or Mety alone had no significant effect on the expression of cardiac CYP1A1, but the protein level of CYP2J2 was significantly reduced in the presence of the P450 inhibitors ($P < 0.05$ vs saline control). These results indicate that cocaine caused an induction of cardiac CYP2J2 and CYP1A1. The cocaine-induced P450 increase was abolished or significantly attenuated by pretreatment of P450 inhibitors either Cime or Mety.

Discussion

In the present study, we found that cocaine increased cardiac and serum TNF-α levels in the long-term cocaine-treated mouse. TNF-α is a proinflammatory cytokine that has been implicated in the pathogenesis of cardiovascular disease, including acute myocardial infarction, chronic heart failure, atherosclerosis, viral myocarditis, and sepsis-associated cardiac dysfunction (15–17). Also, TNF-α mediates myocardial cell apoptosis via the TNF-α receptor I or nitric oxide and Bcl-x (18). Therefore, the increase in cardiac TNF-α found in this study may reflect certain myocardial injuries in cocaine-treated mice. These injuries were not easily detected with gross sections under light microscopy. In addition, we found that pretreatment with the P450 inhibitors Cime and Mety reduced the increase of cardiac

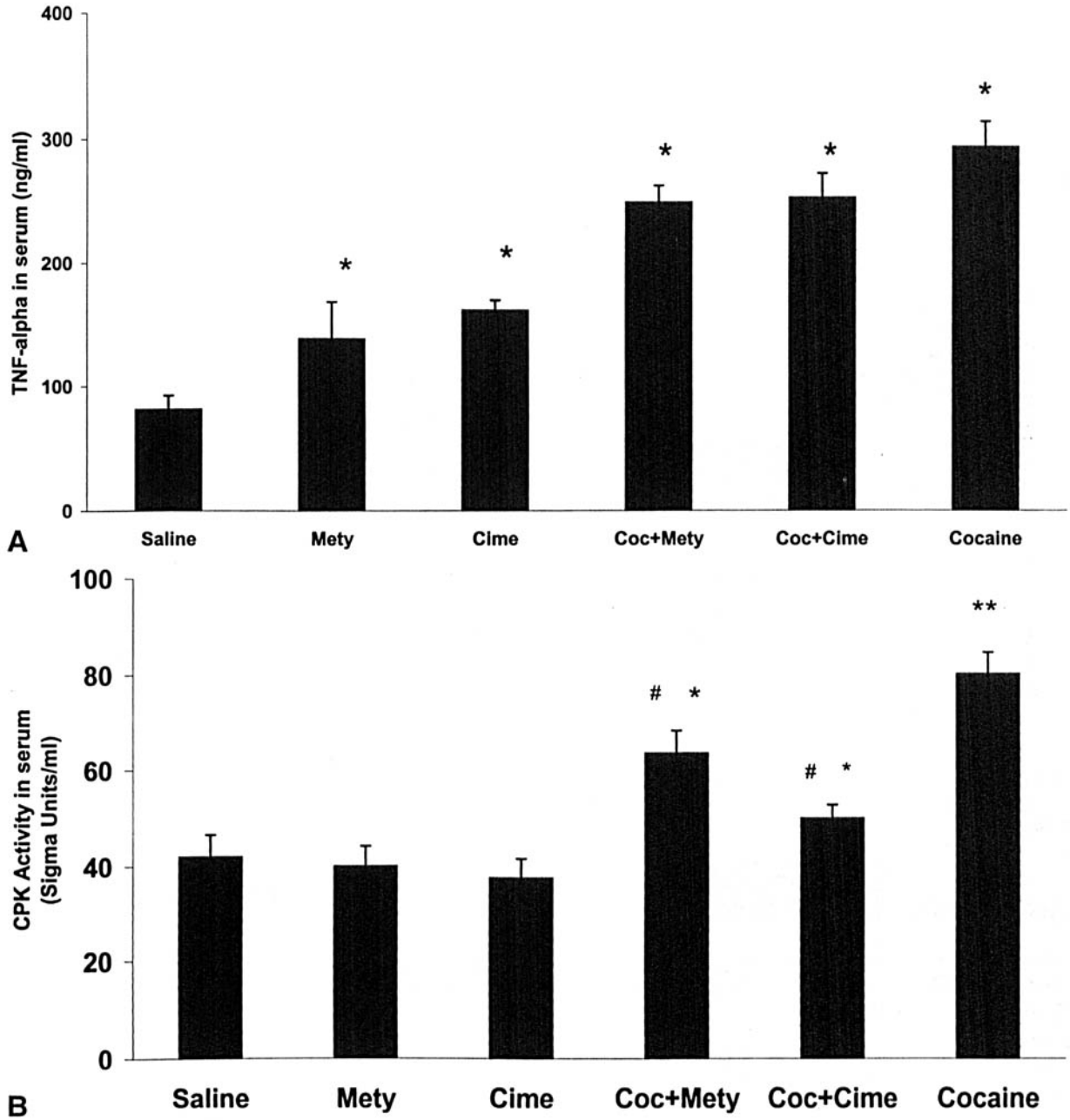

Figure 2. Effects of cocaine on serum TNF-α production and CPK activity in long-term cocaine-treated mice. (A and B) The changes in serum TNF-α production and CPK activity, respectively, after treatment with saline, cocaine, and cocaine plus P450 inhibitors. Mety, 40 mg/kg; Cime, 50 mg/kg; Coc + Mety, 30 mg/kg + 40 mg/kg; Coc + Cime, 30 mg/kg + 50 mg/kg. Each experimental group consisted of 10 mice. $^{*}P < 0.05$; $^{**}P < 0.01$ vs saline control. $\#P < 0.05$ vs cocaine alone.

TNF-α production in cocaine-treated mice hearts. These results suggest that the P450 system may be involved with the increase of TNF-α and cardiotoxicity of cocaine. The suppression of the cocaine-increased cardiac TNF-α production by P450 inhibitors may have clinical significance for treatment of cocaine-induced cardiac complications.

Other important findings in our study are the expression of CYP1A1, CYP1A2, and CYP2J2 in normal mouse cardiac tissues and the induction of cardiac CYP1A1 and CYP2J2 in cocaine-treated mice. Furthermore, the P450 inhibitors Cime and Mety abolished or significantly attenuated the induction of cardiac CYP1A1 and CYP2J2 in the long-term cocaine-administered mice. P450 has been identified in microsomal fractions prepared from heart tissues of different species including rat, rabbit, guinea pig, fish, and pig (14, 19, 20). It is a crucial enzyme system for the cardiac AA metabolic cascade. Epoxyeicosatrienoic acids (EETs) are the major metabolites of AA catalyzed by cytochrome P450 and are important factors in modulating cardiac function under both physiological and pathophysiological conditions. In newborn rat lung, CYP1A1 was undetectable, but when its activity was induced, the toxic effect of oxygen on the lung was potentiated (21). The biological significance and functional roles of another isoform from the CYP1A

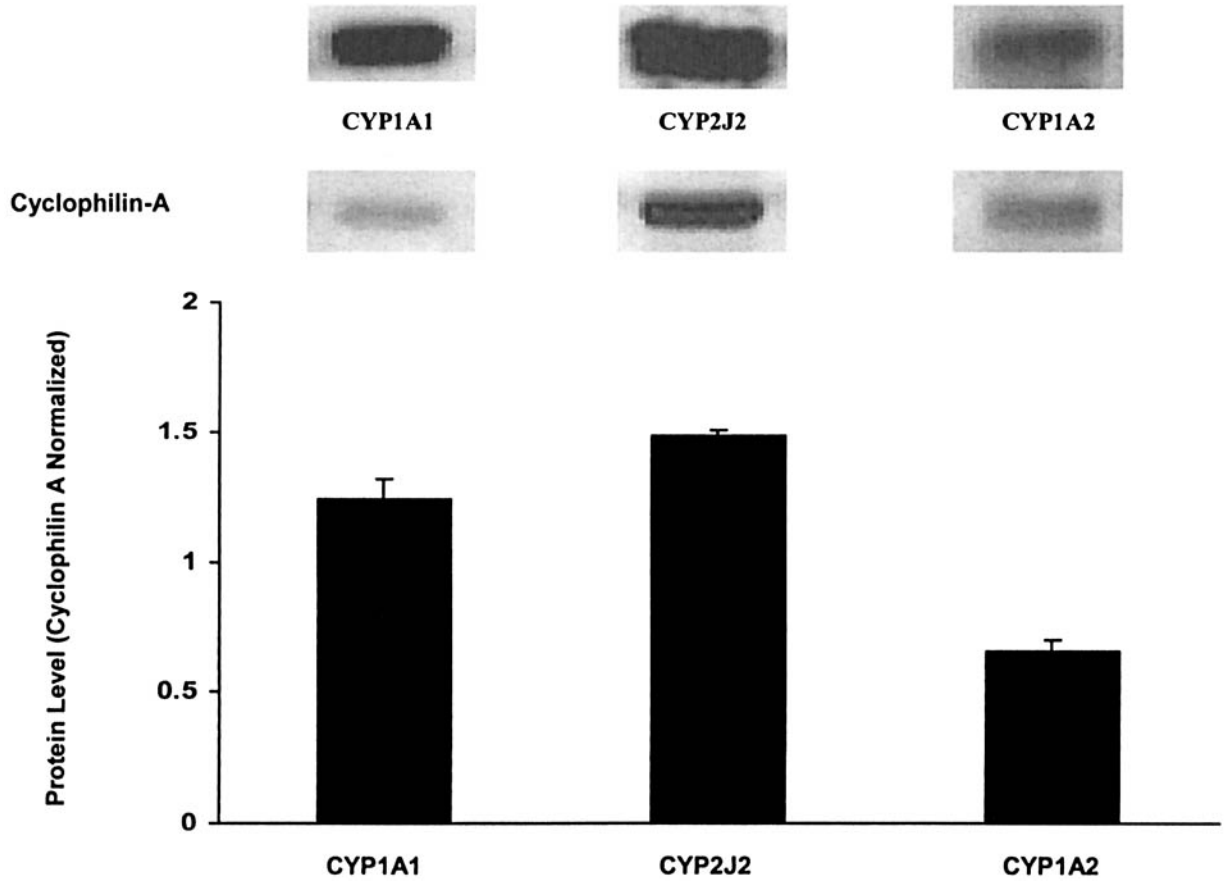

Figure 3. Expression of the P450 isoforms in mouse hearts. The P450 protein levels of CYP1A1, CYP2J2, and CYP1A2 were normalized to their corresponding internal levels with cyclopholin-A, which was added to the samples during Western blot analysis. Each group consisted of seven mice.

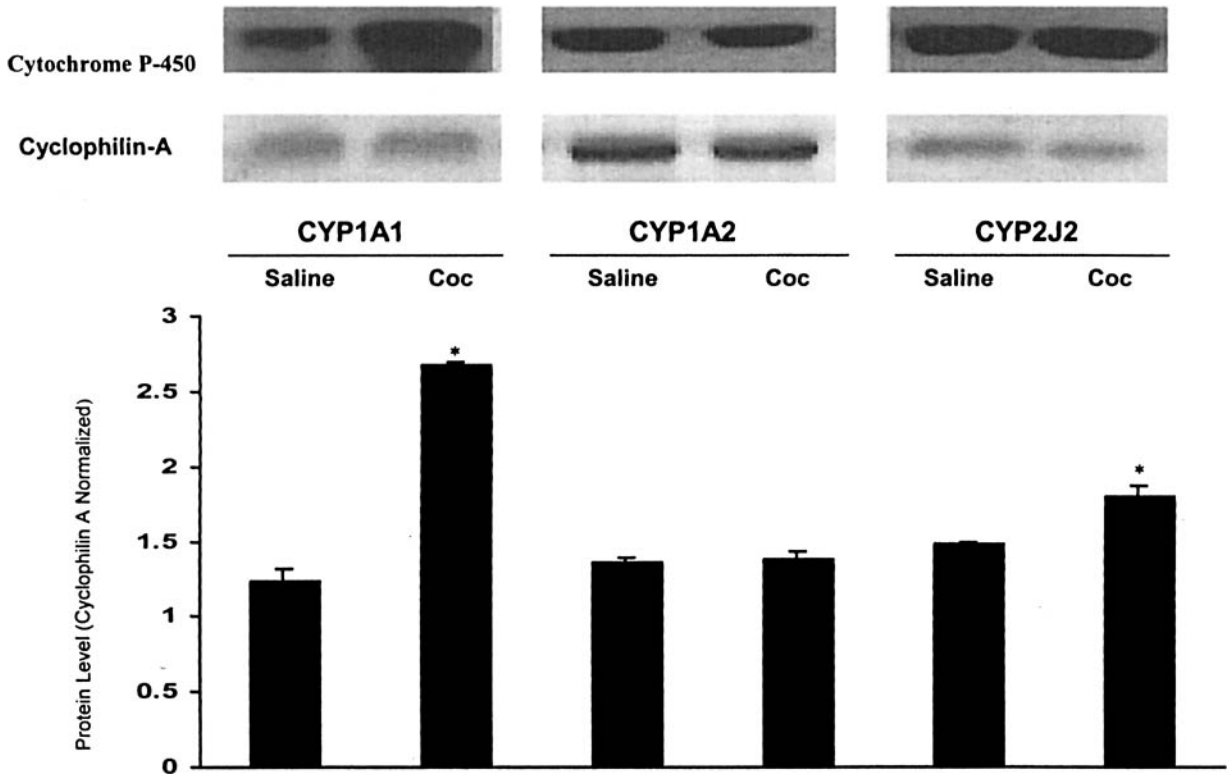

Figure 4. Induction of the P450 isoforms in cardiac tissues of long-term cocaine-treated mice. Each group consisted of seven mice. $^{*}P < 0.05$; $^{**}P < 0.01$ vs saline control.

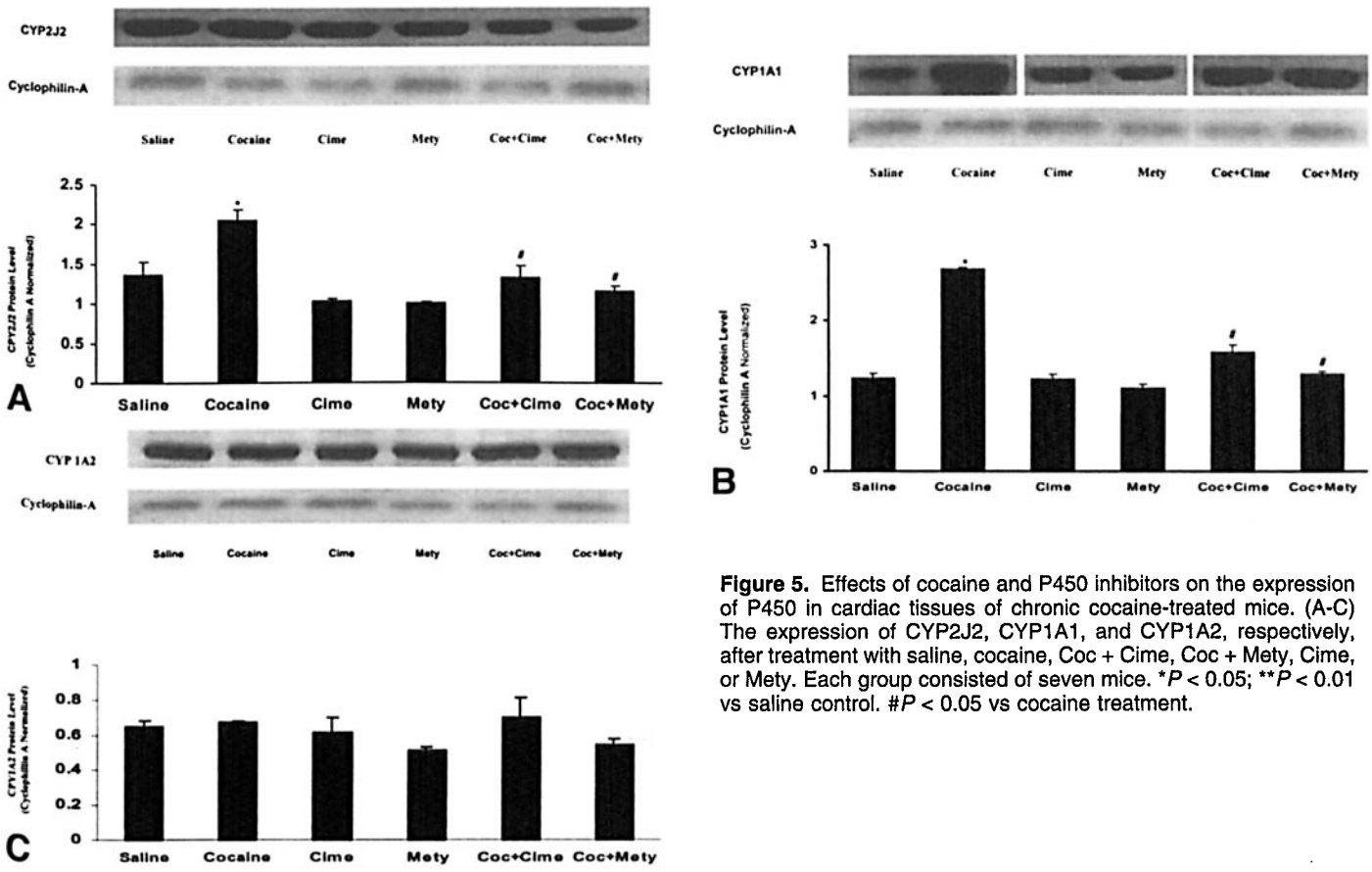

Figure 5. Effects of cocaine and P450 inhibitors on the expression of P450 in cardiac tissues of chronic cocaine-treated mice. (A-C) The expression of CYP2J2, CYP1A1, and CYP1A2, respectively, after treatment with saline, cocaine, Coc + Cime, Coc + Mety, Cime, or Mety. Each group consisted of seven mice. $^{*}P < 0.05$; $^{**}P < 0.01$ vs saline control. $\#P < 0.05$ vs cocaine treatment.

subfamily, CYP1A2, remains unclear. In the present study, Western blot analysis provides evidence of the presence of three different P450 isoforms in mouse cardiac tissues. The protein levels of CYP1A1 and CYP2J2 are much higher than that of CYP1A2 in mouse hearts. The significance of these differences in cardiac physiological functions is unknown. It has been shown that P450 and P450-mediated metabolites of AA play important roles in some diseases. For example, P450 in the kidney acts as a key factor for development of spontaneous or salt-induced hypertension in rats (3, 4). Our results show an increase in cardiac TNF-α accompanied by an induction of cardiac P450 in cocaine-treated mice; therefore, it is possible that myocardial injuries may result from cocaine-induced increase of P450 itself or the P450-catalyzed metabolites of cocaine.

The CYP2J subfamily of P450 catalyzes the NADPH-dependent oxidation of AA to several unique eicosanoids that possess numerous biological activities, including modulation of ion transport, control of bronchial and vascular smooth muscle tone, and stimulation of hormone secretion (14). Also, they demonstrated that CYP2J2 is one of the predominant enzymes responsible for the epoxidation of endogenous AA pools in human heart. Our results show that CYP2J2 is a major isoform of cytochrome P450 in mouse hearts. The protein level of CYP2J2 was highest among the three P450 isoforms examined in our current study. Cocaine produced an increase of this protein. Therefore, CYP2J2 may play an important role in regulation of cardiac function and cardiotoxicity of cocaine.

Previous data showed that cocaine's hepatotoxicity and immunosuppression were well correlated with P450 activity (13, 21, 22). P450 inducers (e.g., Phenobarbital and β-naphthoflavone) potentiated cocaine-induced hepatotoxicity and oxygen-induced lung damage (21). Conversely, the liver toxicity of cocaine was diminished when mice were pretreated with P450 inhibitors such as SKF-525-A, Cime, or Mety. Furthermore, Pellinen and co-workers (22) demonstrated that two isoforms of cytochrome P450, CYP2B10 and CYP3A, were induced in animal liver by daily administration of cocaine, and cocaine's hepatotoxicity was potentiated. In addition, both immunosuppression and hepatotoxicity in animals exposed to cocaine were associated with the induction of another isoform, CYP2B1/2 (13).

Recently, the role of P450 in cardiovascular physiology and pathophysiology has been emphasized. CYP2J2 was considered one of the predominant enzymes responsible for the epoxidation of endogenous cardiac AA (14). The P450-mediated metabolite of AA, EETs, can cause vessel constriction, which may relate to the development of hypertension. EETs may also exacerbate myocardial injuries during ischemia and reperfusion by an increase in Ca^{2+} influx via voltage-dependent channels, or by an inhibition

of Na^+,K^+-ATPase (23). All of these effects may cause an intracellular Ca^{2+} overload and may damage myocardium during ischemia and reperfusion. Therefore, the induction of CYP2J2 and CYP1A1 by cocaine may link to its cardiac toxicity.

Several studies have demonstrated that Cime and Mety are able to reduce the acute pulmonary toxicity of 100% oxygen and prevent the hepatotoxicity of cocaine (13, 21). In the present experiments, the expression of cardiac CYP2J2 and CYP1A1 was increased and Cime or Mety suppressed the increase in cocaine-treated mice. In the liver and immune system, cocaine-induced injuries are believed to result from either an induction of cytochrome P450 or the toxic metabolites of cocaine catalyzed by P450. However, it is undetermined whether cocaine's cardiotoxicity is due to its induction of P450 or its toxic metabolites. Based on our results, we speculate that induction of the cardiac P450 isoforms may be a potential mechanism for cocaine's cardiotoxicity. The cardiac isoform CYP2J2 may be a major isoform of P450 involved in the toxicity of cocaine.

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