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Original Article

Cocaine residue in plasma, cardiac and tracheal tissues of chronic cocaine-treated guinea-pigs

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Abstract

Supersensitivity of adrenoceptors to catecholamines is one of the mechanisms of cocaine-related cardiac complication. The precise mechanism of cocaine enhancing supersensitivity of adrenoceptors is unconcluded. The aim of this study was to determine the levels of cocaine in plasma, cardiac and tracheal tissues in order to correlate with the supersensitivity of adrenoceptors to catecholamines. In this study, two groups of ten guinea-pigs each were injected with 2.5 mg/kg cocaine or normal saline solution intraperitoneally twice daily for 14 days. After 24 hours of cocaine cessation, the cocaine levels in plasma, cardiac and tracheal tissues were determined using high performance liquid chromatography. The results showed that the cocaine levels in plasma and tracheal smooth muscle were 5.08±0.63 ng/ml and 2.8±0.41 ng/mg, respectively, while those in atria and ventricle were lower than 17.5 ng/g and 3.8 ng/g, respectively. These levels were less than the level that had been reported to block norepinephrine uptake (more than 30.34 ng/ml). Moreover, it had been demonstrated that cocaine treatment in the same condition as the present study produced supersensitivity to norepinephrine and epinephrine in isolated guinea-pig atria as well as in trachea which is almost entirely not innervated by the adrenergic nerves. In addition, supersensitivity to oxymetazoline, isoproterenol and salbutamol which are not the substrates of neuronal reuptake were also demonstrated. All these data support the postsynaptic mechanism of cocaine enhancing supersensitivity which might be correlated with cardiac complication in chronic cocaine use.

Keywords: cocaine, supersensitivity, adrenoceptor, HPLC

1. Introduction

Cocaine abuse is one of the major public health problems in many countries. It is the most commonly used illegal drug among subjects seeking care in hospital emergency departments or drug-treatment centers. In addition, it is the most frequent cause of drug-related deaths reported by medical examiners (Lange and Hillis, 2001).

Cocaine has been shown to be associated with myocardial ischemia and myocardial infarction (MI) independently of the administration route, the amount ingested and the frequency of use (Kloner and Rezkalla, 2003). Most patients with cocaine-related acute MI are young, male, have a low coronary risk factor profile for atherosclerosis and have previously normal epicardial coronary arteries (Lange and Hillis, 2001). The pathogenesis of cocaine-related MI has been reported to be multifactorial including increased myocardial oxygen demand, marked vasoconstriction of the coronary arteries, enhanced platelet aggregation and thrombus formation and accelerated atherosclerosis (Erwin, *et al.*, 2004). A number of hypotheses have been proposed to explain the occurrence of cardiac complications of cocaine.

Supersensitivity of adrenoceptors to catecholamines is one of the mechanisms of cocaine-related cardiac complication. The precise mechanism of cocaine enhancing supersensitivity of adrenoceptors is unconcluded. At present,

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presynaptic and/or postsynaptic mechanisms have been hypothesized. For the presynaptic mechanism, cocaine block the uptake of norepinephrine at the presynaptic nerve terminal resulting in the increasing concentration and prolonging duration of the amine at the synaptic cleft (Jain *et al.*, 1990). For the postsynaptic mechanism, cocaine may directly acts at the postsynaptic effectors (Alburges *et al.*, 1996; Trendelenburg *et al.*,1972).

However, more data are required to elucidate the mechanism any involed. Thus, the aim of the present study was to determine the levels of cocaine in plasma, cardiac and tracheal tissues in order to correlate with supersensitivities of adrenoreceptors to catecholamines. The results from this study may lead to more understanding of the actions of cocaine-induced supersensitivity in β-adrenoceptors and the mechanisms of its related toxicity to cardiovascular system.

2. Materials and Methods

2.1 Experimental animals

All experiments were carried out in adult guinea-pigs and performed in accordance with the Guidelines for Animal Experiments of the Prince of Songkla University. Guinea-pigs of either sex, weighing 400-600 g were supplied from the animal house, Faculty of Science, Prince of Songkla University. They were housed in an air-conditioned room (24-26°C) with a 12 hr light/dark cycle and the relative air humidity (50±5%) was strictly regulated. Food and water were available *ad libitum*.

2.2 Drug pretreatment

Guinea-pigs were divided into two groups (N=10 for each group) as follows:

2.2.1 Control group

The animals received 1 ml/kg of 0.9% w/v sodium chloride solution via intraperitoneal (i.p.) injection twice a day for 14 days.

2.2.2 Cocaine treated group

Guinea-pigs were chronically treated with cocaine hydrochloride as described by Darmani *et al.* (1992). They were injected with cocaine hydrochloride 2.5 mg/kg i.p. twice a day for 14 days, in the same way as control group.

2.3 Experimental protocol

After cessation of cocaine or sodium chloride solution, animals were killed by cervical dislocation at 24 hr. Five milliliters of blood was collected from the heart and put into a heparinized glass tube containing 250 units of heparin sodium. The plasma was separated by centrifugation at 3,500 rpm for 15 minutes. The heart and trachea were also dissected. Atria (200 mg) and ventricle (1g) were separated and homogenized by grinder tissue homogenizer in 5 ml of 0.9% sodium chloride, while total tracheal smooth muscle (858 mg) were homogenized in 1 ml of 0.9% sodium chloride (Robert *et al.*, 1991). One hundred milligrams of sodium fluoride was added into plasma, cardiac tissue and tracheal smooth muscle homogenates in order to maintain the stability of cocaine for at least 55 days as confirmed by Isenchmid *et al.* (1989) and Sukbuntherng *et al.* (1995). All samples were stored at -20°C. The analysis were performed within two weeks.

2.4 Chemicals and reagents

Cocaine hydrochloride was obtained from Diosynth (Apeldoom, Netherlands). Acetonitrile (HPLC grade), sodium fluoride (analytical grade) and disodium hydrogen phosphate (analytical grade) were purchased form JT Baker Inc. (Philipsburg. NJ, U.S.A.). Analytical grade acetic acid, sodium dihydrogen phosphate, ammonium acetate, chloroform and 2-propanol were purchased from Merck (Darmstadt, F.R. Germany). Sterile water was obtained by deionization and filtration through a Milli-Q and Nanopure system (Millipore, Molsheim, France), respectively.

2.5 Instrumentation and chromatographic condition

The HPLC system consisted of a liquid chromatograph, an automatically pump (Waters 515 HPLC pump), an automatically injector (717 plus autosampler) and an ultraviolet detector (Waters 2487). Detection was made with the variable wavelength UV detector set at 235 nm and peak area was measured with the csw32 program of Waters. Chromatographic separation was performed on a Novapak C_{18} column, particle size 4.5 mm, 3.9x150 mm I.D. (Waters Associated, Milford, U.S.A.).

2.6 Analytical method

Cocaine concentration in plasma, atrial, ventricular, and tracheal tissues were assayed by a high performance liquid chromatographic (HPLC) method modified from that described by Lim and Peters (1984).

2.6.1 Mobile phase

The mobile phase consisted of 300 ml of acetonitrlie and 700 ml of water containing 0.1 M ammonium acetate (adjusted to pH 5.15 with glacial acetic acid). The mobile phase was freshly prepared, filtered through 0.45 μm filter paper (Peerce, Rock Ford, IL, U.S.A.) and degassed before use. The flow rate was 1.0 ml/min, which gave the pressure of 1,800-2,000 psi. All analyses were performed at room temperature (23-27°C).

2.6.2 Stock solution

Stock solution of cocaine (1 mg/ml) was prepared and stored at -20°C. To assay precision and the percentage recovery, working standard solutions of cocaine (6.25, 25 and 100 ng/ml) were prepared by diluting the stock standard solution with deionized water, drug-free plasma, atrial or ventricular tissue homogenates. The addition of sodium fluoride (NaF) to all samples to prevent cocaine hydrolysis was necessary.

2.6.3 Extraction procedure

Two hundred microlitters of plasma, atrial, ventricular tissue homogenates were extracted by adding 150 µl of 0.1 M Na_2HPO_4 (pH 8.9) and 5 ml of chloroform: 2-propanol (9:1). After vortex mixing for 2 min and centifuging at 3,500 rpm (about 700 g) for 10 min, 4 ml of the organic phase were evaporate to dryness under air stream and the residue was reconstituted with 500 µl of 0.05 M NaH_2PO_4 (pH 5.2) and the content was transferred to a polypropylene microcentifuge tube. After centrifugation for 10 min, 100 µl of the supernatant was injected onto the HPLC system (Tagliaro *et al.*, 1994).

2.6.4 Calibration procedure

Standard calibration was run on each day of analysis. Calibration samples of cocaine were obtained by adding standard cocaine hydrochloride solution to drug-free plasma, atrial, ventricular and tracheal tissue homogenates and adjusting cocaine concentration to 6.25, 12.50, 25, 50 and 100 ng/ml. The spiked plasma, atrial, ventricular and tracheal tissue samples were extracted and assayed as described above. The peak areas obtained from the chromatograms were plotted against cocaine concentration.

2.6.5 Limit of Detection (LOD)

The detection limits (the lowest concentration of cocaine in plasma and tissue homogenates that can be detected) were calculated as LOD = $3.3\sigma/S$ (σ = the standard deviation of the response (S.D.), S = the slope of calibration curve).

2.6.6 Limit of Quantitation (LOQ)

The limit of quantitations (the lowest concentration of cocaine in plasma and tissue homogenates that can be determined with acceptable precision and accuracy under the stated operational conditions of the method) were calculated as LOQ = $10\sigma/S$ (σ = the standard deviation of the response (S.D.), S = the slope of calibration curve).

2.6.7 Assay precision and variability

The intra-day, inter-day precision and variability were determined using plasma, atrial, ventricular and tracheal tissues spiked with cocaine at low, medium and high concentration within a linear range of the calibration curve. Deviation of each cocaine concentration should be within ± 10 % of the spiked values. The coefficient of variation (CV) at each concentration of direct injection should be less than 5% and those of the extraction should be less than 10% .

2.6.8 Extraction recovery

The extraction recovery was determined by comparing the peak area obtained from the extracted samples containing a known amount of cocaine with those obtained from a direct injection of the same concentration. The percentage recovery was calculated as (peak area obtained from extracted sample/ peak area obtained from direct injection) x 100.

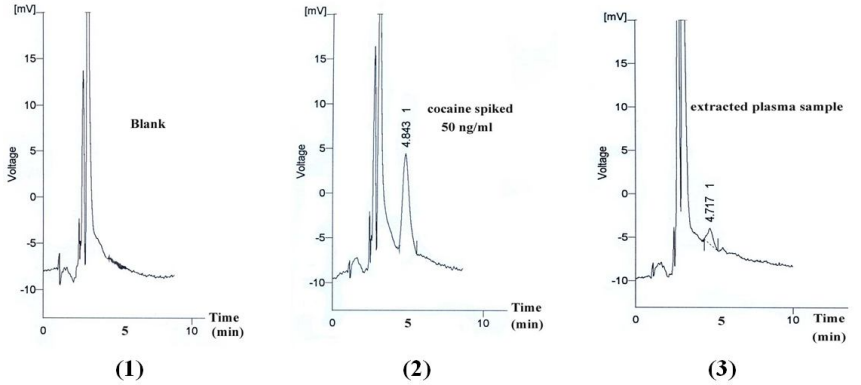
2.7 Data analysis

The concentrations of cocaine in plasma, cardiac, and tracheal tissues were expressed as means ± standard error of means ($\bar{X}$ ± S.E.M). All data were analyzed using unpaired Student *t*-test. Difference was considered significant if *p* value < 0.05.

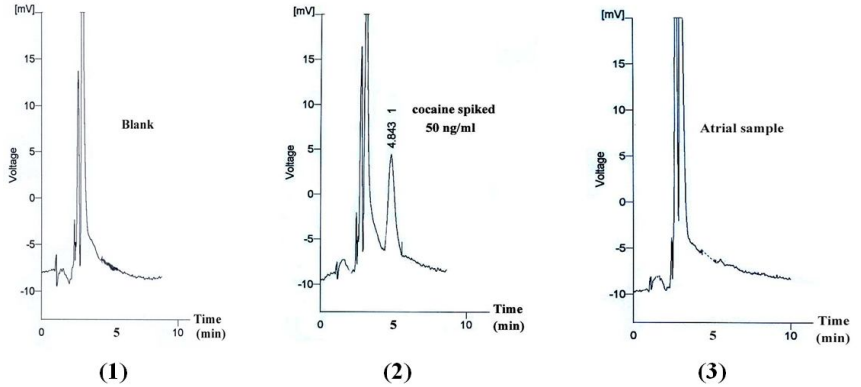
3. Results

Calibration plots of peak areas versus cocaine concentration (6.25-100 ng/ml) were linear with *r* values of 0.999 which indicate good linearity. Lower limit of detections of cocaine in plasma, atria, ventricle and tracheal smooth muscle were 0.69 ng/ml, 17.5 ng/g, 3.8 ng/g and 0.8 ng/g, respectively. Lower limit of quantifications of cocaine in plasma, atria, ventricle and tracheal smooth muscle were 2.1 ng/ml, 52.75 ng/g, 11.6 ng/g and 2.4 ng/g, respectively. The extraction recoveries of all samples were more than 95%. The intra-day and inter-day coefficient of variation of cocaine in plasma, atria, ventricle and tracheal smooth muscle were ranged between 0.8-4.33 % which were within the acceptable limits of less than 10%. The data showed good reproducibility (Table 1). The representative chromatograms of cocaine in plasma, atria, ventricle, and tracheal tissues were shown in Figure 1.

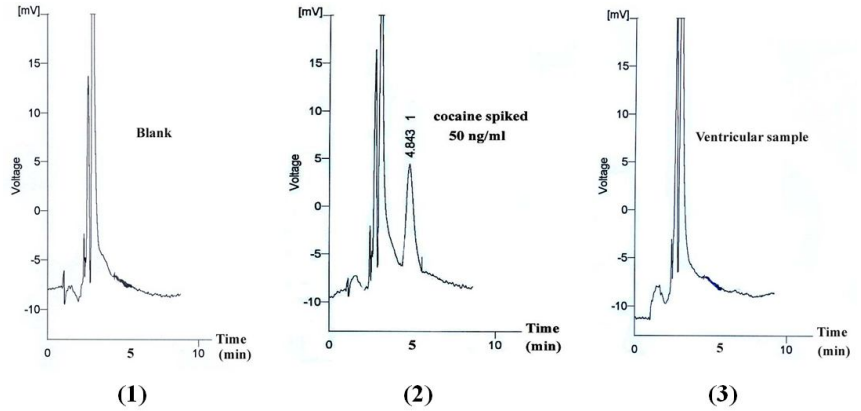
The concentrations of cocaine in plasma and smooth muscle of trachea taken from guinea-pigs at 24 hr after cessation of cocaine were 5.08±0.63 ng/ml and 2.8±0.41 ng/g, respectively, but it could not be detected in atrial and ventricular muscle, where as the lower limit of detection were 17.5 ng/g and 3.8 ng/g, respectively (Table 2).



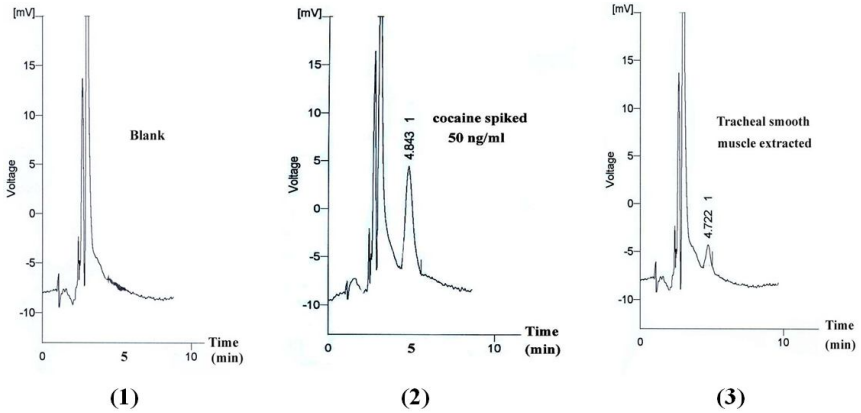
(a) Chromatogram of plasma



(b) Chromatogram of atria



(c) Chromatogram of ventricle



(d) Chromatogram of tracheal smooth muscle

Figure 1. The representative chromatograms of cocaine in plasma, atria, ventricle, and tracheal tissues; (1) blank (2) standard cocaine spiked 50 ng/ml and (3) sample taken from plasma, atria, ventricular, and tracheal smooth muscle in cocaine-treated guinea-pig at 24 hr after cocaine cessation.

Table 1. The validation parameters of cocaine concentrations in guinea-pig plasma, atria, ventricle and tracheal smooth muscle.

Validation parameters	Plasma Concentration (ng/ml)			Atrial Concentration (ng/ml)			Ventricular Concentration (ng/ml)			Tracheal Concentration (ng/ml)		
	6.25	25	100	6.25	25	100	6.25	25	100	6.25	25	100
Precision (%CV)												
- Intra-day	3.39	1.19	0.80	3.25	1.01	0.98	4.33	1.04	0.84	3.21	1.35	0.80
- Inter-day	4.13	1.22	1.78	4.09	1.56	0.84	3.18	1.45	1.58	3.14	2.1	0.78
Recovery (%)	95.15	95.89	96.73	99.54	98.71	98.12	99.18	99.30	98.95	99.07	98.29	97.41
Linearity	r =0.999			r =0.999			r =0.999			r =0.999		
LOD (ng/ml)	0.69			0.70 (17.5 ng/g)			0.76 (3.8 ng/g)			0.69 (0.80 ng/g)		
LOQ (ng/ml)	2.10			2.11 (52.75 ng/g)			2.32 (11.6 ng/g)			2.10 (2.4 ng/g)		

Table 2. Concentration of cocaine in plasma, tracheal smooth muscle, atria and ventricle following 2.5 mg/kg of cocaine administration at 24 hr after cocaine cessation

	Concentration of cocaine in			
	Plasma (ng/ml)	Tracheal smooth muscle (ng/g)	Atria (ng/g)	Ventricle (ng/g)
Mean±SE	5.08±0.63	2.8±0.41	ND*	ND**

* Not detectable, LOD = 17.5 ng/g **Not detectable, LOD = 3.8 ng/g

4. Discussion and Conclusion

Cocaine has been known to sensitize responses of autonomic effector organs to catecholamines. The sensitization is first hypothesized to be due solely to presynaptic mechanism. This classical accepted mechanism of action involves inhibition of peripheral neuronal amines reuptake resulting in increasing the synaptic concentration of these amines available for binding to adrenoceptors and enhancing the effect of exogenously administered norepinephrine and epinephrine (Jain *et al.*, 1990; Kalsner, 1993). However, many investigators have questioned this unitary hypothesis of cocaine action and provided evidences for other neuronal or nonneuronal or postsynaptic sites of sensitization unrelated to amine disposition as follows: 1). The maximum response of the tissue to the amine should not be altered if the sole event mediated by cocaine is the blockade of neuronal uptake mechanism. However, Greenberg and Long (1971) demonstrated that cocaine enhanced the maximum responses of rat vas deferens to norepinephrine. 2) Cocaine potentiated norepinephrine induced contraction responses in isolated vas deferens which is blocked by an irreversible α_1-receptor blocking agent, phenoxybenzamine (Nakatsu and Reiffenstein, 1968). 3) Chronic cocaine treatment (5-25 mg, i.p. tid. for 14 days) did not change the concentration of norepinephrine and its metabolites at certain areas of rat brain (Alburges and Wamsley, 1996; Alburges *et al.*, 1996) and Sunbhanich (1980) has demonstrated that biophase concentrations of epinephrine and norepinephrine were decreased in guinea-pig isolated atria in the presence of cocaine when compared to those of control. 4) Desmethylimipramine, a more potent catecholamine uptake blocker than cocaine, failed to produce potentiation responses to oxymetazoline in isolated splenic capsular strip of the cat (Summers and Tillman, 1979). 5) Cocaine induced supersensitivity in isolated human umbilical artery and guinea-pig tracheal smooth muscle which are devoid of adrenergic innervation (Reiffenstein and Triggle, 1974 and Chouykool, 1998). 6) Cocaine produced the leftward shifts of the dose- response curves to oxymetazoline, isoproterenol and salbutamol, which are not substrates for neuronal uptake, in various isolated tissues such as splenic capsular strip of the cat, guinea-pig atria and trachea (Summers and Tillman, 1979; Chouykool, 1998; Nima, 2007).

Since the pre-and/or postsynaptic mechanism of cocaine is still controversial. The aim of this study was to determine cocaine concentrations in plasma and other tissues such as atria, ventricle and trachea in order to correlate with

the supersensitivity to catecholamines occurred in such tissues after the cessation of chronic cocaine treatment. On the basis that if supersensitivity occurred in spite of the lower than threshold concentration of cocaine that could block amine reuptake, the presynaptic mechanism might not be involved. The method used in this study is the indirect way with the advantage of avoiding determination of amine concentration by using radioactive amine which is hazardous, difficult to obtain and handle.

The method for quantitation of cocaine used in this study showed good linearlity (R = 0.999) and precision (% CV less than 5), yielded high recovery (more than 95%) as compared to many reports using various methods such as gas chromatography, high performance liquid chromatography and liquid chromatography with mass spectroscopy which showed the % CV range of 3-10 and % recovery of 78-89 (Sukbuntherng *et al.*, 1995; Pan and Hedaya, 1997; Maurer, H.H., 1998). The sensitivity, expressed in term of lower limit of detection (LOD), in plasma, trachea and ventricle is quite high (0.69 ng/ml, 0.8 ng/g and 3.8 ng/g, respectively) compared with those of 2-40 ng/ml as previously reported by the investigators mentioned above. The sensitivity in atria is lower (LOD=17.5 ng/g) than other tissues but comparable to that of 6 ng/g in mice brain determined by GC/MS (McCarthy *et al.*, 2004). The LOQ in atria seem rather high (52.75 ng/g), but it is in the same range of 50-81 ng/ml in plasma determined by GC/MS and HPLC (Moeller *et al.*, 1998 and Pan and Hedaya, 1997). It is notable that cocaine could not be detected in atria and ventricle in this study which suggested that, although existed, the concentration should be less than the LOD. Normally, internal standards are used when the quantity of sample analyzed or the instrument response varies from run to run and difficult to control, also when sample loss is unavoidable during sample preparation steps prior to analysis. Instruments or techniques that are fully or partially automated tend to reduce these variations and thus improve precision. In this study, we could get good results (linearlity, precision and recovery), as shown above, without using internal standard.

The present study showed that cocaine concentrations in plasma and smooth muscle of trachea taken from guinea-pigs at 24 hr after cessation of cocaine were 5.08±0.63 ng/ml and 2.8±0.41 ng/g, respectively, while those in atrial and ventricular muscle were lower than 17.5 ng/g and 3.8 ng/g, respectively. According to Trendelenburg *et al.* (1972), the level of cocaine that caused neuronal uptake blockade of [^{3}H]-norepinephrine in cat nictitating membrane was higher than 10^{-7} M (30.34 ng/ml). Reiffenstein and Triggle (1974) also reported that cocaine in the concentration of $3.3x10^{-7}$ - $3.3x10^{-5}$ M (101.12 ng/ml - 10.11 µg/ml) could not block the uptake process of catecholamines. Although the amines uptake at the presynaptic site were not inhibited or determined in the present study, many investigators have shown that chronic cocaine treatment did not change or even decreased the amines in various tissues (Alburges and Wamsley, 1996, Alburges *et al.*, 1996; Sunbhanich, 1980). Thus, it was infered that the level of cocaine in plasma, atria, ventricle and trachea, after 24 hr of cocaine cessation, in the present study, were less than the levels that could block norepinephrine uptake at pre-synaptic site. However, Chouykool (1998) and Nima (2007) have demonstrated that cocaine treatment in the same condition as the present study produced supersensitivity to norepinephrine and epinephrine in isolated guinea-pig atria and trachea. Reiffenstein and Triggle (1974) also reported the supersensitivity responses in isolated human umbilical arteries induced by cocaine at the concentration which could not block the uptake process of catecholamines. Thus, it is considered, by the indirect way we used in our study, that cocaine-induced supersensitivity to catecholamines might not be related to the presynaptic mechanism. In addition, many investigators suggested that cocaine increased influx of Ca^{2+} into the cells (Greenberg and Long, 1971; Kalsner, 1992; Premkumar, 1999; Sabra *et al.*, 2003). Premkumar (1999) also reported that cocaine potentiated the L-type calcium channel currents by cardiac myocyte. It was suggested that cocaine directly bound and facilitated the opening of L-type calcium channels resulting in elevation of intracellular Ca^{2+} and this mechanism of cocaine played an important role in many of the actions in the cardiovascular and central nervous systems.

In conclusion, the results of the present study and the previous evidences of other investigators support the post-synaptic mechanism of cocaine enhancing supersensitivity which might be correlated with cardiac complication in chronic cocaine use.

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