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Cocaine induces apoptosis in cerebral vascular muscle cells: potential roles in strokes and brain damage

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Abstract

Cocaine abuse is known to induce different types of brain-microvascular damage and many adverse cerebrovascular effects, including cerebral vasculitis, intracranial hemorrhage, cerebral infarction and stroke. A major physiological event leading to these pathophysiological actions of cocaine could be apoptosis. Whether cocaine can cause brain-microvascular pathology and vascular toxicity by inducing apoptosis of cerebral vascular smooth muscle cells is not known. This study, using several different methods to discern apoptosis, was designed to investigate if primary cultured canine cerebral vascular smooth muscle cells can undergo apoptosis when treated with cocaine. After treatment with cocaine (10^{-6}-10^{-3} M) for 12-24 h, the death rates of cerebral vascular smooth muscle cells increased in a concentration-dependent manner compared with controls. Morphological analysis of cerebral vascular smooth muscle cells using confocal fluoresence microscopy showed that the percentage of apoptotic cerebral vascular smooth muscle cells increased after cocaine (10^{-6}-10^{-3} M) treatment in a concentration-dependent manner. TUNEL assays also showed positive results for cerebral vascular smooth muscle cells treated with cocaine. These results clearly demonstrate that cerebral vascular smooth muscle cells can undergo rapid apoptosis in response to cocaine in a concentration-dependent manner. Cocaine-induced apoptosis may thus play a major role in brain-microvascular damage, cerebral vascular toxicity and strokes.

Keywords: Cocaine; Apoptosis; Cerebral vascular smooth muscle cell; Strokes

1. Introduction

During the past decade, cocaine abuse has increased dramatically and has attracted public and scientific attention on the acute and chronic toxic effects of this drug of abuse. Long-term ingestion of cocaine is known to cause several types of brain-microvascular pathology (for review, see Neiman et al., 2000). Among the many adverse effects, cerebrovascular complications seem to very prominent, especially compared with other controlled drugs (Gold, 1993). As for cerebral vascular toxicity, cocaine is known to induce cerebral vasculitis (Frederichs and McQuiuen, 1991), intracranial hemorrhage (Mangiardi et al., 1988; Nolte and Gelman, 1989), cerebral infarction (Daras et al., 1994), and subarachnoid hemorrhage, which may lead to strokes or sudden death (Polis et al., 1987; Altura and Grupta, 1992). Mechanisms of cerebral vascular toxicity include cocaine-induced cerebral vessel spasm (Altura et al., 1985; He et al., 1993), increased intracellular free ion calcium overload (Zhang et al., 1996) coupled with decreased intracellular free magnesium ions (Huang et al., 1990; Altura and Gupta, 1992; Altura et al., 1993), decreased brain cellular bioenergetics (Altura and Gupta, 1992) and a compromise of the mitochondrial cytochrome oxidase system (Barbour et al., 1993).

Apoptosis (also known as programmed cell death) was first identified and named by Kerr in 1972 (Kerr et al.,

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1972). It is classically characterized by chromatin condensation and margination, cell shrinkage, and membrane blebbing. It is a kind of cell suicide quite different from necrosis, without inflammatory cell infiltration. Apoptosis is well-programmed by an intrinsic cell–suicide machinery, involving activation of a special group of enzymes called caspases, cutting normal DNA molecules into small fragments and disintegration of the cell into apoptotic bodies (for recent review, see Zimmermann et al., 2001).

Apoptosis is not only a physiological process during embryonic development, but it also plays an important role in the pathophysiology and treatment of many diseases, such as cancers (Kerr et al., 1994). As for blood vessels, apoptosis has been found in neonatal lamb arteries (Cho et al., 1995) and human incisal arteries (Thorball et al., 1985). Evidence also has been accumulating, since 1994, to show that apoptosis occurs in peripheral and coronary atherosclerosis (Geng and Libby, 1995), restenosis after coronary angioplasty (Isner et al., 1995), after carotid artery injury (Han et al., 1995) and in hypertension (Devlin et al., 2000). Both endothelial cells and peripheral vascular smooth muscle cells can undergo apoptosis (Araki et al., 1990; Bennett et al., 1994). Several investigators have begun to study specific mechanisms of vascular smooth muscle cell apoptosis in aortic vascular smooth muscle cells and coronary vascular smooth muscle cells, but no study has appeared on cerebral vascular smooth muscle cells.

It has been reported very recently that cocaine can induce apoptosis in cultured human coronary endothelial cells (He et al., 2001), myocardial myocytes (Xiao et al., 2000), hepatocytes (Wang et al., 2001), thymocytes (Wu et al., 2003), neuronal cells (Sharan et al., 2003) and rat testes (Li et al., 2003). Cocaine-induced apoptosis could be related to its cerebrovascular complications. In this study, using several different cytochemical and biochemical methods, we hypothesized and found that cocaine can induce apoptosis in cerebral vascular smooth muscle cells. We found that cocaine can induce apoptosis in cerebral vascular smooth muscle cells in a concentration-dependent manner; the greater the concentration of cocaine, the more rapid and the more intense was the apoptotic event, and this may be one of the primary mechanisms of stroke and brain-vascular damage related to cocaine abuse.

2. Materials and methods

2.1. Cell isolation and primary cell culture

Smooth muscle cells of canine cerebral basilar arteries were obtained from pentobarbital sodium-anesthetized (40 mg/kg, i.v.) male mongrel dogs (25-35 kg; 10-12 months). The procedure employed to isolate the arterial smooth muscle cells has been reported (Zhang, et al., 1992). Briefly, vascular smooth muscle cells were cultured in Dulbecco's modified Eagle's medium, mixed 1:1 with Ham's nutrient mixture F-12, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% L-glutamine and 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator composed of 95% air 5% CO_2 (Zhang et al.,1992; Altura et al., 1993; He et al., 1993). Morphological examination of confluent cultures revealed vascular smooth muscle cells exhibiting a crisscross pattern, hills-and valleys, and nodular structures when examined by phase-contrast microscopy. Immunohistochemical staining with a monoclonal antibody recognizing exclusively–smooth muscle actin (Sigma, St. Louis, MO, USA) indicated that over 97% of the cultures were pure vascular smooth muscle cells (Altura et al., 1993; Zhang et al., 1996). No fibroblasts were noted in our cultures. All the cells used for the experiments were passage 3-6 and were at 80 ~ 90% confluence. After 24 h of culture in serum-free medium, the primary cells were treated with cocaine HCl (10^{-6}-10^{-3} M) in the medium with 1% fetal bovine serum for 12, 24 and 48 h, respectively.

2.2. Cell viability

Trypan blue exclusion was used to determine cell viability. The experiments were performed in 24-well plates. At the end of each experiment, the cells were harvested using trypsin (0.05%). The cells were then centrifuged at 1200 × g for 10 min at 4 °C, examined by adding an equivalent volume of a 0.4% trypan blue solution to an aliquot of the resuspended cells and incubated for 5 min. The stained and unstained cells were counted by means of a hemacytometer under 100 × microscopy. Six fields were examined.

The mean values obtained represent data of triplicates from each separate experiment.

2.3. Quantitative morphological analysis

To confirm that cell death was occurring via apoptosis, cells were grown on coverslips. After they reached 80 ~ 90% confluence, the cells were cultured in serum-free medium for 24 h, and treated with cocaine HCl (10^{-6}-10^{-3} M) in medium with 1% fetal bovine serum for 12, 24 and 48 h,respectively The cells were then fixed with formaldehyde for 10 min, rinsed with phosphate buffered saline (PBS) for 5 min, 0.2 ml of RNAse A stock solution (1 mg/ml) was added at 37 °C for 30 min, cells washed with PBS for 5 min, and then incubated with 10 μg/ml of propidium iodine or 10 μg/ml acridine orange for 15 ~ 20 min. After decolorization with distilled water, the cells were viewed with an ultraviolet-fluorescence microscope and an ultraviolet-laser confocal microscope. Cells with typical features of nuclear fragmentation and/or marked condensation of chromatin with reduced nuclear size were interpreted as apoptotic cells (He et al., 2001). The number of apoptotic cells and total cells were counted in six randomly selected high-power fields. The percentage of

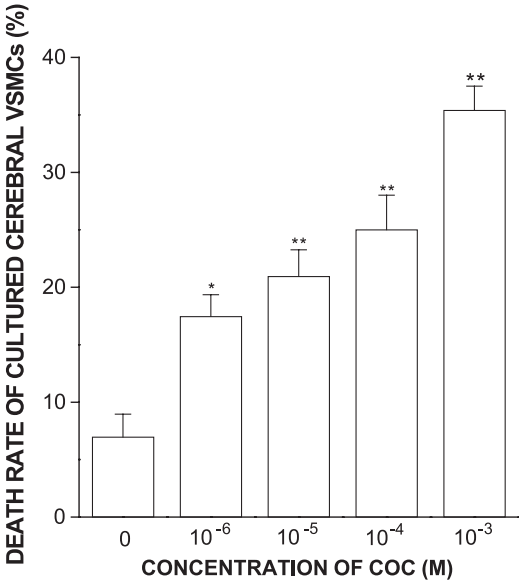

Fig. 1. Cocaine HCl (10^{-6}–10^{-3} M) increased the death rates of primary cultured canine cerebral vascular smooth muscle cells (n=6) as determined by trypan blue exclusion. *P<0.05, **P<0.01 vs. Controls.

apoptotic cells was calculated as the number of apoptotic cells/number of total cells × 100%.

2.4. Tunel assay

The terminal deoxyribonucleotidyl transferase(TDT)-mediated dUTP nick-end labeling (TUNEL) assay was used to detect DNA fragmentation in situ according to kit instructions (Gavrieli et al., 1992). Briefly, cells were seeded on coverslips and treated with cocaine HCl for 24 h, fixed with 4% paraformaldehyde in PBS for 1 h at 25 °C, rinsed with phosphate buffered saline (PBS) for 5 min, incubated in permeabilization solution (1% triton X-100 in 0.1% sodium citrate) for 2 min on ice, and the TUNEL reaction mixture added for 1 h at 37 °C in a humidified atmosphere in the dark. Negative controls were set up by adding labeling solution without terminal transferase instead of the TUNEL reaction mixture. After rinsing 3 times with phosphate buffered saline (PBS), samples were analyzed in a drop of PBS under fluorescence using an excitation wavelength wave length in the range of 450-500 nm and detection wave length in the range of 515-565 nm.

2.5. Materials

Cocaine HCl was purchased from The National Institute of Drug Abuse. Propidium iodide, acridine orange and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). In situ apoptosis kits were purchased from Roche (Mannheim, Germany). All other chemicals were of highest purity and purchased from Fisher Scientific (New Jersey).

2.6. Statistics

Where appropriate, results are expressed as means ± S.E.M and were examined for statistical significance by means of Student t-tests and ANOVA. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Cocaine induces death of cerebral vascular smooth muscle cells

The death rate of cultured cerebral vascular smooth muscle cells treated with cocaine (10^{-6}-10^{-3} M), as determined by trypan blue exclusion, increased when compared with controls (Fig. 1).

3.2. Cocaine causes nuclear changes characteristic of apoptosis in cerebral vascular smooth muscle cells

In order to investigate whether the increased death of cerebral vascular smooth muscle cells, induced by cocaine, is partly due to apoptosis or necrosis, propidium iodine or acridine orange was used to study the morphology of the nuclei. The percentage of apoptotic cells was increased by cocaine (10^{-6}-10^{-3} M) at 24 h in a concentration-dependent manner (Fig. 2.). Cerebral vascular smooth muscle cells treated with cocaine 10^{-5} M for 12, 24, and 48 h all showed increased apoptotic rates compared with controls

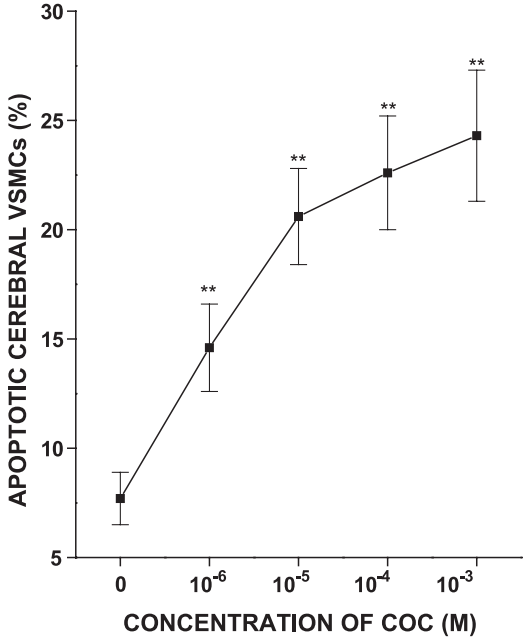

Fig. 2. Apoptotic rates of cultured canine cerebral vascular smooth muscle cells treated with cocaine HCl (10^{-6}–10^{-3} M) for 24 h were increased when compared to control (n=6). **P<0.01 vs. Controls.

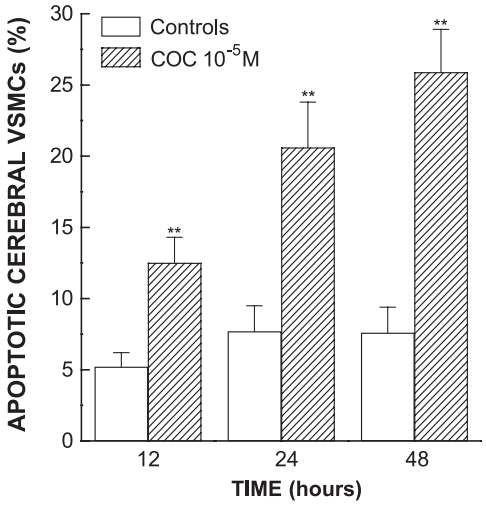

Fig. 3. Cultured canine cerebral vascular smooth muscle cells treated with cocaine HCl 10^{-5} M for 12, 24 and 48 h show increased percentages of apoptotic cells (n=6). **P<0.01 vs. Controls.

(Fig. 3.); this also took place in a concentration-and time-dependent manner.

3.3. Results of TUNEL assays

Cerebral vascular smooth muscle cells treated with 10^{-5} M cocaine HCl for 24 h showed positive results with the TUNEL assay (Fig. 4). Although not shown, the results with the TUNEL assay indicated that cocaine-induced DNA-fragmentation occurred in a concentration-and time-dependent manner (n = 6), similar to that observed for the nuclear changes using propidium iodide (Fig. 3) Untreated control cells failed to show positive nuclei reactions with the TUNEL assay (n = 6).

4. Discussion

Apoptosis, a word taken from the Greek language, was first used by Kerr in 1972, to describe a kind of cell death

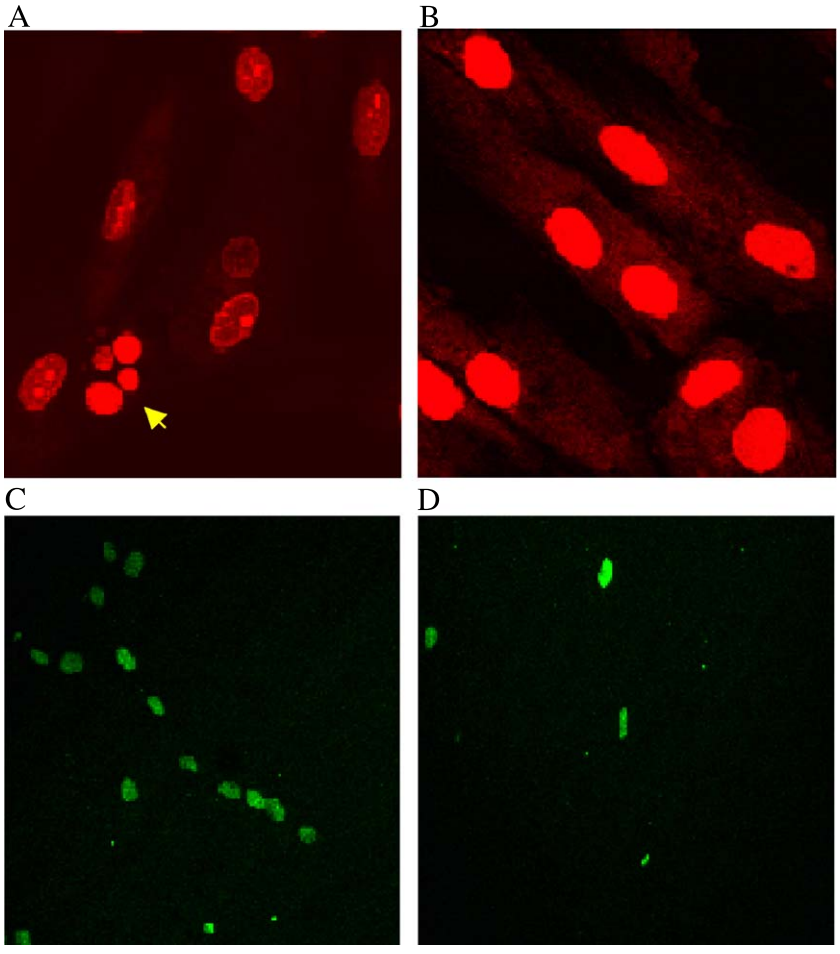

Fig. 4. Cultured canine cerebral vascular smooth muscle cells treated with 10^{-5} M cocaine HCl for 24 h underwent apoptosis. (A) Morphological changes of cerebral vascular smooth muscle cell nuclei treated with 10^{-5} M cocaine observed under confocal fluoresence microscope (400×) after propidium iodide dye treatment. Arrow shows a cell nucleus with condensation and fragmentation. (B) Control. (C) TUNEL result of cerebral vascular smooth muscle cells treated with 10^{-5} M cocaine is positive (200×). (D) Control for TUNEL assay is negative.

which is quite different from the morphological events of necrosis (Kerr et al., 1994). It is determined to be very important in cell dynamics, together with cell proliferation. The net balance between cell proliferation and cell apoptosis determines the extent of cell growth (Kerr et al., 1994). It is natural and necessary for body homeostasis as a whole. But under pathophysiological conditions, apoptosis can also initiate disease mechanisms. Previous studies have shown that apoptosis is involved in a number critical events occurring during normal development and plays key roles in a wide variety of diseases including vascular-related diseases of diverse types and strokes (Hetts, 1998; Mallat and Tedgui et al. 2000).

Although apoptosis has been thought to play an important role in blood vessel dynamics, only during the past ten years have researchers begun to get interested in the role of apoptosis in vascular diseases. Apoptotic vascular smooth muscle cells can be found in coronary or peripheral atherosclerosis (Geng and Libby1995), restenosis (Isner et al., 1995) and hypertension (Devlin et al., 2000). This may help to explain why the number of vascular smooth muscle cells remains to be almost the same level at 2 weeks and 3 days after angioplasty, despite the very active proliferation of vascular smooth muscle cells after endothelium injury. Apoptosis may also be one of the factors that contributes to the rupture of plaques (Bennett et al., 1997). Some recent in vitro experiments suggest that aortic or coronary vascular smooth muscle cells can undergo apoptosis if induced by nitric oxide (NO), PDGF, low serum concentration, c-myc gene transfection, or angiotensin II, which suggests that apoptosis of at least peripheral vascular smooth muscle cells is regulated by many factors in vivo, thus playing roles in the pathogenesis of several vascular diseases (Pollman et al., 1996; Bennett et al., 1994). In the present study, we demonstrate, for the first time, that primary cultured cerebral vascular smooth muscle cells can also undergo apoptosis, like coronary or aortic vascular smooth muscle cells. It is thus, likely, that this physiological event is important in brain infarction, intracranial haemorrhages, and other types of stroke.

It has been reported that plasma levels of cocaine after intravenous use in cocaine-related deaths are approximately 6×10^{-7} M to 3×10^{-4} M (Polis et al., 1987). The blood levels required to produce euphoric effects are approximately 10^{-7} M to 10^{-5} M (Van Dyke et al.,1976). Cocaine is well-known for its ability to induce brain-vascular cerebral damage such as infarctions or intracranial haemorrhage and strokes (Mangiardi et al., 1988; Nolte and Gelman, 1989; Altura and Gupta, 1992; He et al., 1993).This study clearly shows, for the first time, that cocaine (10^{-6}-10^{-3} M) will cause apoptosis of primary cultured cerebral vascular smooth muscle cells in a concentration-dependent manner, and at levels found in the blood of cocaine users. This programmed cell death, therefore, may be one of the major mechanisms of cocaine-induced central nervous system toxicity.

It has been reported that a calcium ion increase can, itself, induce apoptosis by activating Ca^{2+}-Mg^{2+}-dependent endonucleases [Woo,et,al,1996,]. Previous studies from our laboratory have documented the effects of cocaine on cerebral vascular smooth muscle cells including a rapid and progressive $[Ca^{2+}]_i$ overload (Zhang et al., 1996) and $[Mg^{2+}]_i$ depletion (Altura and Gupta, 1992; Altura et al., 1993) coupled to subsequent cerebral ischemia, reduced microvascular blood flows (Huang et al, 1990) and a compromise of brain cellular and mitochondrial bioenergetics (Altura and Gupta, 1992; Barbour et al., 1993). These results suggest that $[Ca^{2+}]_i$ overload and $[Mg^{2+}]_i$ depletion are most likely early signaling pathways for cocaine-induced apoptosis of cerebral vascular smooth muscle cells. Cocaine-induced apoptosis may, partly, be mediated by the free calcium ion increase which quickly happens after addition of cocaine (Zhang et al., 1996). Although the precise signaling pathway(s) by which cocaine induces apoptosis in cerebral vascular smooth muscle cells needs to be investigated, the identification of both pro-apoptotic and anti-apoptotic modulators could lead to new therapeutic approaches for the treatment of cocaine-induced brain-microvascular damage and strokes.

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