Neurotoxicity of Anhydroecgonine Methyl Ester, a Crack Cocaine Pyrolysis Product

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Smoking crack cocaine involves the inhalation of cocaine and its pyrolysis product, anhydroecgonine methyl ester (AEME). Although there is evidence that cocaine is neurotoxic, the neurotoxicity of AEME has never been evaluated. AEME seems to have cholinergic agonist properties in the cardiovascular system; however, there are no reports on its effects in the central nervous system. The aim of this study was to investigate the neurotoxicity of AEME and its possible cholinergetic effects in rat primary hippocampal cell cultures that were exposed to different concentrations of AEME, cocaine, and a cocaine–AEME combination. We also evaluated the involvement of muscarinic cholinergic receptors in the neuronal death induced by these treatments using concomitant incubation of the cells with atropine. Neuronal injury was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays. The results of the viability assays showed that AEME is a neurotoxic agent that has greater neurotoxic potential than cocaine after 24 and 48 h of exposure. We also showed that incubation for 48 h with a combination of both compounds in equipotent concentrations had an additive neurotoxic effect. Although both substances decreased cell viability in the MTT assay, only cocaine increased LDH release. Caspase-3 activity was increased after 3 and 6 h of incubation with 1mM cocaine and after 6 h of 0.1 and 1.0mM AEME exposure. Atropine prevented the AEME-induced neurotoxicity, which suggests that muscarinic cholinergic receptors are involved in AEME’s effects. In addition, binding experiments confirmed that AEME has an affinity for muscarinic cholinergic receptors. Nevertheless, atropine was not able to prevent the neurotoxicity produced by cocaine and the cocaine–AEME combination, suggesting that these treatments activated other neuronal death pathways. Our results suggest a higher risk for neurotoxicity after smoking crack cocaine than after cocaine use alone.

Key Words: anhydroecgonine methyl ester; neurotoxicity; crack cocaine; rat primary hippocampal cell culture.

Crack cocaine, the smoked form of cocaine, is a highly addictive drug, and its use was first reported in the 1980s in Europe and the United States (Hamid, 1992). Since then, its use has been increasing worldwide, and cocaine addiction is considered a major public health problem in several countries (Darke et al., 2002; Fischer et al., 2006; Haasen et al., 2004; Marsden et al., 2009; Oliveira and Nappo, 2008; Pavarin, 2006).

Crack is cocaine in its freebase form, which has a lower melting point (96°C–98°C) than cocaine hydrochloride (198°C). Therefore, the heating of freebase cocaine produces rapid cocaine volatilization and absorption by the lungs, and the drug reaches the brain faster than by any other route. This process results in the formation of anhydroecgonine methyl ester (AEME), also called methylecgonidinone, a cocaine pyrolysis product that also has rapid pulmonary absorption (Fandiño et al., 2002). According to Martin et al. (1989) and Nakahara and Ishigami (1991), 50–80% of cocaine is converted to AEME at temperatures of 255°C–420°C, and at 650°C, this conversion is greater than 80%. Nevertheless, it is very difficult to predict the amount of cocaine converted to AEME due to variations in the temperature used to volatilize it, the smoking devices used, and the purity of freebase cocaine (Paul et al., 2005).

Use of crack cocaine can lead to loss of control over decision making and produces harmful neurological, psychiatric, cardiovascular, and respiratory consequences. It has been associated with more violent behavior and aggression compared with the use of powdered cocaine (Cornish and O’Brien, 1996; Haasen et al., 2005). The neurological and psychiatric effects of crack use include the development of seizures, intracerebral hemorrhage, vascular spasm (Brown et al., 2009; Oliveira and Nappo, 2008; Pavarin, 2006).

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2002). Although crack cocaine reaches the brain very quickly, its short-term effects make users to smoke at a high frequency, which leads to a rapid development of dependence.

Cocaine in its hydrochloride or base forms has been associated with brain atrophy (Sim et al., 2007), in addition to abnormalities of brain metabolic (Volkow et al., 2004) and electrical activities (Reid et al., 2006). Smith et al. (1993) demonstrated that high doses of cocaine could inhibit hippocampal long-term potentiation, a process involved in memory formation. The relationship between these brain alterations and an individual’s neurocognitive function has also been investigated. In fact, neurocognitive dysfunctions, such as disruption of verbal memory and attention, have been reported in crack users (Cunha et al., 2004; De Oliveira et al., 2009; Di Sclafani et al., 2002).

Cocaine neurotoxicity has been demonstrated in several biological models, both in vivo and in vitro. For instance, excitotoxicity has been demonstrated in cocaine-exposed rats (Huber et al., 2001; Schilström et al., 2006), cocaine activates apoptosis in a dopaminergic cell line (PC12 cells) and in primary neuron cultures (Imam et al., 2005; Lepsch et al., 2009), and cocaine induces oxidative stress in human neuronal progenitor cells (Poon et al., 2007). Cocaine has also been shown to potentiate the neurotoxicity induced by TAT, a human immunodeficiency virus (HIV) protein with an important role in the pathogenesis of HIV dementia (Aksenov et al., 2006). Although some results indicate cocaine neurotoxicity, Goodman and Slevitzer (1993) were unable to find any evidence of cocaine-induced neuronal damage in rats after acute and chronic treatments.

Understanding the possible neurotoxicity of AEME would raise a new hypothesis about the neurotoxic potential of crack cocaine. The few studies available on the effects of AEME have been conducted in the peripheral nervous system. Erzouki et al. (1995) showed that intravenous administration of AEME in rabbits reduced the blood pressure and heart rate and increased the respiratory rate. Moreover, AEME showed a negative inotropic effect in cultures derived from human ventricular trabeculae due to a decrease in calcium availability during muscular contraction. This effect is possibly mediated through muscarinic cholinergic receptors, a process that is reversed by atropine, a nonspecific muscarinic receptor antagonist (Woelf et al., 1997). Scheidweiler et al. (2003) showed that, in sheep, AEME produces cardiovascular effects that are also consistent with a muscarinic cholinergic effect. Significant hypotension and tachycardia occurred in all sheep, and these effects were antagonized by intravenous treatment with atropine.

According to Bradley et al. (2003), activation of the cholinergic M$_1$-muscarinic subtype of receptors (G1 protein-coupled receptors) may lead to an increase in intracellular calcium, which can activate the caspase pathways, resulting in neuronal death. In fact, Shih et al. (2010) demonstrated that arecoline, a muscarinic cholinergic receptor agonist used as a therapy in patients with Alzheimer’s disease, induced neuronal apoptotic death through attenuation of the antioxidant system, which enhanced oxidative stress.

Our aim was to investigate the effects of AEME in rat primary hippocampal cell cultures because the hippocampus is one of the most important regions involved in learning and memory, and it is rich in muscarinic cholinergic receptors. Thus, we evaluated the following questions: (1) whether AEME has cholinergic agonist properties, (2) the neurotoxicity of AEME, (3) the contribution of the cocaine–AEME combination to neurotoxicity, and (4) whether this neurotoxicity is dependent on muscarinic cholinergic receptors.

Importantly, this is the first study to evaluate the neurotoxicity of AEME. Furthermore, this is also the first study to verify cocaine neurotoxicity in rat primary hippocampal cell cultures.

MATERIALS AND METHODS

Animals. Pregnant Wistar rats weighing 230–250 g were obtained from the Butantan Institute, São Paulo, Brazil. They were housed in plastic cages and maintained in a room with constant temperature (22±1°C) on a 12:12-h light/dark cycle (lights on at 7:00 A.M.). Food and water were provided ad libitum. This study was performed according to National Institutes of Health guidelines and approved by the Animal Use Ethics Committee of the Butantan Institute and the Animal Experimentation Ethics Committee of the School of Pharmaceutical Sciences at the University of São Paulo.

Synthesis of AEME. AEME was synthesized using cocaine that was generously donated by the Criminal Institute of São Paulo to the Laboratory of Toxicological Analyses (School of Pharmaceutical Sciences, University of São Paulo) for research purposes. The cocaine was purified to 95% purity.

Briefly, a 5mM (1.70 g) cocaine hydrochloride solution was refluxed in 50 ml of concentrated hydrochloric acid for 24 h. Then, the mixture was cooled to room temperature, diluted with 30 ml of water and extracted with diethyl ether (2 × 255 ml) to remove benzoic acid. The aqueous phase was then evaporated under vacuum to dryness. The white solid was further dried under vacuum at 100°C for 24 h (Zhang et al., 1997). This produced crude anhydroecgonine hydrochloride (0.96 g, 95%), which was used in the next step without further purification. The product was confirmed by its melting point of 239°C–244°C. A solution prepared with 50 ml of dry methanol and 3mM (0.61 g) of the previously synthesized anhydroecgonine hydrochloride was stirred and bubbled with dry gaseous HCl for 4 h. Thereafter, the mixture was refluxed for 6 h, and the solvent was evaporated under vacuum. The yellow oil that remained was dried under vacuum for 24 h. This produced crude AEME (0.50 g, 99%) that was used without further purification. The product was evaluated by proton nuclear magnetic resonance (’H-NMR) and electrospray ionization–mass spectrometry (ESI–MS) in accordance with the literature. ’H-NMR (300.13 MHz, CDCl$_3$, tetramethylsilane; 0 ppm): 6.93, C=O–H (1H, s); 3.79, OCH$_3$ (3H, s); 2.75, NCH$_3$ (3H, s); 2.78–1.93 (8H, m); ESI–MS (m/z): 182 [M + H]$^+$; 151 [M – OCH$_3$ + H]$^+$; 122 [M – COOCH$_3$]$^-$ (Kline et al., 1990).

Rat primary hippocampal cell culture. Hippocampal neurons were dissociated from the hippocampi of E18–E19 Wistar rat embryos. Pregnant rats were anesthetized with pentobarbital (45 mg/kg), and the fetuses were rapidly euthanized by decapitation to remove their hippocampi. The tissues were placed into a Petri dish containing 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) in cooled neurobasal medium (Gibco). The hippocampi were placed into a Petri dish containing 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) in cooled neurobasal medium (Gibco). The hippocampi were washed with Hank’s Balanced Salt Solution and subjected to mechanical fragmentation. The isolation of hippocampus cells was performed using proteolytic digestion with trypsin (Sigma; Jahr and Stevens, 1987; Silva et al., 2006). The hippocampus fragments were transferred to 0.25% trypsin in Earl’s Balanced Salt Solution (EBSS) solution, pH 7.2–7.4, and were incubated for 10 min at 37°C. Following trypsinization, the cells were washed with an
EBSS solution containing 277.5 U/ml DNase (Sigma) and 10% fetal bovine serum (Gibco). After dispersion with Pasteur pipettes of different diameters, the tissue was resuspended in neurobasal medium (Gibco) supplemented with 0.5mM L-glutamine, 25µM L-glutamic acid, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2% B27 supplement (Gibco) to reduce glial cell proliferation (Brewer et al., 1993; Silva et al., 2006). Then, the cells were seeded onto 0.01% poly-L-lysine-coated multiwell culture plates and maintained at 37°C in a humidified atmosphere of 5% CO₂ for 7–8 days, the time required for maturation of hippocampal neurons. On the second day, half of the old medium was replaced with the same volume of a fresh medium of the same composition. On the seventh day, the cells were incubated with cocaine and/or AEME at several concentrations for 3, 6, 12, 24, or 48 h, depending on the experiment. For the assessment of cell viability, hippocampal neurons were plated on poly-L-lysine-coated 96-well culture plates at a density of 2 × 10⁵ cells/cm².

Characterization of cultured cell types by immunohistochemistry. Immunofluorescent labeling was used to characterize the cell types present in the culture. Coverslips with attached cells were removed from the culture medium with forceps and washed once for 5 min in ice-cold phosphate-buffered saline (PBS). Then, the cells were fixed in a mixture of acetone and methanol (1:1) for 10 min at −20°C. Prior to staining, the cells were permeabilized in PBS containing 0.2% Triton for 30 min at room temperature. The coverslips were blocked to prevent nonspecific antibody binding by incubating them at room temperature for 30 minutes in PBS containing 2% normal goat serum, 4% bovine serum albumin, and 0.2% Triton on a rocker set at 12 cycles/min. The neurons were labeled with mouse anti-microtubule-associated-protein 2 (MAP-2) primary antibody (1:2000, Sigma) overnight at 4°C, and the astroglia with rabbit anti-glial fibrillary acidic protein (GFAP) primary antibody (1:1500, Sigma) for 1 h at room temperature. Afterward, the coverslips containing the cells were washed thrice for 5 min and incubated for an additional 1 h with secondary anti-mouse (fluorescein, 1:200, Jackson) and anti-rabbit antibodies (Texas Red, 1:200, Jackson). After washing thrice for 5 min, the dishes were mounted with mounting media containing 4,6-diamidino-2-phenylindole (DAPI; Vector), which labels the nuclei of all the cells present in the culture (total cells). Finally, the cells were analyzed under a fluorescence microscope (Nikon eclipse TE300).

MTT-reduction assay. Cell viability was evaluated using the 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Liu et al., 1997; Mosmann, 1983). Briefly, the yellow compound is reduced to formazan, a purple product, by the mitochondrial reductase enzymes of viable cells, and the absorbance of the cells was measured at 570 nm. In all cell viability tests, 250mM KCl was used as a positive control.

After each period of incubation with cocaine, AEME, or a cocaine–AEME combination, all the medium was removed, and 100 µl of MTT solution (5 mg/ml MTT in PBS and neurobasal medium without phenol red [1:9, v/v/l]) were added. After 3 h of incubation with MTT at 37°C in a humidified atmosphere of 5% CO₂, 100 µl of dimethyl sulfoxide was added to each well. After 30 min of shaking, the absorbance at 570 nm was measured in a multiwell plate reader (SpectraMax Plus, Molecular Devices) at intervals of 20 s for 15–20 min or until complete stability of the readings. Then, 10 µl of a 20-mM pyruvate solution was added to each well, and the absorbance at 340 nm was measured at intervals of 20 s for at least 5 min. The kinetic curve was traced at a linear location on the graph that contained measurements taken for at least 3 min (Jayalakshmi et al., 2005). This assay was performed in quintuplicate, and the results were expressed as pmol of NADH consumption/minute/cell.

Caspase-3 activity. Caspase-3 activity was assayed as described by Melo et al. (2010), with minor modifications. The cells were centrifuged at 1000 x g for 15 min at 4°C, and the pellet obtained was resuspended in 200 µl of lysis buffer containing 10% sucrose, 0.1% 3-(3-cholamido-propyl)dimethylammonio)-1-propanesulfonic acid (CHAPS), 100mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.4), 10 mg/ml leupeptin, 10 mg/ml aprotinin, 200mM phenylmethylsulfonyl fluoride (PMSF), and 10mM dithiothreitol (DTT). The cells were incubated for 30 min at 4°C and centrifuged at 12,000 x g for 30 min at 4°C. The supernatant was collected, and the protein content was determined by the Bradford (1976) method. Caspase protease activity was determined by incubating the lysate (20 µg of total protein) with 50mM of the fluorescent substrate acetyl Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC; for caspase-3) in a buffer containing 10% sucrose, 100mM HEPES (pH 7.4), 10 mg/ml leupeptin, 10 mg/ml aprotinin, 200mM PMSF, and 10mM DTT. Caspase activity was assessed by measuring, at 5-min intervals, the fluorescence of 7-amino-4-trifluoromethylcoumarin released over a period of 30 min using a spectrofluorometer (Molecular Devices SpectraMax Gemini XS, CA).

[3H]Quinuclidinyl benzilate binding assay. Hippocampal membranes, obtained from six animals for each experiment, were prepared as described by Cardoso et al. (2004). Hippocampi were isolated from rats, minced, and homogenized in 25mM Tris–HCl, pH 7.4 (containing 0.3 M sucrose, 5mM MgCl₂, 1mM EDTA, and 1mM PMSF), with an Ultra-Turrax homogenizer (T25, Ika Labotechnik, Staufen, Germany). The homogenate was centrifuged at 1000 x g for 10 min. The supernatant was filtered through two layers of gauze and then centrifuged at 100,000 x g for 1 h. The final 100,000 x g pellet was resuspended in 1 ml of 25mM Tris–HCl, pH 7.4 (containing 5mM MgCl₂, 1mM EDTA, and 1mM PMSF), with a Ultra-Turrax homogenizer and stored at −80°C. All procedures were carried out at 4°C, and all solutions contained freshly added 1mM PMSF to inhibit proteolysis. The protein concentration of the membrane preparations was determined with a protein reagent assay (Bio-Rad Laboratories Inc., Hercules, CA). Competition-binding experiments were performed as described by Abdalla et al. (2004). Briefly, the hippocampus membrane preparation (80 µg protein/ml) was incubated for 1 h with [3H] quinuclidinyl benzilate (QNB; specific activity: 44 Ci/mmol; New England Nuclear, Boston, MA) at a concentration near the distribution coefficients, Kᵦ, values, reported by Cardoso et al. (2004) at 30°C in the absence and presence of increasing concentrations of AEME (or atropine, used as a control for the assay). The competition-binding data were analyzed using the weighted nonlinear least-squares interactive curve-fitting program of GraphPad Prism (GraphPad Prism Software Inc., San Diego, CA). A mathematical model for one or two binding sites was applied. The inhibition constant (Kᵦ) was determined from the competition-binding curves using the Cheng and Prusoff equation (Cheng and Prusoff, 1973). The potency was expressed by the negative logarithm of the Kᵦ value (pKᵦ).

Statistical analysis. Assays were performed in quintuplicate and were repeated three times (MTT) and two times (LDH). Caspase-3 assays were performed twice in quadruplicate. Data of the binding assays were representative of four independent experiments performed in duplicate. The data were expressed as mean ± SEM and were analyzed by ANOVA followed by Newman–Keuls post hoc test. A significance level of 95% (p < 0.05) was accepted.
RESULTS

Characterization of the Cultured Cell Types by Immunohistochemistry

The cultured cells were immunohistochemically characterized using MAP-2, a neuronal marker, and GFAP, an astrocytic marker. A predominance of neurons (92%) was observed, with 8% of the cells being astrocytes (Fig. 1).

Evaluation of AEME- and Cocaine-induced Neurotoxicity

After incubating the cell cultures with cocaine or AEME for 24 or 48 h each, determination of neuronal viability using the MTT test showed a decrease in mitochondrial metabolism after 24 h of exposure to concentrations higher than 10^{-3} M AEME [F(10,154) = 41.17, p < 0.001] and 2 mM cocaine [F(9,136) = 40.80, p < 0.001]; the percentage of viable cells was 64.6 ± 8.2% and 67.3 ± 7.1%, respectively (Figs. 2A and 2C). After 48 h of exposure, a reduction in neuronal viability was observed for concentrations higher than 10^{-3} M AEME [F(10,154) = 112.0, p < 0.001] and 2 mM cocaine [F(9,140) = 117.6, p < 0.001]; here, the percentage of viable cells was 64.3 ± 3.9% and 61.4 ± 6.9%, respectively (Figs. 2B and 2D).

An elevation in the LDH content was observed after 24 and 48 h of exposure to 2 mM cocaine (21.8 ± 0.5 pmol of NADH consumption/minute/cell [F(9,90) = 452.0, p < 0.001]; 32.1 ± 1.6 pmol of NADH consumption/minute/cell [F(9,90) = 241.9, p < 0.001], respectively). There was no alteration in the LDH release after AEME treatments with any concentration or exposure time (Fig. 3).

Neurotoxicity of a Cocaine–AEME Combination

The concentrations used for these experiments were 1 mM AEME and 2 mM cocaine. These AEME and cocaine concentrations were chosen based on the MTT assay, which were able to reduce cell viability by 50% (LC_{50}). Thus, these experiments were conducted with four groups: control, AEME, cocaine, and the cocaine–AEME combination.

Cocaine, AEME, or the cocaine–AEME combination did not promote neuronal death after 3 or 6 h of exposure. After 12 h of exposure, only the cocaine–AEME combination group showed neuronal death; 68.9 ± 5.6% [F(4,66) = 86.99, p < 0.001] of the cells survived (Fig. 4). After 24 h of exposure, neuronal death occurred in the cells incubated with cocaine, AEME, and the cocaine–AEME combination; the percentages of surviving cells were 77.0 ± 2.4% [F(4,69) = 231.4, p < 0.001], 76.8 ± 3.9% [F(4,69) = 231.4, p < 0.001], and 73.3 ± 2.5%; [F(4,69) = 231.4, p < 0.001], respectively. After 48 h of exposure, the percentage of viable cells in the cultures treated with cocaine and AEME decreased to 58.6 ± 5.3% [F(4,70) = 192.1, p < 0.001] and 61.5 ± 3.6% [F(4,70) =...
192.1, p < 0.001], which corresponds to 41.4 and 38.5% neuronal death, respectively. The cocaine–AEME combination produced 78.5% cellular death [F(4,70) = 192.1, p < 0.001], suggesting an additive effect between cocaine and AEME because the sum of the individual responses (79.9% neuronal death) is very similar to the combination response (78.5%).

LDH was assayed only after 24 and 48 h of exposure to both drugs because neuronal death was not observed before 24 h of incubation with 1mM AEME or 2mM cocaine (Fig. 5). The cocaine–AEME combination increased LDH activity after 24 (19.3 ± 2.1 pmol of NADH consumption/minute/cell) [F(4,45)=266.0, p < 0.001] and 48 h of exposure (29.4 ± 0.6 pmol of NADH consumption/minute/cell) [F(4,45) = 64.1, p < 0.001].

Caspase-3 activity was evaluated after 3 and 6 h of exposure to cocaine and AEME at different concentrations, as well as after exposure to the cocaine–AEME combination. We observed that 0.1 and 1mM AEME increased caspase-3 activity after 6 h of exposure [F(2,20) = 5.09, p < 0.05]. Additionally, 1mM cocaine increased caspase-3 activity after 3 [F(2,20) = 9.13, p < 0.01] and 6 h [F(2,21) = 9.28, p < 0.01] of exposure (Fig. 6). However, there was no difference between the effects of 1 and 2mM cocaine on caspase-3 activity after 6 h of exposure. In contrast, after 3 h of exposure, there was a decrease in caspase-3 activity in the 2-mM cocaine group compared with the 1-mM cocaine group. There was no change in caspase-3 activity in cells incubated with the cocaine–AEME combination for 3 h. After 6 h of exposure, the cocaine–AEME combination group showed a decrease in this enzyme’s activity [F(3,28) = 6.76, p < 0.01].

FIG. 2. The MTT cell viability results, expressed as the percentage of cells that chemically reduced MTT to formazan compared with control (CTRL; n = five wells per group in each of the three independent assays). Here, 250mM KCl was used as the positive control. (A) 24 h of AEME exposure, (B) 48 h of AEME exposure, (C) 24 h of cocaine exposure, and (D) 48 h of cocaine exposure. CTRL, control and AEME, anhydroecgonine methyl ester. *p < 0.05, **p < 0.01, and ***p < 0.001, compared with CTRL (ANOVA and Newman–Keuls multiple comparison).
Involvement of the Muscarinic Cholinergic System in AEME Neurotoxicity

To evaluate whether AEME binds to the muscarinic cholinergic system, a competition-binding assay was performed.

Effect of AEME on [3H]QNB Binding in Rat Hippocampus. The displacement curves for [3H]QNB bound to the hippocampal membranes are shown in Figure 7. The profiles of displacement of the [3H]QNB binding by AEME and atropine (positive control) in the hippocampus samples from male rats are shown in the figure. The analysis indicated a statistical preference for a one-site, rather than a two-site, fit, with a pKᵢ of 4.15 ± 0.15, n = 4, for AEME; and a pKᵢ of 8.51 ± 0.11, n = 4, for atropine.

MTT Assay After Incubation With Atropine. Figure 8 shows the neuronal viability test after incubating the cell cultures for 24 h with cocaine, AEME, or cocaine–AEME combination in the absence and presence of atropine at different concentrations (1 [F(8,126) = 14.53, p < 0.001], 10 [F(8,125) = 20.21, p < 0.001], or 50µM [F(8,126) = 29.89, p < 0.001]). Although all concentrations of atropine were able to prevent AEME neurotoxicity, these concentrations showed no effect in the groups exposed to cocaine and the cocaine–AEME combination.

DISCUSSION

The most interesting finding of this study is that AEME is even more toxic than cocaine in rat primary hippocampal cell cultures. Moreover, the cocaine–AEME combination had an additive neurotoxic effect, as observed using the MTT assay. We also suggest that neuronal death caused by AEME may be
mediated by muscarinic receptors because we demonstrated both muscarinic-receptor binding by AEME and atropine protection of the hippocampal cells from AEME-induced neuronal death.

LDH release occurred only after exposure to cocaine and a cocaine–AEME combination. The increase in LDH release after these treatments is probably due to a more intense loss in membrane integrity caused by cocaine. Although LDH release is not enough to identify a necrotic process, we suggest that the induction of cell death by AEME involves apoptosis triggered by caspase-3 activation, whereas cocaine toxicity is probably characterized by membrane injury at the concentration used.

The neurotoxicity of drugs of abuse is usually associated with oxidative stress and mitochondrial dysfunction, leading to cell death by apoptosis and/or necrosis (Cunha-Oliveira et al., 2008). This is the first work to evaluate the neurotoxicity of AEME and the first study to address cocaine neurotoxicity in hippocampal cells. Our results corroborate those presented by Poon et al. (2007), which showed increases in LDH activity in human neuronal progenitor cells after cocaine incubation, suggesting that necrosis is one of the mechanisms underlying cocaine-induced neuronal death. Cocaine’s neurotoxic effects include massive dopamine release, which can be oxidized either spontaneously or by enzymatic processes. Consequently, oxidative species, such as superoxide anions, hydrogen peroxide, and quinones, are generated, leading to cellular damage of lipids, proteins, and DNA (Bisaglia et al., 2010). In fact, the involvement of mitochondrial damage and the participation of reactive oxygen species (ROS) in dopamine-mediated cocaine cytotoxicity has already been described (Kovacic, 2005). Other studies have reported cocaine induction of mitochondrial dysfunction and activation of the mitochondria-dependent intrinsic caspase cascade in cortical neurons (Cunha-Oliveira et al., 2006, 2010). Furthermore, inhibition of complex I of the mitochondrial respiratory chain in myocardial cells (Yuan and Acosta, 2000) and downregulation of mitochondrial gene expression in the rat cingulate cortex (Dietrich et al., 2004) have also been reported. The activation of caspase-3, described in this study, is in agreement with the results presented by Cunha-Oliveira et al. (2006, 2010). Moreover, AEME also seems to be an inducer of apoptosis, as caspase-3 activity was increased in the study. However, after exposure to the cocaine–AEME combination, the decrease in caspase-3 activity suggests a change in the neuronal death mechanism. Caspase-3 inhibition has been reported to induce cellular death by activation of necrotic pathways (Seo et al., 2009).

To investigate the combined neurotoxicity of cocaine and AEME, we performed the MTT and LDH assays with equipotent concentrations of cocaine and AEME after different periods of exposure. The neurotoxicity observed using the MTT assay was time-dependent. Although cocaine, AEME,
and the cocaine–AEME combination treatments decreased neuronal viability after 12 to 48 h of exposure, an additive effect occurred only at 48 h. It is important to mention that the concentrations of 1mM AEME and 2mM cocaine used in these assays were the previously determined LC50 values. The cocaine concentration was in the range used by other groups (50–2500µM) in different cell types, such as embryonic brain cells, myocardial cells, hepatocytes, and PC12 cells (Cunha-Oliveira et al., 2010; Imam et al., 2005; Yuan and Acosta, 2000; Zaragoza et al., 2001).
FIG. 7. The displacement curves induced by AEME and the muscarinic acetylcholine receptor (mAChR) antagonist atropine for [$^3$H]QNB bound to the hippocampal membranes from male rats. The data are plotted as percentages of binding in the absence of AEME or atropine. Each point and vertical line represent the mean ± SEM of four different experiments performed in duplicate. AEME, anhydroecgonine methyl ester.

FIG. 8. The MTT cell viability results, expressed as the percentage of cells that chemically reduced MTT to formazan, compared with CTRL after 24 h of exposure ($n$ = five wells per group in each of the three independent assays). In this test, 250mM KCl was used as the positive control. Neuronal cells were incubated in the absence and presence of 1µM (A), 10µM (B), and 50µM atropine (C). CTRL, control; AEME, anhydroecgonine methyl ester; AT, atropine; cocaine-AEME, combination of 2mM cocaine and 1mM AEME. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, compared with CTRL. a$p < 0.001$ and b$p < 0.01$, compared with 1mM AEME (ANOVA and Newman–Keuls multiple comparison).
The main neurotransmitters in hippocampus are the cholinergic and glutamatergic systems (Lendvai and Vizi, 2008). The hippocampus receives dopaminergic input from both the substantia nigra and the ventral tegmental area that projects into the hilus area and the CA1-subiculum region that expresses some dopaminergic receptors, such as D1, D2, and D5 subtypes, as well as the enzymatic machinery associated with this neurotransmitter (Otmaakhova and Lisman, 1996). While dopamine is present in lower concentrations than acetylcholine in the hippocampus, cocaine-induced neurotoxicity in hippocampal cells may be due to toxic dopamine metabolites, which lead to oxidative stress (Dietrich et al., 2005; Poon et al., 2007).

Because AEME seems to have a peripheral muscarinic agonist effect (Erzouki et al., 1995; Scheidweiler et al., 2003; Woolf et al., 1997), we hypothesized that AEME’s effects could be mediated by muscarinic receptors. In fact, atropine, an unspecific muscarinic antagonist, was able to prevent AEME-induced neurotoxicity, suggesting that muscarinic cholinergic activation is one of the mechanisms underlying neuronal death. Arecoline, a muscarinic cholinergic receptor agonist, produced neuronal apoptotic death at concentrations from 50 to 200μM (Shih et al., 2010). According to this study, arecoline depressed antioxidant defenses and increased ROS as well as caspase-3 activity. Nevertheless, atropine was not able to prevent the neuronal death induced by cocaine and the cocaine–AEME combination. However, direct interactions of cocaine with the muscarinic system have also been described, with cocaine acting on both M1 and M4, as well as nicotinic receptors (Flynn et al., 1992; Niu et al., 1995). Our data did not confirm the interaction between cocaine and muscarinic receptors because our results did not show that atropine could reverse the effects of cocaine.

To characterize the effect of AEME on muscarinic acetylcholine receptors, different concentrations of AEME were examined for their ability to compete with [3H]QNB for its binding sites in the rat hippocampus. The binding curve of the competition-binding experiments with AEME was suggestive of a single [3H]QNB binding site, indicating that muscarinic receptors may be the target proteins of AEME. The pKᵢ value obtained for AEME (4.15 ± 0.15) is very similar to the pKᵢ values reported for arecoline, a classical muscarinic cholinergic agonist that has a similar chemical structure to AEME, in binding studies on cloned M₁–M₅ muscarinic receptors (4.55 ± 0.15, 4.75 ± 0.18, 4.11 ± 0.14, 4.14 ± 0.11, and 4.83 ± 0.12, respectively; Dong et al., 1995). To validate the competition-binding assay, the nonselective muscarinic antagonist atropine was used as a positive control. The pKᵢ value for atropine obtained in this study is also similar to those reported in the literature in binding studies on cloned M₁–M₅ muscarinic acetylcholine receptor (mAChRs; Dong et al., 1995).

In conclusion, we suggest that AEME is not just a biological marker of crack cocaine use. Its neurotoxicity, combined with that of cocaine, may predispose crack cocaine users to more serious deleterious outcomes compared with other cocaine-administration routes.

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