We examined the potential neurotoxicity of caffeine in vivo and in vitro. Intraperitoneal administration of caffeine (50 mg/kg, 3 times a day) produced neuronal death in various brain areas of neonatal rats 24 h later. Caffeine at doses >300 μM was also neurotoxic in murine cortical cell cultures. Caffeine-induced neuronal death was accompanied by cell body shrinkage and attenuated by anti-apoptotic drugs including cycloheximide, high potassium, and growth factors. Two necrotic pathways, excitotoxicity and oxidative stress, did not mediate caffeine neurotoxicity. The pro-apoptotic protease caspase-3 was activated to mediate neuronal death following exposure to caffeine. The present findings suggest that caffeine may cause caspase-3-dependent neuronal cell apoptosis in neonatal rat as well as in vitro.

**Key words**: Apoptosis; Caffeine; Caspase; Excitotoxicity; Neuron; Oxidative stress

**INTRODUCTION**

Caffeine (1,3,7 trimethyl xanthine) is the most widely used psychoactive drug and is a mild stimulant. Caffeine increases ventilation and is frequently prescribed to treat apnea in preterm infants. The pharmacological actions of caffeine occur by means of antagonizing adenosine receptors, inhibiting phosphodiesterases, activating the ryanodine receptor, an intracellular Ca\(^{2+}\)-release channel, and antagonizing GAB\(_A\) receptors [1,2].

In addition to the stimulatory actions, a wealth of evidence has reported that caffeine can have neurotoxic side effects. Caffeine intake before and during pregnancy appears to increase the risk of fetal loss [3]. The systemic administration of caffeine can cause seizures in animals as well as humans [4,5]. The direct neurotoxic actions of caffeine have been shown in cerebellar cell cultures [6]. A brief exposure to caffeine can disturb neurogenesis by regulating death and proliferation at early embryonic stages [7]. In the present study, we studied the possibility that administration of caffeine would cause neuronal death in neonatal rats. We also performed experiments to determine patterns and mechanisms of caffeine-induced neuronal death in cortical cell cultures.

**MATERIALS AND METHODS**

**Caffeine toxicity in vivo**: Seven-day-old rat pups received three doses of caffeine in saline (50 mg/kg, i.p.) at 5 h intervals and were sacrificed 1 day after the first caffeine injection. Brains were removed and sectioned at 18 μm in a cryostat. Every fourth section was stained with terminal-deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) as described previously [8]. In brief, brain sections were fixed in 3% paraformaldehyde, washed in PBS, and post-fixed in ethanol/acidic acid (2:1) at −20°C. Endogenous peroxidase was inactivated by incubating the sections with 2% H\(_2\)O\(_2\) for 5 min. Sections were reacted with digoxigenin-dUTP and terminal deoxynucleotidyl transferase at 37°C for 1 h, incubated with anti-digoxigenin-peroxidase antibody at room temperature for 2 h, and DNA was visualized using 0.05% diaminobenzidine and methyl green. Cell death was analyzed by counting TUNEL-positive cells/mm\(^2\) in various brain areas (5–15 sections per brain area) under light microscopy at ×200.

**Primary cortical cell cultures**: Mouse cortical cell cultures were prepared as described previously [9]. All animals were handled in accordance with a protocol approved by our institutional animal care committee. Cerebral cortices were removed from brains of the 14-day-old fetal mice, gently triturated with a Pasteur pipette, and plated on 6- or 24-well plates (five hemispheres/plate, ~2.5 × 10\(^5\) cells/plate) precoated with 100 μg/ml poly-D-lysine and 4 μg/ml laminine. Plating media consisted of Eagle’s minimal essential media (MEM, Earle’s salts, supplied glutamine-free) supplemented with 5% horse serum, 5% fetal bovine serum, 2 mM glutamine, and 21 mM glucose. Cultures were
maintained at 37°C in a humidified 5% CO₂ atmosphere. Cytosine arabinofuranoside (final concentration, 10µM) was added to cultures at 7–9 days in vitro (DIV 7–9) where glia became confluent underneath neurons. Two days later, cultures were shifted into a growth medium identical to the plating medium but lacking fetal serum. All experiments were carried out at DIV 10–12.

**Caffeine toxicity in cortical cell cultures:** Co-cultures of neurons and glia (DIV 10–12) were continuously exposed to caffeine in MEM (Earle’s salts, glutamine-free, bicarbonate-free) supplemented with 21 mM glucose, 26.5 mM sodium bicarbonate, and 100 µM trolox, a vitamin E analogue. Neuronal death was analyzed 24 h later by counting viable neurons excluding trypan blue. Apoptotic cell death was further assessed by TUNEL under light microscopy at ×400 magnification.

**Western blotting:** Cells were lysed in a lysis buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P40, 0.1% sodium dodecyl sulfate (SDS), 1 mM PMSF, 100 µg/ml leupeptin. Cell lysates were centrifuged at 13,000 r.p.m. for 10 min. The supernatants were collected, subjected to electrophoresis on a 12% SDS-polyacrylamide gel, and then transferred to a nitrocellulose membrane. The blot was incubated in 5% non-fat dry milk for 30 min, reacted with a rabbit polyclonal antibody for activated caspase-3 or overnight at 4°C, and then incubated with biotinylated goat anti-rabbit IgG for 2 h. Signals were detected using the Vectastatin ABC kit and luminol as an enhanced chemiluminescence substrate and signals were detected using the Vectastatin ABC kit and luminol as an enhanced chemiluminescence substrate. Levels of immunoreactive bands were quantified using densitometric analysis using an Image Gauge (Fuji Photo film Co., Ltd).

**Immunohistochemistry:** Mixed cortical cultures were fixed in 4% paraformaldehyde, treated with 0.05% Triton X-100 for 10 min, and incubated in 4% bovine serum albumin for 1 h. Cultures were then reacted with anti-NeuN (1:300), a neuronal marker protein, or anti-activated caspase-3 antibody (1:200) at room temperature for 2 h. For triple staining, cultures were then incubated in a rhodamine (for activated caspase-3) or AMCA (for NeuN) conjugated secondary antibodies for activated caspase-3 and NeuN revealed caspase-3 (Fig. 3b). Immunohistochemistry staining using anti-digoxigenin-fluorescein antibody instead of neuronal cell necrosis in cortical cell cultures, mediates caffeine-induced neuronal apoptosis. Western blot analysis of caspase-3, a pro-apoptotic protease, would mediate caffeine-neurotoxicity [9,13]. Additional experiments were performed to test whether caspase-3, a pro-apoptotic protease, would mediate caffeine-induced neuronal apoptosis. Western blot analysis of activated caspase-3 revealed that caspase-3 was activated in cortical cell cultures exposed to caffeine for 12 h. Caffeine-induced neuronal apoptosis was significantly prevented by high K⁺, BDNF or insulin that selectively attenuate neuronal cell apoptosis (Fig. 2c) [11,12]. Caffeine neurotoxicity was induced in the presence of the anti-oxidant trolox and not prevented by addition of antagonists of ionotropic glutamate receptors, MK-801 antagonizing NMDA receptors or CNQX antagonizing AMPA/kainate receptors. This implies that neither oxidative stress nor glutamate neurotoxicity, the main cause of neuronal cell necrosis in cortical cell cultures, mediates caffeine neurotoxicity [9,13].

**Materials:** Caffeine, cycloheximide, insulin and anti-actin antibody were purchased from Sigma (St. Louis, MO). MK-801 and 6-cyano-7-nitroquinolinoxaline-2,3-dione (CQX) were obtained from RBI (Natick, MA). BDNF was obtained from R and D (Minneapolis, MN). Trolox was obtained from Aldrich (Milwaukee, WI). z-VAD-fmk was obtained from Enzyme systems Products (Livermore, CA). Trypan blue stain 0.4% was purchased from Gibco-BRL (Grand island, NY). Apoptosis detection kit (ApopTaq) was purchased from Oncor (Gaithersburg, MD). Activated caspase-3 and NeuN antibody was obtained from New England Biolabs (Beverly, MA) and Chemicon (Temecula, CA), respectively. Rhodamine, AMCA conjugated antibody and ABC kit were purchased from Vector laboratories (Burlingame, CA).

**RESULTS**

We first examined whether administration of caffeine would produce neuronal death in 7-day-old rat pups. TUNEL-positive cells were rarely observed in saline-treated normal rat brain but markedly increased in various brain areas following i.p. injections of caffeine (Fig. 1). In particular, the number of TUNEL-positive cells was significantly increased in perialretal cortex, temporal cortex, caudate putamen, dentate gyrus granule cells, thalamus, and hypothalamus (Fig. 1b). Condensation and margination of nuclear chromatin in TUNEL-positive cells suggest that caffeine causes apoptotic cell death in neonatal rat brain (Fig. 1a) [10].

We next examined patterns of caffeine-induced neuronal death in cortical cell cultures. Exposure of mixed cortical cell cultures (DIV 10–12) to 150–1000 µM caffeine resulted in decreased number of process-bearing cortical neurons that grow on glial cell layer. It produced neuronal cell death in a dose-dependent manner (Fig. 2a). Glial cells (e.g. astrocytes) in mixed cell cultures or pure glial cell cultures remained intact 24 h following exposure to caffeine. The caffeine-induced neuronal death was accompanied by shrinkage of cell body and DNA fragmentation (TUNEL-positive cells) exhibiting condensed granulation and marginated labeling, which are the morphological criterion of apoptosis. This neuronal death was blocked by addition of cycloheximide, a protein synthesis inhibitor known to block neuronal cell apoptosis (Fig. 2b) [9]. Thus, caffeine appears to cause neuronal cell apoptosis. This was further supported by prevention of caffeine neurotoxicity by high K⁺, BDNF or insulin that selectively attenuate neuronal cell apoptosis (Fig. 2c) [11,12]. Caffeine neurotoxicity was induced in the presence of the anti-oxidant trolox and not prevented by addition of antagonists of ionotropic glutamate receptors, MK-801 antagonizing NMDA receptors or CNQX antagonizing AMPA/kainate receptors. This implies that neither oxidative stress nor glutamate neurotoxicity, the main cause of neuronal cell necrosis in cortical cell cultures, mediates caffeine neurotoxicity [9,13].
reduced with addition of zVAD-fmk, a broad inhibitor of caspases (Fig. 3c).

DISCUSSION
We provide evidence that the systemic administration of caffeine can cause widespread apoptotic cell death in various brain areas including neocortex and thalamus in neonatal rat brain. The differential vulnerability to caffeine may be due to differential sensitivity of the cortical and thalamic region to caffeine or related with neurogenesis. Experiments in cortical cell cultures demonstrate that caffeine selectively injures neurons primarily through apoptosis regardless of excitotoxicity and oxidative stress, which is mediated through activation of caspase-3. In mice, systemic administration of caffeine at doses of 97, 125 or 194 mg/kg/day resulted in mean plasma concentration of caffeine up to 2.7, 4.9 and 7.1 μg/ml 7 days later: is comparable to that in human studies of chronic caffeine treatment [14–17]. In the present study, neonatal rats received i.p. caffeine at similar doses applied in mice and human. While high doses of caffeine can cause seizure in infants, administration of caffeine produced neuronal death without producing behavioral seizure. Caffeine also causes neuronal death in cortical cell cultures dose-dependently in the range 300–1000 μM, corresponding to the plasma concentrations (~500 μM) in humans during chronic caffeine treatment [18]. Apoptosis and necrosis are morphologically defined by shrinkage apoptosis and swelling necrosis, respectively, and comprise of two distinctive patterns of neuronal death occurring in various neurological diseases. Evidence is being accumulated that signaling pathways underlying apoptosis and necrosis can be mutually exclusive. For example, neurotrophins, insulin-like growth factors, and gangliosides prevent neuronal cell apoptosis but markedly potentiate free radical-induced neuronal cell necrosis [12,19,20]. This implies that neuroprotective molecules should be applied to appropriately protect neurons from pathological insults based upon the patterns of neuronal death. The present findings suggest that caffeine-induced neuronal cell death occurs by apoptosis. First, neuronal death exposed to caffeine was accompanied by cell body shrinkage and TUNEL staining characteristic of apoptosis. Second, high potassium and cycloheximide, the pharmacological criteria used to define neuronal cell apoptosis, prevent caffeine-induced neuronal death. Third, BDNF and insulin, the neurotrophic factors shown to selectively prevent apoptosis in cortical cell cultures, also prevent caffeine-induced neuronal death. Finally, neither oxidative stress nor excitotoxicity, the major routes leading to neuronal cell necrosis, mediates neuronal death following exposure to caffeine.
Caspases, a family of the cysteine proteases, have been proposed as common mediators for apoptosis of neuronal cell as well as non-neuronal cells. Caspase-3, a final downstream caspase for execution of apoptosis, is activated selectively in neurons following exposure of mixed cortical cultures containing neurons and glia to caffeine, which mediates caffeine-induced neuronal cell apoptosis. Further study will be needed to delineate upstream signals underlying caffeine-induced activation of caspase-3 that are classified into mitochondria-dependent intrinsic apoptotic pathways involving cytochrome c release from mitochondria and extrinsic apoptotic pathways involving interaction of Fas and Fas ligand [21,22]. Caffeine may trigger caspase-dependent apoptosis possibly by interfering with activity of adenylate cyclase or intracellular Ca\textsuperscript{2+} homeostasis that can cause neuronal cell apoptosis [6,14,23,24].

Fig. 2. Caffeine-induced neuronal death in cortical cell cultures. (a) Mixed cortical cell cultures (DIV 10–12) were exposed to indicated doses of caffeine in the presence of 100 μM trolox, a vitamin E analogue. Neuronal death was analyzed 24 h later by counting neurons excluding trypan blue (values are mean ± s.e.m., n = 8–16 fields randomly chosen from 4–8 culture wells/condition). (b) Phase contrast photomicrographs (upper panel) and TUNEL staining (lower panel) of mixed cortical cell cultures (DIV II) taken 24 h after exposure to sham control (a), 500 μM caffeine (b), or 500 μM caffeine plus 1 μg/ml cycloheximide (c,f) in the presence of 100 μM trolox. Note representative apoptotic neurons (arrows) showing features of cell body shrinkage and DNA fragmentation (TUNEL-positive cells). Bar = 30 μm. (c) Cortical cell cultures (DIV 10–12) were exposed to 500 μM caffeine, alone or with 10 μM MK-801 (MK), 50 μM CNQX, 1 μg/ml cycloheximide (CHX), 25 mM K+, 100 ng/ml BDNF or 100 ng/ml insulin. Neuronal death was analyzed 24 h later by counting neurons excluding trypan blue (values are mean ± s.e.m., n = 4–6 fields randomly chosen from 8 culture wells/condition). *Significant difference from the sham control, p < 0.05, ANOVA and Student–Neuman–Keuls' test.
CONCLUSION

Caffeine, the most common psychostimulant drug, can cause caspase-dependent neuronal cell apoptosis irrespective of oxidative stress and excitotoxicity. The pro-apoptotic effects of caffeine may contribute to undesirable consequences including developmental malformation in the nervous system and fatality. Thus, the therapeutic potential of caffeine should be considered with respect to its neurotoxicity through the activation of caspases.

REFERENCES


Fig. 3. Caspase-3 mediates caffeine induced-neuronal death. (a) Western blot analysis showing activation of caspase-3 using anti-activated caspase-3 antibody in cortical cell cultures (DIV 11) exposed to 500 µM caffeine for indicated points of time. Actin was also probed to confirm equal protein loading. Levels of activated caspase-3 were quantified by densitometric analysis (values are mean ± s.e.m., n = 4 for each condition). *Significant difference from the sham control (BL), p < 0.05, ANOVA and Student–Neuman–Keuls' test. (b) Fluorescence photomicrographs of cortical cell cultures (DIV 11) co-localized with NeuN (blue, left panel), activated caspase-3 (red, middle panel) antibody and TUNEL-positive (green, right panel) neurons following 12 h exposure to a sham control (a–d) or 500 µM caffeine (e–h). Arrows indicate triple-labeled neurons with NeuN, activated caspase-3 and TUNEL. Bar = 30 µm. (c) Cortical cell cultures were exposed to 500 µM caffeine, alone or with 100 µM zVAD-fmk (zVAD). Neuronal death was analyzed 24 h later by counting neurons excluding trypan blue (values are mean ± s.e.m., n = 4–6 fields randomly chosen from 8 culture wells/condition). *Significant difference from caffeine alone, p < 0.01, Student's t-test.

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