Caffeic acid phenethyl ester prevents neonatal hypoxic–ischaemic brain injury

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Summary
Neonatal hypoxic–ischaemic (HI) brain injury resulting in encephalopathy is a leading cause of morbidity and mortality with no effective treatment. Here we show that caffeic acid phenethyl ester (CAPE), an active component of propolis, administered either before or after an HI insult, significantly prevents HI-induced neonatal rat brain damage in the cortex, hippocampus and thalamus. In addition to blocking HI-induced caspase 3 activation, CAPE also inhibits HI-mediated expression of inducible nitric oxide synthase and caspase 1 in vivo and potently blocks nitric oxide-induced neurotoxicity in vitro. Furthermore, CAPE directly inhibits Ca2+ -induced cytochrome c release from isolated brain mitochondria. Thus, CAPE induces neuroprotection against HI-induced neuronal death, possibly by blocking HI-induced inflammation and/or directly inhibiting the HI-induced neuronal death pathway. CAPE may therefore be a novel effective therapy for preventing neonatal HI injury.

Keywords: brain injury; hypoxia–ischaemia; caffeic acid phenethyl ester; rat

Abbreviations: CAPE = caffeic acid phenethyl ester; CGN = cerebellar granule neuron(s); HI = hypoxic–ischaemic, hypoxia–ischaemia; iNOS = inducible nitric oxide synthase; NO = nitric oxide.


Introduction
Hypoxic–ischaemic (HI) encephalopathy in the prenatal and perinatal periods is a major cause of damage to the fetal and neonatal brain, resulting in considerable morbidity and mortality (Han et al., 2002; Cowan et al., 2003). This insult to the nervous system is often associated with clinical syndromes of neurological disability, such as seizures, intellectual impairment and cerebral palsy. Although systemic and cerebrovascular physiological factors play important roles in the initial phases of the process, the intrinsic death pathways of specific neurons and/or systems may be more important in determining the final pattern of neuronal damage and dysfunction. A key mechanism which is believed to be responsible for hypoxic–ischaemic neuronal damage is excitotoxicity, a phenomenon which is caused by overstimulation of excitatory transmitter receptors, mainly glutamate receptors (Johnston et al., 2001). When excessively stimulated by elevated synaptic levels of glutamate and membrane depolarization associated with ischaemia, channels in these receptors allow a lethal flood of Ca2+ and sodium to enter neurons. In the developing brain, HI insults can trigger prominent delayed programmed cell death or apoptosis as well as necrosis (Cheng et al., 1998). Mitochondria appear to play an essential role in determining the fate of cells subjected to HI (Gillard et al., 1998). Disrupted mitochondrial function during HI can lead to cytochrome c protein release and triggers the activation of caspase 3 and/or other caspase-related apoptotic pathways (Cheng et al., 1998). Furthermore, cytokines, including interleukin 1β and nitric oxide (NO), are also involved in HI-induced brain injury (Liu et al., 1999; Peeters-Scholte et al., 2002). The prominence of both apoptosis and
necrosis as well as neuroinflammation in neurodegeneration after HI in the immature brain suggests that it will be important to better understand the roles and relationships of these processes to develop effective neuroprotective strategies. In this study, we used the well-characterized Levine model (unilateral carotid ligation followed by exposure to hypoxia), in which features of both apoptosis and necrosis are prominent for this model in neonatal rats and mice (Vanucci et al., 1990; Han et al., 2001). Inhibition of caspase 3 partially protects against brain injury after neonatal HI injury (Han et al., 2002; Le et al., 2002). These data suggest that activation of apoptosis-executing caspase 3 plays an important role in neonatal HI injury. Furthermore, HI-induced activation of proapoptotic pathways may also be linked to activation of caspase 1 (Peeters-Scholte et al., 2002) and iNOS (Liu et al., 1999). Most recently, minocycline has been shown to provide dramatic neuroprotection in this model, suggesting that a compound which can inhibit multiple apoptotic targets involved in HI injury may provide a better therapeutic effect than specific inhibitors.

Caffeic acid phenethyl ester (CAPE), an antioxidant flavonoid, is the active component of the propolis purified from the hives of honeybees. It has antiviral, anti-inflammatory, anti-oxidant and immunomodulatory properties (Grunberger et al., 1988; Su et al., 1991, 1994). It has been demonstrated that CAPE is a potent and specific inhibitor suppressing NF-κB activation (Natarajan et al., 1996), lipid peroxidation (Sud‘ina et al., 1993), lipoxygenase activities (Laranjinha et al., 1995), protein tyrosine kinase (Kimura et al., 1985) and ornithine decarboxylase (Zheng et al., 1995). Most recently, it has been demonstrated that CAPE is able to block ischaemia- and low potassium-induced neuronal death (Amadio et al., 2003; Irmak et al., 2003). We now report that administration of CAPE effectively blocks HI-induced degeneration of neurons in the cortex, hippocampus and thalamus. In a mechanistic study, we have found that CAPE treatment also inhibits HI-mediated iNOS and caspase 1 expression in vivo and potently blocks NO-induced neurotoxicity in vitro. Additionally, CAPE is able to protect isolated mitochondria against Ca 2+ -induced cytochrome c release and HI-induced caspase 3 activation. Thus, both indirect (block HI-induced inflammation) and/or direct inhibition of HI-mediated neurotoxicity may underlie CAPE’s neuroprotective properties.

Material and methods

Animals and treatment

HI brain damage was induced as described previously (Han et al., 2002). All animal procedures were approved by the Indiana University animal committee. Briefly, 7-day-old Sprague–Dawley rat pups (Harlan Laboratories, Indianapolis, IN, USA) were anaesthetized with 2.5% halothane and the left carotid artery was permanently ligated. After surgery, the rat pups were allowed to recover for 2 h with their dams. Hypoxic exposure was then achieved by placing pups in a 2.0-litre airtight plastic chamber submerged in a 37.0°C water bath and flushed for 2.5 h with a humidified mixture of 8% oxygen and 92% nitrogen. Pups were then returned to their dams and housed under a 12-h light and 12-h dark cycle with food and water until they were killed. Pups (5–7 per group) received intraperitoneal injections of CAPE (40 mg/kg/day) dissolved in corn oil or vehicle before and/or after the hypoxic insult.

Tissue preparation and assessment of brain infarct volume

Following treatment, rats were anaesthetized by halothane inhalation and perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After postfixation and cryoprotection in 30% sucrose/phosphate buffer, brains were frozen in liquid nitrogen and sectioned serially (50 μm). Coronal sections from the genu of the corpus callosum to the end of the dorsal hippocampus were stained with cresyl violet as described previously (Han et al., 2002). For Western blotting and caspase 3 activity assay, some rats were decapitated and each side of the cerebral cortex and hippocampus was dissected on a dry ice-cooled glass plate. After adding 1.5 ml of buffer (0.1 M potassium phosphate, pH 7.5, 20 mM ethylenediamine tetraacetate (EDTA), the tissue was homogenized in ice. The supernatant was obtained by centrifugation (14 000 r.p.m. for 20 min). To determine infarct volume, briefly, the cross-sectional areas of the cortex, hippocampus and thalamus in each of eight equally spaced reference planes were photo-scanned, and the area of each brain region was calculated using the SPOT software (Diagnostic Instrument, VA, USA). The sections used for quantification corresponded approximately to plates 12, 15, 17, 20, 23, 28, 31 and 34 in the rat brain atlas (Han et al., 2002). The percentage volume occupied by HI damage in the cortex and the hippocampus was obtained by dividing the sum of the damaged areas ipsilateral to the carotid ligation (left side) by the sum of the ipsilateral areas of the corresponding normal area (right side). For statistical analysis, one-way analysis of variance (ANOVA) was used to compare groups with a p < 0.05 significance cut-off.

Primary rat CGN neuronal cultures and neurotoxicity assays

Cerebellar granule neurons (CGN) were prepared from 8-day-old Sprague–Dawley rat pups (Harlan Laboratories) as described previously (Du et al., 1997). Briefly, freshly dissected cerebellum were dissociated and the cells were seeded at a density of 1.2 to 1.5 × 10^6 cells/ml on poly-L-lysine-coated dishes in basal medium Eagle supplemented with 10% fetal bovine serum, 25 mM KCl and gentamicin (0.1 mg/ml). Cytosine arabinoside (10 μM) was added to the culture medium 24 h after initial plating. All experiments used neurons after 7–8 days in vitro. Viable neurons were quantified by counting fluorescein-positive (green) cells, which result from the de-esterification of fluorescein diacetate by living cells. Briefly, cultures were incubated with fluorescein diacetate (10 μg/ml) for 5 min and examined and photographed using UV light microscopy, and the numbers of neurons from representative low-power fields were counted as described previously (Du et al., 1997). Propidium iodide, which interacts with nuclear DNA to produce a red fluorescence, was used to identify dead neurons. For propidium iodide staining, cultures were incubated with propidium iodide (5 μg/ml), examined, and photographed using UV light microscopy as described previously (Du et al., 1997).

Caspase 3 activity assay (Du et al., 1997)

Brain tissues were collected and homogenized with 20 strokes of a B-type pestle in precooled buffer (50 mM Tris–HC1 pH 7.4,
1 mM EDTA, 10 mM ethyleneglycol-bis-(β-aminoethylether)-N, N-tetraacetic acid (EGTA), 1mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/µl aprotinin. Lysates were centrifuged at 15 000 r.p.m. at 4°C for 20 min and protein concentrations were determined (Pierce, Rockford, IL, USA) (Du et al., 1997). Extracts were either used immediately or stored at –80°C. Aliquots of protein (30 µg) were incubated with 100 µM caspase 3 substrate (Ac-DEVD-pNA; Calbiochem, La Jolla, CA, USA) in a total volume of 1.0 ml at 37°C. The colorimetric release of p-nitroaniline from the Ac-DEVD-pNA substrate was recorded at 60 min and 405 nm. Enzymatic activity for caspase 3 was linear over the range of protein concentrations used to calculate specific activity.

**Western blot analysis**

Western blot analysis was performed on rat brain extracts from selected regions and cell cytoplasmic extracts. Extracts were prepared by lysing tissues/cells in a buffer containing 1% Nonidot P-40, 0.1% sodium dodecyl sulphate (SDS), 50 mM Tris (pH 8.0), 50 mM NaCl, 0.05% deoxycholate and protease inhibitor (Roche, Indianapolis, IN, USA). Proteins (10 µg) were size-fractionated on a 4–12% polyacrylamide gradient gel (SDS-NuPAGE) and transferred onto nitrocellulose (Hybond N; Amersham, CA, USA). Blots were then probed with polyclonal or monoclonal antibodies, followed by a secondary antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, PA, USA) and visualized using enhanced chemiluminescence.

**Mitochondrial isolation and cytochrome c assay**

Rat brain and liver mitochondria were prepared from 7-day-old Sprague–Dawley rats. Briefly, brains and livers were homogenized in ice-cold buffer containing 250 mM mannitol, 75 mM sucrose and 10 µM K-HEPES (pH 7.4), and homogenates were centrifuged at 1000 g for 10 min. Supernatants were then centrifuged at 10 000 g for 15 min. Pellets that were washed three times were used immediately in experiments. For in vitro cytochrome c assay, an aliquot of 12.5 µg liver and brain mitochondria (25 µl) was pretreated with CAPE for 5 min following challenge with 100 µM of CaCl2 for 30 min at 30°C. After centrifugation, the supernatant was evaluated by western blotting. To assay neuronal cytochrome c release, CGN were washed once with ice-cold phosphate-buffered saline and harvested in 500 µl ice-cold buffer A (50 mM Tris–HCl, pH 7.4; 1 mM EDTA; 1 mM DTT; complete protease inhibitor (Roche); 250 mM sucrose). The cells were disrupted by douncing 10 times with a pestle in a 7-ml Wheaton douncer. After centrifugation in a microcentrifuge at 1000 g for 10 min at 4°C, the supernatants were further centrifuged at 12000 g for 40 min. The resulting supernatants were used for immunoblot analysis.

**Results**

**CAPE markedly attenuates HI-induced brain damage**

To investigate the neuroprotective effects of CAPE on HI-induced neuronal death in vivo, we treated 7-day-old rats with CAPE (40 mg/kg intraperitoneally) or solvent (control) once a day for 7 days. Rats were exposed to HI injury 30 min after the first drug treatment. Seven days after HI injury, the brains were analysed histologically to quantify the amount of damage in the cortex, hippocampus and thalamus (Han et al., 2002). HI injury resulted in approximately 41, 54 and 32% of tissue loss in the cortex, hippocampus and thalamus, respectively, compared with non-injured controls (p < 0.001) (Fig. 1A and B). Rats that received CAPE showed significantly reduced tissue damage in these areas compared with solvent-treated animals (cortex, 26% damage, p < 0.001; hippocampus, 19% damage, p < 0.001; thalamus, 14% damage, p < 0.001) (Fig. 1A and B). In contrast, we found no evidence of brain injury in sham-operated animals exposed only to hypoxia (data not shown).

We next treated animals with CAPE (40 mg/kg, intraperitoneally) 4 h after an HI insult had been completed. Interestingly, CAPE treatment significantly protected neurons against HI-induced neuronal death in the cortex and hippocampus even when given 4 h after HI insult. Rats that received CAPE 4 h after an HI insult showed a significantly greater volume of remaining tissue in the cortex and hippocampus than solvent-treated rats (cortex, 51 versus 66%, p < 0.001; hippocampus, 40 versus 66%, p < 0.001) (Fig. 1C). CAPE treatments 4 h after HI injury also exerted a neuroprotective effect in the thalamus, although not significantly (64 versus 75%) (Fig. 1C).

The neuroprotective effects of CAPE observed in our experiments could not be attributed to hypothermia because the temperature of animals treated with CAPE for 60 min did not differ from that of animals treated with vehicle (vehicle, 36.2 ± 0.2°C; CAPE, 36.6 ± 0.6°C, eight animals per group, p > 0.05).

**CAPE pretreatments attenuate HI-induced caspase 3 activation**

The underlying cellular mechanisms by which CAPE induces neuroprotection in the neonatal HI model remain unclear. Previous studies have shown that HI-induced neuronal death has features of apoptosis (Hill et al., 1995) with prominent caspase 3 activation (Cheng et al., 1998; Liu et al., 1999). Therefore, we asked whether CAPE pretreatment blocks HI-induced caspase 3 activity in the neonatal brain. In brain lysates derived from animals treated with CAPE, no induction of caspase 3 activity was detected in the ipsilateral cortex, which was subjected to HI treatments. In contrast, consistent with previous reports, lysates from animals treated with vehicle showed markedly increased caspase 3 activity in the cortex ipsilateral to carotid ligation. CAPE pretreatments significantly blocked HI-induced caspase 3 activation (Fig. 2).

**CAPE blocks Ca2+-induced cytochrome c release**

Since both intracellular Ca2+ overload (Delivoria-Papadopoulos and Mishra, 2000) and caspase 3 activation play important roles in HI-induced neurotoxicity, we examined Ca2+-induced release of cytochrome c, an apoptotic initiator which directly activates caspase 3, to investigate the primary target of CAPE. As shown in Fig. 3, 100 µM Ca2+ was able to induce cytochrome c release from mitochondria isolated from neonatal rat brains, and CAPE significantly blocked Ca2+-induced cytochrome c release starting at 20 µM,
which is a concentration 10 times lower than that producing a similar effect with minocycline (Zhu et al., 2002). A similar result has been observed in experiments using liver mitochondria.

**CAPE pretreatments markedly attenuate HI-induced iNOS and caspase 1 overexpression, as well as NO-induced neurotoxicity**

Since NO synthases and caspase 1 have been proposed to mediate (at least in part) HI-induced neuronal death (Liu et al., 1999; Peeters-Scholte et al., 2002), we measured both iNOS and caspase 1 in hippocampal homogenates of rats treated with HI (Fig. 4). Twenty four hours after HI treatment, both iNOS and caspase 1 were upregulated in hippocampal homogenates, as determined by western blotting. This increase was partially blocked by pretreatment with CAPE (Fig. 4). In contrast, neither HI injury nor CAPE had any effect on nNOS expression in these same samples (Fig. 4). Furthermore, since NO has been shown to play an important role in HI-induced neurotoxicity (Peeters-Scholte et al., 2002), we went on to determine whether CAPE affected NO-mediated injury. We asked whether CAPE could directly block NO-induced neurotoxicity in cultured neurons. Treatment of CGN with the NO donor sodium nitroprusside resulted in significant neuronal death in CGN. This cell death was almost completely blocked by CAPE at 10 μM (Fig. 5).

**Discussion**

The results in this report provide the first evidence, to our knowledge, that CAPE given before or 4 h after a neonatal HI insult can effectively protect against neuronal and tissue loss in the cortex, hippocampus and thalamus in vivo. The finding is in agreement with previous studies showing that CAPE protects against ischaemia–reperfusion injury of the spinal cord (Ilhan et al., 1999) and brain (Irmak et al., 2003). Prior studies suggest that caspase 3 is the important factor contributing to delayed caspase-dependent cell death after neonatal HI. Caspase 3 inhibitors block caspase 3 activation and cleavage of its substrates, resulting in significant neuroprotection against HI-induced brain injury. These results are in line with our finding that HI-induced caspase 3 activation is inhibited by CAPE. Since CAPE does not directly inhibit caspase 3 enzyme activity (data not shown), we decided to evaluate its effect on cytochrome c release as cytochrome released from mitochondria is a potent stimulus for caspase 3 activation (Liu et al., 1996). Our results clearly demonstrate that inhibition of cytochrome c release is one of the primary targets of CAPE. Given that the concentration required for the

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**Fig. 1 CAPE prevents neuronal loss after hypoxic–ischaemic injury.** (A) Representative coronal sections of postnatal day 14 (P14) rat brains demonstrate that 7 days after unilateral (left) carotid ligation and exposure to hypoxia for 2.5 h at P7 (see Han et al., 2002 for details) with or without treatment with CAPE at 40 mg/kg daily. (a) Untreated. (b) Ischaemia treated with corn oil. (c) Ischaemia treated after 0.5 h of pretreatment with CAPE at 40 mg/kg. Note the moderate or severe damage in the hemisphere ipsilateral to carotid ligation in most animals (compare a with b) and the significant protection by CAPE (compare b with c). Arrows indicate lesioned sides. (B, C) Assessment of brain infarct tissue loss in the cortex, hippocampus and thalamus was carried out as described in the text. The area of tissue in the hippocampus ipsilateral to the lesioned hemisphere was compared in the same animal with the area of tissue remaining in the matching brain region contralateral to the unlesioned hemisphere. The percentage area loss was then determined in each animal, and data are presented as the mean ± SEM for each group. (B) CAPE at 40 mg/kg 0.5 h before hypoxia significantly protects against volume losses of cortex, hippocampus and thalamus induced by ischaemic injury (59 ± 2 versus 84 ± 3, 46 ± 3 versus 81 ± 5, and 68 ± 3 versus 86 ± 2, respectively; n = 13, one-way ANOVA, ***p < 0.001). (C) CAPE at 40 mg/kg 4 h after hypoxia also protects against HI-induced volume losses in cortex, hippocampus and thalamus (51 ± 4 versus 66 ± 4, 40 ± 4 versus 66 ± 5, and 64 ± 5 versus 75 ± 3, respectively; n = 8 mice/group, one-way ANOVA, ***p < 0.001) (see text for details).
analyses of Zhu and colleagues, the concentrations required of minocycline and CAPE have both been overstated, since very high concentrations of isolated mitochondria have been used in experiments. The physiologically relevant concentrations of CAPE may be at least 100 times less than those used in experiments (0.2 μM), according to the adjustments of Zhu and colleagues. How much CAPE is really required to protect mitochondria from the release of cytochrome c in the physiological conditions, however, remains to be determined.

Additionally, CAPE treatment reduced the induction of caspase 1 to 38% when estimated by western blotting 24 h after HI treatments. Caspase 1 cleaves inactive precursor, prointerleukin-1β (pro-IL-1β) to the mature form of IL-1β, which is a proinflammatory cytokine (Yrjanheikki et al., 1998). Mice deficient in caspase 1 are resistant to neonatal hypoxic–ischaemic brain damage (Xu et al., 2001), suggesting that inhibition of caspase 1 induction contributes to neuroprotection by CAPE treatment.

In the HI model, it is also suggested that neuronal nitric oxide synthase (nNOS) and iNOS correlate with neurotoxic effect by release of NO under uncontrolled circumstances, whereas endothelial nitric oxide synthase (eNOS) has a neuroprotective effect (Bolanos and Almeida, 1999). Inhibitors of these two forms of NOS protect against HI-induced brain damage (Peeters-Scholte et al., 2002). Interestingly, in addition to reducing iNOS induction, we demonstrate that CAPE treatment can directly block NO-induced neuronal death, suggesting that the neuroprotective effects of CAPE may also be related to the ability of CAPE to directly inhibit iNOS expression and NO-mediated neuronal death. Inhibition of iNOS by CAPE may be able to directly or indirectly prevent mitochondria damage and the resulting neuronal death by attenuation of ATP depletion, glutamate release and the formation of reactive derivatives such as peroxynitrite, NO2,N2O3 and S-nitrosothiols (Brown and Bal-Price, 2003).

In this study, however, we cannot rule out CAPE, as an NF-κB inhibitor may inhibit NF-κB-induced neurotoxicity in ischaemic injury, as suggested by several reports (Schneider et al., 1999; Nurmi et al., 2004), although the exact role of NF-κB in the regulation of neuronal death in HI-induced injury remains unclear (Lipton, 1997; Matsushita et al., 2003). Blockade of NF-κB activation by CAPE may provide additive neuroprotection against neonatal HI. However, it has been reported that NF-κB in the same neurons behaves as a stimulus-dependent, anti-apoptotic or pro-apoptotic factor (Kaltschmidt et al., 2002), so further investigation is required to confirm the role of NF-κB in CAPE-induced neuroprotection on neonatal HI models. Since both iNOS and caspase 1 have been reported to be involved in ischaemic injury, our data suggest that the anti-inflammatory effects of CAPE may act by synergic neuroprotection together with a ‘direct’ neuroprotective action on neurons to protect the brain against brain injury. Furthermore, since inflammation, the release of cytochrome c and caspase 3 activation are shared features for a variety of neurodegenerative disorders, inhibition of these factors simultaneously by CAPE may contribute to the...
broad spectrum of effects of this drug in the inhibition of neuronal death. However, before CAPE becomes a novel effective therapy for preventing neonatal HI injury, further studies on its ability to cross the blood–brain barrier and its human safety are required.

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Fig. 4 CAPE pretreatments block HI-induced iNOS and caspase 1 expression in vivo. Immunoblot analyses were performed with polyclonal antibodies against iNOS, nNOS and caspase 1 (Santa Cruz). Ischaemia treatment increases iNOS and caspase 1 expression by 24 h after treatment. CAPE (CA-I) blocks the increase in both iNOS and caspase 1 after HI treatment (I). Note that HI and CAPE treatments fail to alter nNOS expression in these same samples. C = control. Group data (n = 3) on protein levels of caspase 1 and iNOS were normalized for nNOS.

Fig. 5 CAPE significantly inhibits NO-induced CGN neuronal death. CGN were exposed to CAPE (10 μM) for 24 h in the presence or absence of sodium nitroprusside (SNP; 50 μM; ***p < 0.001, one-way ANOVA). Neuronal viability was assessed by staining with fluorescein diacetate and propidium iodide and visual counting of viable neurons from representative photomicrographs. Values are expressed as percentages of control cultures for each experiment and the data represent the mean ± SE of triplicate determinations from a representative experiment repeated at least three times with similar results.

References
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