Pomegranate Polyphenols and Resveratrol Protect the Neonatal Brain against Hypoxic-Ischemic Injury

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\textbf{Introduction}

Neonatal hypoxia-ischemia (H-I) is a major cause of morbidity and mortality in human newborns with motor and cognitive sequelae frequently seen in survivors [Robertson et al., 1989; Shankaran et al., 1991; Volpe, 1995; Back, 2001]. Recent advances in neonatal care have improved the survival of severely prematurely born babies. These very low birth weight babies have a greatly increased risk of perinatal brain injury, including hypoxic-ischemic brain injury [Back and Rivkees, 2004; Gieron-Korthals and Colon, 2005]. Neonatal H-I has been associated with increases in reactive oxygen species, and recent studies suggest that new avenues of treatments of neonatal H-I should include selective responses to the generation of oxidative damage [Singh et al., 1999; Hamrick and Ferriero, 2003; Gulcan et al., 2005]. Current clinical treatments that appear successful in initial studies include hypothermia, which is thought to slow down the neuronal cell death processes and attenuate the generation of reactive oxygen species [Kil et al., 1996; Thoresen et al., 1997; Hashimoto et al., 2003; Gluckman et al., 2005; Shankaran et al., 2005].

Our lab and others have utilized an animal model of neonatal H-I in mice to determine mechanisms of injury as well as address potential treatments [Gibson et al., 2001; Han et al., 2001; West et al., 2006]. In a recent study using this model, we found that continuous pre- and postinjury ingestion of pomegranate juice by the dam
leads to significant protection of the neonatal brain [Loren et al., 2005]. The mechanism of neuroprotection afforded by pomegranate juice is unknown but it could potentially involve the antioxidant or other properties of polyphenols.

One of the most well studied polyphenols is resveratrol which is found in grapes, several types of nuts and kojihon (Japanese Knotweed), an oriental medicine used to treat diseases of the blood vessels [Sato et al., 1997; Faustino et al., 2003; Tokusoglu et al., 2005]. Resveratrol is an antioxidant but its in vivo effects reach far beyond that of other antioxidants. Most famously, resveratrol is thought to be the compound responsible for the low incidence of heart disease in the French population due to the high intake of red wine in France [Kopp, 1998]. Besides cardiovascular effects, resveratrol may have prophylactic effects against other human diseases, such as cancer [Bianchini and Vainio, 2003] and dementia [Leibovici et al., 1999; Truelsen et al., 2002]. In rats, resveratrol has been studied extensively in adult models of stroke and has been found to protect the brain when the rodents have been pre-treated for 3 weeks or longer with resveratrol containing drinking water [Virgili and Contestabile, 2000] or when resveratrol is administered by interperitoneal injection [Huang et al., 2001; Gupta et al., 2002; Sinha et al., 2002]. However, there have to our knowledge been no studies looking at the neuroprotective effects of resveratrol in neonatal brain injury.

In this study, we found that pomegranate polyphenol extract (PPE) as well as resveratrol can protect the neonatal rodent brain against H-I brain injury. When administered before injury, resveratrol can protect against both caspase-3 activation at 24 h after the injury and tissue loss at 7 days after injury. These studies suggest that polyphenols should be considered for further evaluation as potential treatments to lessen the effects of neonatal H-I brain injury.

Materials and Methods

Animals and Surgical Procedures

All rats and mice were kept under 12/12 light/dark cycles with ad libitum access to food and water. For studies of polyphenol extract of pomegranates and resveratrol in mice, male and female C57BL/6-J mice were interbred. For pups used in H-I experiments, the date of delivery was noted and at postnatal day 7 (P7) the pups underwent hypoxia-ischemia as described [Gibson et al., 2001; Han et al., 2001; West et al., 2006]. Briefly, mice were anaesthetized by inhalation of 5% halothane (balance room air) for induction and 1.5% for maintenance. An incision was made on the left side of the neck and the carotid artery isolated, exposed and permanently ligated. The pups were then put at 37°C to wake up and finally returned to the dam for a 2 h recovery period. Hypoxia was induced by putting pups in temperature-controlled chambers (37°C) through which humidified 8% oxygen flowed for 45 min. A similar protocol was used to induce H-I injury in P7 Sprague-Dawley rats; however, for the surgery on rats 2% halothane was required for maintenance of anesthesia and the rat pups were subjected to 8% oxygen for 2.5 h.

PPE Ingestion

The pomegranate polyphenol-enriched extract (PPE) was obtained from Pom Wonderful. PPE was produced from the skin and the aril of Punica granatum L. Wonderful variety and contains 0.9 mg polyphenols per mg powder. 96 mg of PPE was diluted in 100 ml of sugar water containing the same sugar composition as used in the previous experiments with pomegranate juice (a 1:160 dilution of 12.4% sucrose, 1.1% fructose and 1.1% glucose) [Loren et al., 2005]. This corresponds to an adult mouse ingesting 4.8 mg polyphenols per day (each mouse drinks about 5 ml of water per day). This dose of polyphenols was estimated to be similar to the amount of polyphenols ingested by mice drinking pomegranate juice in our previous study [Loren et al., 2005]. PPE diluted in sugar water or straight sugar water alone was given to the pregnant females as the sole source of drinking water throughout pregnancy and following the delivery, for the duration of the life of the pups. Drinking water was prepared fresh twice a week and kept in UV opaque bottles to prevent breakdown of the polyphenols. Since littermate controls were not possible for this experiment, we used 4 litters for each group to minimize the effect of litter to litter variability.

Resveratrol and Vehicle Injections

Before each experiment, resveratrol (Cayman Chemicals) was freshly dissolved in 100% dimethyl sulfoxide (DMSO, Sigma) at a final concentration of 70 mg/ml. At this concentration, a 3.5-gram mouse would receive a 1-μl injection to give a final concentration of 20 mg/kg (high dose). For 200-μg/kg (medium-dose) and 2-μg/kg (low-dose) injections the 70 mg/ml stock was diluted 1:100 and 1:10,000, respectively. Drug or vehicle was injected into the intraperitoneal space using a 26-gauge needle and a 5-μl Hamilton syringe (Hamilton). For initial studies in mice, 3 different time points of injection were chosen: 24 h before the start of the hypoxic episode, 10 min before hypoxia or 3 h after completion of the hypoxic episode. At each time point, half the litter would be injected with one concentration of resveratrol and the other half injected with vehicle (DMSO) alone. All rats were injected with 20 mg/kg at 10 min before injury, or 3 h after injury using a freshly prepared 70 mg/ml stock as for mice.

Tissue Lysis and DEVD Cleavage Activity

At 24 h following the end of the hypoxic episode, mice and rats were sacrificed by lethal injection of 200 mg/kg pentobarbital. Following transcardial perfusion with heparinized saline, the brains were extracted and the left and right hippocampi dissected on ice and frozen using dry ice. For each individual experiment, left and right hippocampi from all animals were lysed on the same day in the same lysis buffer (Cell Signaling) containing protease inhibitor cocktail (Roche). Lysates were cleared by spinning at 20,000 g for 15 min at 4°C. Protein concentration in each lysate was determined using a BCA kit (Pierce) and DEVD cleavage ac-
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Western Blotting
Remainder tissue lysates from the hippocampi that had been used for DEVD cleavage assays were pooled together into groups containing lysates from 4 individual mice. Based on protein concentration, each pool contains the same amount of protein lysate from the left or right hippocampus of 4 different mice. This allows for comparison of lysates from several different mice on the same gel. For Western blotting, 20 μg of total protein per lane was separated on a 4–12% Bis-Tris NuPage gel (Invitrogen). Proteins were transferred onto Immobilon P membranes (Millipore) and blocked in 2% ECL blocking reagent (Amersham). Membranes were then incubated with primary antibody overnight. Antibodies used were: Spectrin (1: 1k, Chemicon), tubulin (1:4k, Sigma). Membranes were then incubated with HRP-labeled secondary antibody, developed using SuperSignal (Pierce), and visualized on an ImageStation (Kodak).

Histology and Tissue Loss Calculation
For histological assessment of the extent of H-I injury, mice were sacrificed at P14 and their brains extracted and sectioned into 50-μm sections. Slices 300-μm apart were mounted, stained with cresyl violet and digitized on an Expression 1680 transparency scanner (Epson). Percentage volume loss in the hippocampus, cortex and striatum was calculated by comparing the area of remaining tissue in the injured and noninjured hemispheres as described [West et al., 2006]. For hippocampal tissue loss, 6 consecutive sections were measured, for cortical tissue loss 4 consecutive sections were measured and for striatal tissue loss 2 consecutive sections were measured.

Statistics
All data are presented as mean ± SEM and comparisons between drug and vehicle groups were done using a t test if the data were parametric, or a Mann-Whitney U test for nonparametric data. Statistical significance was set at p < 0.05. Statistics were performed using GraphPad Prism (GraphPad Software, Inc.).

Results
Ingestion of PPE by the Dam Lowers Caspase-3 Activation in Hippocampus of Pups following Neonatal H-I
Pregnant female mice were given either vehicle (sugar water) or PPE in vehicle as the sole supply of drinking water during pregnancy and following delivery. Neonatal H-I was performed on the pups at postnatal day 7 (P7) and the pups were sacrificed 24 h following the injury. Caspase-3 activity (measured as rate of DEVD cleavage) was measured in the left and right hippocampus. In this model of neonatal H-I in our prior studies, the majority of tissue loss and cell death is in the hippocampus with ~40% tissue loss 7 days after H-I, ~20% tissue loss in the striatum, and ~5% in the cortex [Han et al., 2001; West et al., 2006]. In previous studies using this model, caspase-3 activation in the hippocampus has been shown to correlate well with injury in other brain regions [Han et al., 2001; West et al., 2006]. Caspase-3 activation is a reliable and readily quantifiable measure of the extent of apoptotic neuronal cell death in response to neonatal H-I injury [Han et al., 2000; Gibson et al., 2001]. Thus, we assessed caspase-3 activation in the hippocampus after H-I. Pups of dams that have been administered PPE had significantly less caspase-3 activity in the hippocampus than did pups of dams drinking sugar water (VEH, n = 21). ** p = 0.0024 comparing PPE vs. vehicle-treated mice.

Resveratrol Protects against Caspase-3 and Calpain Activation in Mice in a Dose- and Time-Dependent Manner
To further explore the role of polyphenols in protecting the neonatal brain following neonatal H-I, we inves-
tigated the effect of the specific polyphenol resveratrol. Previous studies using resveratrol in adult rat stroke models have focused on high concentrations and the effect of treatment before the injury. Since the effect of resveratrol has not been tested in neonatal H-I model, we wanted to investigate the dose and time dependence of protection by resveratrol in this injury paradigm in mice. We tested 3 different concentrations of resveratrol: 20 mg/kg, 200 μg/kg and 2 μg/kg. These concentrations were administered at 3 different time points: 24 h before the start of hypoxia, 10 min before the start of hypoxia, and 3 h after hypoxia. As an indicator of the level of injury we measured caspase-3 activity at 24 h after hypoxia. When administered 24 h before the hypoxic insult, resveratrol decreased DEVD cleavage activity in the injured hippocampus at 24 h following neonatal H-I injury given at P7. The number of animals in each treatment group is indicated inside each bar in the graph: 55 pups were given either resveratrol or vehicle at 24 h before injury, 74 pups were given resveratrol or vehicle 10 min before injury, and 64 mice were treated with resveratrol or vehicle 3 h after injury. There is no difference in DEVD cleavage activity in the noninjured hippocampus, data not shown for clarification. 

**a** If injected at 24 h before hypoxia, resveratrol protects against caspase-3 activation in the hippocampus in a dose-dependent manner, when compared to littermate mice injected with vehicle. The high and the medium dose of resveratrol significantly reduce the amount of caspase-3 activation, while the low dose of resveratrol does not provide significant protection. 

**b** If injected 10 min before hypoxia resveratrol reduces caspase-3 activation at the high and medium doses, but not at the low dose. 

**c** When injected at 3 h after injury, resveratrol does not reduce caspase-3 activity at any of the doses utilized. *p < 0.05 and **p < 0.01 resveratrol vs. vehicle-injected littermates.
tered 24 h prior to injury (fig. 2b). However, when administered 3 h after injury, resveratrol has no effect on caspase-3 (fig. 2c). The level of protection is the same at 20 mg/kg and 200 μg/kg, showing that resveratrol could potentially protect at physiologically relevant levels. Interestingly, resveratrol is protective even when given at 24 h before the injury. This could either be due to slow metabolism of resveratrol [Yu et al., 2002] in the mouse pup or that resveratrol can mimic the effects of preconditioning, as shown in a rat brain slice model of ischemia [Raval et al., 2006]. To see if there was a difference in protection afforded by resveratrol administered at different time points, we compared the level of caspase-3 activation between time points by one-way ANOVA. While caspase-3 activation in mice receiving preinjury administration is significantly different from postadministration, there is no statistical difference in the results obtained when resveratrol was administered either 24 h or 10 min prior to H-I.

To further investigate the cell death pathways inhibited by resveratrol, we investigated calpain activation in the injured and noninjured hippocampus following H-I. Spectrin is cleaved by both calpain and caspase-3, giving rise to specific cleavage products, p145 and p150 for calpain and p120 for caspase-3. We have previously shown that calpain cleavage of spectrin is caspase-3 independent [Han et al., 2002; West et al., 2006]. Thus, calpain cleavage of spectrin is a measure of a nonapoptotic and likely necrotic type of cell death. In brain lysates assessed 24 h after H-I, the calpain cleavage products of spectrin were markedly decreased in the hippocampus of mice that had received resveratrol injection, suggesting that calpain activation is prevented by resveratrol. *** p < 0.001 resveratrol vs. vehicle by t test.

**Resveratrol Protects the Neonatal Brain against Tissue Loss following H-I**

To correlate the decrease in molecular markers of apoptosis and necrosis in mice injected with resveratrol with longer term protection of the brain, we measured the percentage tissue loss at P14. For this study, littermate mice received either 20 mg/kg resveratrol or vehicle at 10 min before the start of the hypoxic period at P7. At 7 days after injury (P14), the mice were sacrificed and their brains extracted and processed for histological analysis. Percentage volume tissue loss was calculated by comparing the ipsi- and contralateral areas of the brain in control and resveratrol-treated groups.
secutive coronal sections 300-μm apart. We have previously shown that this is an accurate way to measure the amount of tissue lost following neonatal H-I injury [Cheng et al., 1997; West et al., 2006]. Administration of 20 mg/kg of resveratrol resulted in significant protection against tissue loss in the hippocampus and striatum (fig. 4). There is also a decrease in the cortical tissue loss; however, this is not statistically significant due to the low amount of injury in the cortex.

Resveratrol Reduces Caspase-3 Activation in the Hippocampus of Neonatal Rats following H-I

The patterns and mechanisms of brain injury in the neonatal rat and mouse are similar but not the same. To further investigate the neuroprotective effects of resveratrol, we tested the ability of resveratrol to inhibit caspase-3 activation in a rat model of neonatal H-I. Although neonatal H-I injury in our protocol is performed at P7 in both rats and mice, it is likely that the brain of the two rodents are at slightly different developmental stages at this age [Hagberg et al., 1997]. An example of differences between species is that minocycline has been found to be neuroprotective in the rat but not in the mouse neonatal H-I model [Tsuji et al., 2004]. Also, the time course of caspase-3 activation in rats and mice following neonatal H-I is different [Cheng et al., 1998; Han et al., 2001]. Finally, there is a difference in the fatality rate during exposure to hypoxia between rats and mice. In mice the fatality rate

![Graph showing percent tissue loss in hippocampus, cortex, and striatum.](image-url)
is below 10%, probably because the hypoxic episode is relatively short. However, in our protocol that results in a similar amount of brain injury, rats are hypoxic for 150 min and this is associated with a higher fatality rate. In this study, 4 of 22 (18%) rats injected with resveratrol died during hypoxia while 6 of 22 (27%) of rats injected with vehicle died during hypoxia.

To test if resveratrol specifically protects the neonatal rat brain, we measured caspase-3 activation at 24 h following neonatal H-I in rats. We found that administration of 20 mg/kg resveratrol to rats 10 min before the start of the hypoxic period leads to a significant decrease in caspase-3 activation in rat pups as it did in mice (fig. 5a). We also tested if 20 mg/kg of resveratrol is protective when administered 3 h after the injury since the time course of caspase-3 activation is different between rats and mice and since there has been reports of neuroprotective agents working in the rat when administered after the injury [Wei et al., 2004; Shin et al., 2006]. However, at this time point, resveratrol is not protective against caspase-3 activation (fig. 5b). Thus, it appears that resveratrol protection of the rat brain follows similar time dependence as in the mouse brain.

Discussion

Dietary supplementation with foods rich in polyphenols – pomegranates, blueberries, green tea, and apple juice – has been shown to provide neuroprotection in animal models of focal brain ischemia, of periventricular white matter injury, and of Alzheimer’s disease [Levites et al., 2001; Sweeney et al., 2002; Dajas et al., 2003; Etus et al., 2003; Ortiz and Shea, 2004; Loren et al., 2005; Hartman et al., 2006]. Polyphenols have been found to possess antioxidant properties as well as to have effects on gene expression [Kostrzewa and Segura-Aguilar, 2003]. Specifically, one polyphenol, resveratrol, has been shown to increase activity of members of the sirtuin gene class, blunting p53 action and blocking apoptosis [Latruffe et al., 2002; Hall, 2003; Howitz et al., 2003]. Recent studies indicate that among foods that contain polyphenols, juice extracted from the pomegranate has the highest concentration of measurable polyphenols [Gil et al., 2000; Kela-wala and Ananthanarayan, 2004]. The pharmacologic actions of pomegranate juice include antiatherosclerotic, antibacterial, and antiproliferative properties [Anesini and Perez, 1993; Kim et al., 2002]. We recently found that when the polyphenol-rich pomegranate juice is consumed by the dam polyphenols from the juice are present

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in the effects of polyphenols is via activation of the sirtuins such as SIRT1.

Polyphenols such as resveratrol may have beneficial effects on health via their antioxidant properties, suppression of inflammatory pathways, or other pathways such as activation of the sirtuin pathway [Aggarwal and Shishodia, 2004]. Included in the sirtuin family is SIRT1, a human protein deacetylase that promotes cell survival by mechanisms such as negatively regulating the p53 tumor suppressor [Luo et al., 2001; Vaziri et al., 2001; Langley et al., 2002], deacetylation of transcription factor FOXO3 [Brunet et al., 2004; Motta et al., 2004], repression of PPARγ signaling [Picard et al., 2004], and modulation of NF-κB-dependent transcription [Yeung et al., 2004]. Modulation of these pathways may provide a means to protect the developing brain against neonatal H-I-induced brain damage. Recent studies show that polyphenols, including resveratrol, increase cell survival via activation of SIRT1 [Howitz et al., 2003]. Parker et al. [2005] found that increased sir2 gene dosage or treatment with resveratrol in Caenorhabditis elegans blocked neuronal dysfunction and cell death induced by polyglutamine expansion. Suggesting that resveratrol may act through a similar pathway in mammals, resveratrol protected mammalian neuronal cell lines from mutant huntingtin-induced cell death, and this effect was inhibited by sirtuin inhibitors [Parker et al., 2005]. There is also evidence that resveratrol can block axonal degeneration via SIRT1 in the mammalian peripheral nervous system [Araki et al., 2004]. While increasing evidence suggests that resveratrol and other polyphenols are neuroprotective, whether their protective actions in the CNS in vivo are via SIRT1 has not been directly assessed. Determining the mechanism of protection of resveratrol, pomegranate polyphenols, and other polyphenols may lead to novel insights into both pathogenesis and treatment of neonatal H-I brain injury.

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References


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