PARP-1 gene disruption in mice preferentially protects males from perinatal brain injury

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Abstract
Poly(ADP-ribose) polymerase-1 is over-activated in the adult brain in response to ischemia and contributes to neuronal death, but its role in perinatal brain injury remains uncertain. To address this issue, 7-day-old wild-type (wt) and PARP-1 gene deficient (parp+/− and parp−/−) Sv129/CD-1 hybrid mice were subjected to unilateral hypoxia-ischemia and histologic damage was assessed 10 days later by two evaluators. Poly(ADP-ribose) polymerase-1 knockout produced moderate but significant (p<0.05) protection in the total group of animals, but analysis by sex revealed that males were strongly protected (p<0.05) in contrast to females in which there was no significant effect. Separate experiments demonstrated that PARP-1 was activated over 1–24 h in both females and males after the insult in neonatal wt mice and rats using immunocytochemistry and western blotting for poly(ADP-ribose). Brain levels of NAD+ were also significantly reduced, but the decrease of NAD+ during the early post-hypoxia-ischemia (HI) phase was only seen in males. The results indicate that hypoxia-ischemia activates Poly(ADP-ribose) polymerase-1 in the neonatal brain and that the sex of the animal strongly influences its role in the pathogenesis of brain injury. Keywords: brain injury, hypoxia, ischemia, neonatal, poly(ADP-ribose)polymerase.

The role of PARP-1 in perinatal hypoxia-ischemia may also be limited because it is cleaved by caspase-3, which is more strongly activated by hypoxia-ischemia in the neonatal brain compared with the adult (Cheng et al. 1998; Hu et al. 2000; Nakajima et al. 2000; Zhu et al. 2000; Wang et al. 2001). To address these issues, the aims of the present study were to determine if mice with a complete (−/−) or partial (+/−) deficiency of the parp-1 gene are protected from perinatal hypoxic-ischemic brain injury and the degree to which PARP-1 is activated by perinatal hypoxia-ischemia.

**Materials and methods**

Animal experiments were conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals.

**Experimental protocols**

In one set of experiments, brain injury was assessed in 7-day-old parp gene deficient (+/−, −/−) and wt sv129/CD-1 hybrid mice. In another set of experiments, the time course of PARP-1 activation after hypoxia-ischemia was analyzed indirectly in 7-day-old CD-1 mice (Charles River Laboratories Inc., Wilmington, MA, USA) by the detection of PAR polymers with western blot and measurement of tissue levels of NAD+ or in 7-day-old rats (Rice et al. 1981) using immunohistochemical localization of PAR polymers.

**Parp gene deficient mice**

The PARP-1 knockout (KO) mice used have a disruption at the second exon of the parp gene (Wang et al. 1995). As the founding strain (sv129) is not suitable for hypoxia-ischemia experiments (Sheldon et al. 1998), sv129 PARP-1 knockout mice were backcrossed with CD-1 mice to obtain F3 CD-1 (12.5%) mice. Heterozygous parp (+/−) mice were then bred to obtain litters consisting of homozygous (parp−/−), heterozygous (parp+/−) and wild-type (wt) siblings, which were subjected to hypoxia-ischemia on postnatal day 7. Previous reports indicated that such hybrid strains develop reproducible brain injuries after hypoxia-ischemia and have reasonably low mortality (Sheldon et al. 1998).

**Neonatal hypoxia-ischemia in mice**

Hypoxia-ischemia in CD-1/sv129 hybrid (wt, parp−/−, parp+/−) and CD-1 wild-type (Charles River Laboratories) 7-day-old (weight 2.8–5.8 g) mice was induced as follows: the right common carotid artery was ligated under isoflurane inhalant anesthesia (4% induction and 1% maintenance), the incision was infiltrated with local anesthetic (1% lidocaine, 0.05% epinephrine), the pups were removed from the dam and the temperature of the water used to humidify the gas mixture, oxygen in nitrogen) for 55 min. The temperature in the incubator was allowed to recover for 1–2 h (Bona et al. 1998). Wounds were infiltrated with a local anesthetic, and the pups were allowed to recover for 1–2 h (Bona et al. 1998). The litters were placed in a chamber perfused with a humidified gas mixture (7.7% oxygen in nitrogen) for 55 min. The temperature in the incubator and the temperature of the water used to humidify the gas mixture, was kept at 36°C.

**Genotyping using PCR**

Genotyping was performed independently of histologic analysis. Genomic DNA was isolated from tail samples using phenol-chloroform extraction. The wt PARP-1 allele was amplified using primers (5′-CCACCGCAGCTCAGAGAAGCCA-3′ and 5′-CAT-GTCAGGGGAAGATTCCC-3′) located within the PARP fragment that was replaced by a neomycin resistant gene (neo) in the KO; the mutant allele was amplified using primers located within the neo gene (5′-TTCCATCGGATCCTGCTGCCTC-3′ and 5′-AG-CGCTGCTGACGGTCTACTG-3′). PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide under UV illumination. Wild-type and parp−/− control lanes were included on each gel. The genotypes were identified by single DNA bands of 130 bp (parp+/+, wild-type), single 220 bp (parp−/−, homozygous) or two DNA bands of 130 and 220 bp (parp+/−, heterozygous).

**Neonatal hypoxia-ischemia in rats**

Unilateral hypoxia-ischemia was induced in 7-day-old Wistar rat pups (weight 12.6–15.2 g; Mollegaard, Zealand, Denmark) of either sex (Rice et al. 1981). Rats were anesthetized with halothane (3% induction and 1.0–1.5% maintenance) and the left common carotid artery was cut between double ligatures of prolene suture (6–0). Wounds were infiltrated with a local anesthetic, and the pups were allowed to recover for 1–2 h (Bona et al. 1998). The litters were placed in a chamber perfused with a humidified gas mixture (7.7% oxygen in nitrogen) for 55 min. The temperature in the incubator and the temperature of the water used to humidify the gas mixture, was kept at 36°C. After hypoxic exposure, the pups were returned to their biological dams and were allowed to recover for 1–24 h (see below). Control pups, subjected to neither ligation nor hypoxia, were killed at postnatal day 7. The rat animal experimentation was approved by the Ethical committee of Göteborg, Sweden (270/01).

**Tissue preparation for NAD+ and western blot analysis**

CD-1 mice were decapitated under deep isoflurane anesthesia, either under control conditions (n = 6) or 1 h (n = 5), 4 h (n = 7) and 24 h (n = 6) after hypoxia-ischemia. The head was immediately frozen in liquid nitrogen and kept at −80°C until dissection. Brains were microdissected in a cryostat at −20 to −25°C. The anterior and posterior part of the brain were removed and the central forebrain
was split into one anterior slice (mostly striatum and cerebral cortex) and one posterior slice (mostly thalamus, hippocampus and cerebral cortex). Slices were divided into right (exposed to hypoxia-ischemia) and left (exposed to hypoxia) hemispheres.

Measurement of tissue content of NAD*
Approximately 40 mg of brain tissue was weighed and manually homogenized in 400 µL of 0.5 M HClO₄, 4°C and centrifuged (3000 g, 10 min, −2°C). The pellet was used for protein determination (Bradford) and the supernatant was neutralized with 800 µL of KOH/phosphate-buffered saline (PBS) 1M. The precipitate was removed by centrifugation (3000 g, 10 min, 4°C) and the supernatant was frozen until further analyzed. NAD/NADH was analyzed using a colorimetric method (Jacobson and Jacobson 1997) in which all NAD⁺ is converted to NADH by enzymatic cycling with alcohol dehydrogenase. NADH reduces thiazolyl blue through intermediation of phenazine methosulfate to the corresponding purple formazan. The rate of reduction of thiazolyl blue is proportional to the concentration of NAD⁺/NADH and is evaluated at A562.

PAR polymer western blots
Brain tissue specimens (approximately 40 mg) were homogenized in buffer containing 10 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 2 mM DTT, 0.1 mM PMSF, 10 µg/mL leupeptin, 100 µg/mL benzamidine, and 0.25 mM sucrose. Protein concentrations were determined (Bradford) and equal amounts of protein were loaded on an 8% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) gel (50 µg/lane) and separated overnight at 65 V. The proteins were electrotransferred to a nitrocellulose membrane, and stained with Ponceau S (0.1%) to confirm equal loading. Membranes were washed 3 × with PBS-T, and equal amounts of protein were loaded on an 8% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) gel (50 µg/lane) and separated overnight at 4°C. The proteins were electrotransferred to a nitrocellulose membrane, and stained with Ponceau S (0.1%) to confirm equal loading. Membranes were incubated in blocking buffer (PBS/0.1% Tween/5% dry milk) for 1 h and incubated overnight at 4°C with rabbit anti-PAR polyclonal antibody (1/2000) (Biornol, SA-276) (Affar et al. 1998, 1999) with anti-beta-tubulin antibody used as loading control [1/5000 (Sigma T-5168)]. Membranes were then washed 3 × with PBS-T 5% milk and incubated for 1 h with peroxidase-coupled anti-rabbit or anti-mouse antibodies (Pierce, Rockford, IL, USA). After washing with PBS-T, the bands were visualized by chemiluminescence using Supersignal West Pico (Pierce). The blots were quantified using densitometry and the values are expressed as percentage of control.

Immunohistochemical detection of PAR polymers
Rats were killed at postnatal day 7 (n = 9; controls) and at 1 h (n = 4), 2 h (n = 5), 4 h (n = 8), 8 h (n = 4), 14 h (n = 6) and 24 h (n = 9) after hypoxia-ischemia. Pups were deeply anesthetized and perfusion-fixed with 5% formaldehyde in 0.1M PBS (Histofix, antibody 1:300 (3000 g, 10 min, 4°C) and the supernatant was frozen until further analyzed. NAD/NADH was analyzed using a colorimetric method (Jacobson and Jacobson 1997) in which all NAD⁺ is converted to NADH by enzymatic cycling with alcohol dehydrogenase. NADH reduces thiazolyl blue through intermediation of phenazine methosulfate to the corresponding purple formazan. The rate of reduction of thiazolyl blue is proportional to the concentration of NAD⁺/NADH and is evaluated at A562.

Results
Mortality and brain injury in immature parp gene deficient and wt mice
Mortality was five of 38 (13%) in wt, five of 77 (5.6%) in parp⁺/– and three of 46 (6.5%) in Parp−/− mice; differences in mortality were not statistically significant. A 22-grade scoring system was used for evaluation of brain injury in the cerebral cortex, striatum, hippocampus and thalamus. Regression analysis revealed a moderate protective effect of reduced PARP-1 gene dose on the total injury score for all animals as well as for injury in the cortex, hippocampus, striatum and thalamus (Fig. 1). However, analysis according to sex revealed that the parp genotype was critical in males, but not in females (Figs 2 and 3). The total injury score in parp−/− was reduced in males (p < 0.05), but was not significantly reduced in females compared with wt mice. Brain injury was significantly reduced in all regions of parp−/− deficient mice compared with wt (Fig. 4); this protective effect was most pronounced in the thalamus.

PARP-1 activation after neonatal hypoxia-ischemia
Formation of PAR via nuclear protein modifications is a marker of PARP-1 activity and this was measured in mice.
using western blotting with a highly selective and specific monoclonal antibody to PAR (Fig. 5). We observed increased PAR immunoreactivity also in the immature mouse brain 4–24 h after hypoxia-ischemia. There appeared to be a transient increase of PAR also contralaterally in the hemisphere subjected to hypoxia only, but this change was not statistically significant. PARP-1 catalyzes the synthesis of PAR polymers from NAD⁺; therefore the tissue content of NAD⁺ represents an indirect measure of PARP-1 activation. A significant loss of NAD⁺ occurred 4–24 h after hypoxia-ischemia, which was not observed in the contralateral hemisphere (Fig. 5c). Separate analysis of PAR accumulation in males and females showed a significant increase in PAR accumulation in both females and males during the early post-HI phase (Fig. 6a), whereas a decrease in NAD⁺ content was only detected in males (Fig. 6b).

Immunohistochemistry demonstrated a weak nuclear PAR staining in the cortex and striatum of control rats (Fig. 7). Occasional cells were intensely PAR immuno-positive 1–4 h after hypoxia-ischemia, and there was a progressive increase in the number of intensely positive cells 8–14 h after the insult. There was also increased PAR immunostaining in the striatum, hippocampus and thalamus 8–14 h after hypoxia-ischemia (not shown) and, qualitatively, there appeared to be a modestly increased number of intensely PAR-positive cells in the contralateral hemisphere. We did not find any gender difference in the number of PAR positive cells in the cerebral cortex (female, 54 ± 6; male, 72 ± 5) or in the striatum (female, 119 ± 25; male, 112 ± 19) 4 h after hypoxia-ischemia.

Discussion

The results demonstrate that PARP is activated by hypoxia-ischemia in the neonatal rodent brain and that injury is reduced by a genetic deficiency of PARP-1. This data, as well as results of a previous study using pharmacologic inhibition of PARP activity (Ducrocq et al. 2000), support the hypothesis that brain injury in the immature brain depends on PARP-1 activation. Additional support is provided by our observation that hypoxia-ischemia caused an accumulation of PAR polymers, detected by western blot and immunostaining in injured tissue that paralleled the time course for loss of NAD⁺. The data are in general agreement with several previous studies of PARP-1 activation by ischemia and NMDA administration in adult animals (Eliasson et al. 1997; Endres et al. 1997; Ducrocq et al. 2000; Mandir et al. 2000; Plaschke et al. 2000) but the accumulation of PAR appears to be less robust than in adults.

Protective effects of parp-1 gene disruption are not due to compensatory processes for gene deletion because re-introduction of wild-type PARP-1 using recombinant replication-deficient Sindbis virus into parp-deficient mice completely restores the susceptibility to NMDA toxicity and focal ischemia (Mandir et al. 2000; Goto et al. 2002). Surprisingly, the protective effect of PARP-1 deficiency was strikingly dependent on the sex of the mice, with males preferentially protected compared with females. The effect was not due to a difference in severity of initial brain injury as this was similar for male and female wt mouse pups.
were detected in females. Brain injury score for male and female in all brain regions studied in males, whereas no significant differences 
(not accompanied by a drop in NAD+ (Nagayama 2000, 2004)). Activation of PARP-1 has been suggested to be less toxic if important observation to explain the female response, as 

36 and 17 in female +/+, +/– and –/–, respectively, and 16, 36 and 26 were determined by non-parametric regression analysis with 

–/–) genotypes are presented as mean ± SEM. Levels of significance 
ischemic injury at 7 days of age. Brain injury score for parp (wt, +/–, 

Gene Dose 
PARP 
(+/-) 
(+/-) 
(-/-) 

Male 
 Female 

Total Injury Score 

Fig. 3 Total injury score was reduced by knockout (–/–) of PARP-1 gene in male (p < 0.05) but not female mice with unilateral hypoxic-ischemic injury at 7 days of age. Brain injury score for parp (wt, +/–, 
–/–) genotypes are presented (mean ± SEM) for the cerebral 
cortex, striatum, hippocampus and thalamus. Levels of significance 
were determined by non-parametric regression analysis with n = 17, 
36 and 17 in female +/+; +/– and –/–, respectively, and 16, 36 and 26 in male +/+, +/– and –/–). 

Such a clear sex dependency with respect to PARP-1 involvement in CNS vulnerability has not been reported previously; previous studies published on focal ischemia in adult parp gene mutants were done in male mice (Eliasson et al. 1997; Endres et al. 1997). We presently found that the degree of PAR accumulation during early (1–4 h) post-HI reperfusion was similar in females and males, whereas the drop in NAD⁺ was only found in males. This may be an important observation to explain the female response, as activation of PARP-1 has been suggested to be less toxic if not accompanied by a drop in NAD⁺ (Nagayama et al. 2000; Yu et al. 2003). We do not understand the underlying mechanisms for the difference in NAD⁺ levels but, 

cytotoxic
effect appears to be independent of caspase activation. Synergism between AIF and caspase-dependent cell death could potentially be important in perinatal hypoxia-ischemia (Han et al. 2002; Zhu et al. 2003). The genes for AIF, as well as for several other proteins involved in perinatal hypoxia-ischemia that may be related to PARP-1 (e.g. X-linked inhibitor of apoptosis), are localized on the X chromosome and may, in addition to NAD+, be differentially expressed in males and females. Sex differences have also been reported for hypothermia, which provides more effective long-term protection in female than in male 7-day-old rats (Bona et al. 1998). Genetic background (pure Sv129 vs. Sv129/C57b16) also influenced the protective effect of the same disruption of the parp gene (second exon) used in these experiments in adult mice, but no influence of sex has been reported. In adult rodents, females sustain less injury than males after experimental ischemia (Hurn and Macrae 2000). This resistance is acquired after puberty (Payan and Conrad 1977), depends on the estrous cycle and is lost after menopause in accordance with a putative protective effect of sex steroids, especially estrogen (Hurn and Mcrae 2000; Stein 2001). Differences between males and females in this model are unlikely to involve exposure to hormones, but sex differentiation of the brain occurs in critical phases during embryonic and postnatal life (Hutchison et al. 1995; Becu-Villalobos et al. 1997) in ways that could affect vulnerability to injury.

In conclusion, these studies indicate that PARP-1 activation is an important step in the cascade of events leading to hypoxic-ischemic brain injury in neonates, and that differences in sex-determined brain development influence the expression of neuronal death by this mechanism. The results provide additional insight into the pathogenesis of ischemic brain injury and may have implications for the application of neuroprotective drugs acting on this mechanism.

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References


