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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 immnocytochemistry 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.

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The ubiquitous nuclear enzyme poly (ADP-ribose) polymerase (PARP) is activated in response to cellular stress and facilitates DNA relaxation and repair by catalyzing the synthesis of branched chain polymers of poly(ADP-ribose; PAR) from NAD^+ and transferring them to nuclear proteins including histones, topoisomerases, p53 and PARP itself (Satoh and Lindahl 1992; Eliasson *et al*. 1997; Le Rhun *et al*. 1998; D'Amours *et al*. 1999; Ha and Snyder 2000; Chiarugi and Moskowitz 2002). PARP-1 (EC 2.4.2.30), the most abundant isoform in the brain, has been shown to contribute to the cascade of ischemic neuronal injury in the adult brain through depletion of NAD^+ stores and ATP and activation of microglia inflammatory responses (Berger 1985; Szabo and Dawson 1998; Ullrich *et al*. 2001). Mitochondrial release of apoptotic inducing factor (AIF) has been suggested as an alternative pathway for PARP-mediated chromatolysis and cell death (Yu *et al*. 2002). Although PARP-1 inhibitors or deletion of the *parp-1* gene affords protection against NMDA toxcity and ischemic injury in the adult brain (Eliasson *et al*. 1997; Endres *et al*. 1997; Mandir *et al*. 2000), the importance of PARP-1 activation in perinatal brain injury remains unclear. PARP-1 is expressed more highly in the embryonic and postnatal period than at later times and, according to one study, the PARP-1 inhibitor 3-aminobenzamide reduced injury after focal ischemia in 7-day-old rats (Ducrocq *et al*. 2000). However, other more potent inhibitors with better bioavailability failed to provide neuroprotection (Feng and LeBlanc

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Abbreviations used: AIF, apoptotic inducing factor; Het, heterocygote; HI, hypoxia-ischemia; KO, knockout; MAP, microtubule-associated protein; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose)polymerase; *wt*, wild-type.

2000). 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 localizing 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 back-crossed with CD-1 mice to obtain F3 CD-1 (87.5%)/SV-129 (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 and the animals were allowed to recover for 90 min. The mice were exposed to hypoxia (10% oxygen in nitrogen) for 50 min at 36°C. The animals were allowed to recover and were returned to their dams. The tail was sampled at the time of perfusion or at the time of death during or immediately after hypoxia-ischemia for genotyping using PCR. The mouse hypoxia-ischemia protocol was approved by the Johns Hopkins University Animal Care and Use Committee.

Evaluation of hypoxic-ischemic brain injury in CD-1/sv129 (KO, heterocygote (Het) and wild-type) mice

Animals were anesthetized 10 days after hypoxia-ischemia and were perfused transcardially with paraformaldehyde. After all the mice for the entire experiment had been subjected to hypoxia-ischemia and their brains were sectioned and prepared for histologic analysis, two authors blinded to the experimental group evaluated injury in cresyl violet-stained sections (10/animal). Each investigator rated the neuropathology using dark-field microscopy to screen for injured cells with clumped chromatin (Lange *et al*. 1999) and bright-field microscopy to evaluate neuronal injury. The system for evaluating neuropathologic injury described previously (Nakajima *et al*. 2000) was used in the present study, with minor modifications. Neuropathologic injury in cerebral cortex was scored 0–4 (0, no injury; 1, few small isolated groups of injured cells; 2, several larger groups of injured cells; 3, moderate confluent infarction; 4, extensive confluent infarction) and in hippocampus, striatum and thalamus was scored 0–3 for mild, moderate or severe atrophy and 0–3 for mild, moderate or severe neuronal injury/infarction. The total score (0–22) was the sum of these ratings. The average of the scores of the two investigators was used for statistical analysis.

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′-CCAGCGCAGCTCAGAGAAGCCA-3′ and 5′-CATGTTCGATGGGAAAGTCCC-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′-TTCCATCCGAGTACGTGCTCGCTC-3′ and 5′-AGCTGTGCTCGACGTTGTCACTG-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 (*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 $HClO_4$ 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 thizolyl blue through intermediation of phenazine methosulfate to the corresponding purple formazan. The rate of reduction of thiazylol 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 $MgCl_2$, 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 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 LP96-10 (1/2000) (Biomol, SA-276) (Affar *et al.* 1998, 1999) and 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 chemoluminescence 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 0.1 M PBS (Histofix, Histolab, Goteborg, Sweden). Brains were rapidly removed, post-fixed in 5% formaldehyde at 4°C for 24 h, dehydrated with graded ethanol and xylene, the brains were paraffin-embedded and cut into 5 or 10 μm sections. Sections were deparaffinized in xylene and rehydrated in graded ethanol before staining.

Antigen retrieval was performed by boiling the sections in 10 mM sodium citrate buffer (pH 6.0) for 10 min. Non-specific binding was blocked with 4% horse serum in PBS. Anti-PAR antibody (Kawamitsu *et al.* 1984) was diluted 1 : 500 in PBS containing 0.2% Triton X-100, followed by rinsing in PBS (3 × 5 min). Slides were incubated with secondary biotinylated horse anti-mouse antibody 1 : 300 (3 μg/mL in PBS) for 60 min. Endogenous peroxidase activity was blocked with 0.6% H_2O_2 in methanol for 10 min. Visualization was performed using Vectastain ABC Standard with 0.5 mg/mL 3,3′-diaminobenzidine (DAB) enhanced with 15 mg/mL ammonium nickel sulfate, 2 mg/mL beta-D glucose, 0.4 mg/mL ammonium chloride and 0.01 mg/mL beta-glucose oxidase (Sigma, St Louis, MO, USA). Negative controls, in which the primary antibody was omitted, were completely blank.

PAR positive cells were counted in the cortex and striatum on each section at 400 × magnification (one visual field = 0.196 mm^2) of four males and four females at 4 h after hypoxia-ischemia (HI). Three visual fields within an area displaying loss of MAP-2 were counted and expressed as average number per visual field in both cortex and striatum. This average was used for statistical comparison.

Statistical analyses

To assure adequate statistical power and allow for variability in the model, 19 litters of mice were used in these experiments. After the complete, independent data sets for injury assessment and genotyping on all mice in the hypoxia-ischemia experiments were available, the data were combined to analyze the effect of genotype and sex on injury. Because brain injury scores are ordinal, rank-based non-parametric regression analysis was used to examine the effect of PARP gene dose (+/+ = 2, +/– = 1, –/– = 0) on brain injury score for each region examined. Data for PAR immunoreactivity (western blot) and NAD^+ content of hypoxic-ischemic versus control brain tissue were analyzed using ANOVA and Fisher's PLSD, *post hoc* test. Separate analysis for males or females used ANOVA and Fisher's PLSD and regression analysis. Student's *t*-test was used to compare levels in the left and right hemispheres. Mortality was assessed by chi-square analysis. Differences were considered significant at $p < 0.05$.

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

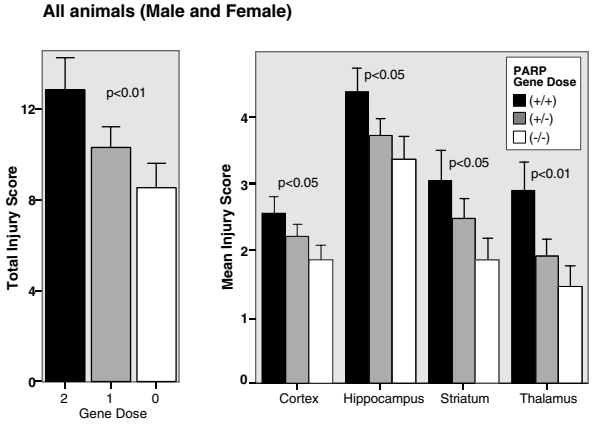

Fig. 1 Brain injury in all mice according to PARP-1 genotype. There was a protective effect of reduced PARP-1 gene dose on the total injury score for the entire group of animals as well as on the regional injury scores. Scores reflect the mean (± SEM) of scores from two of the investigators blinded to the genotype of the animals. Levels of significance were determined by non-parametric regression analysis with $n = 33$ for *wt* (+/+), 72 for *het* (+/–) and 43 for knockouts (–/–).

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.

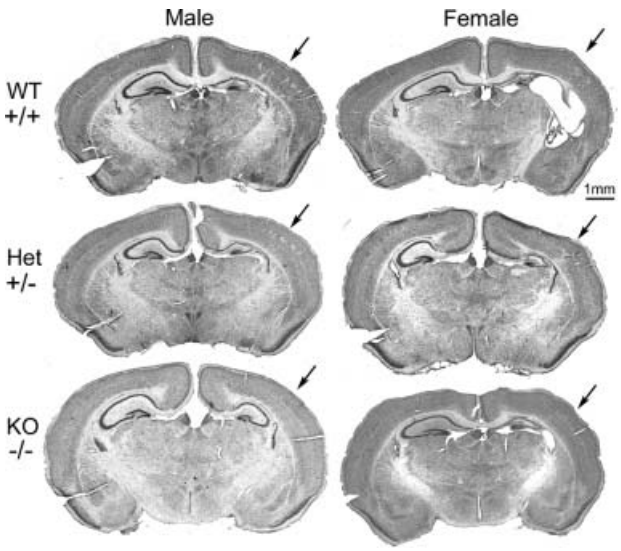

Fig. 2 Nissl stained coronal sections of male and female littermate mice 10 days after unilateral hypoxic-ischemic injury at 7 days of age illustrate that greater protection is imparted by knockout (–/–) of PARP-1 gene in males than in females. Arrows indicate the side of right carotid artery ligation with relative preservation of cortex, hippocampus and thalamus in the male PARP –/– mice.

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.

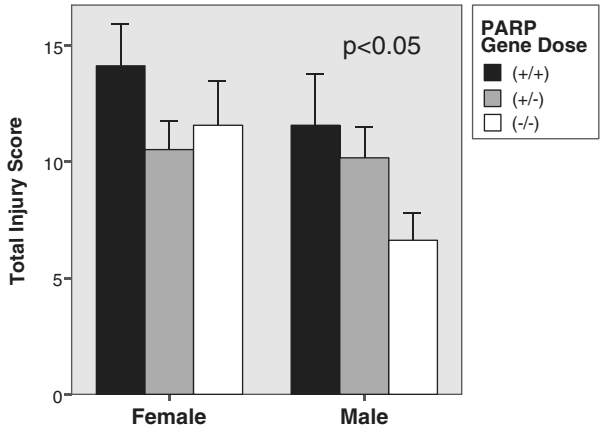

Fig. 3 Total injury score was reduced by knockout (–/–) of PARP-1 gene in male ($p < 0.05$) but not female mice with unilateral hypoxic-ischemic injury at 7 days of age. Brain injury score for parp (wt, +/–, –/–) genotypes are presented as mean ± SEM. Levels of significance were determined by non-parametric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

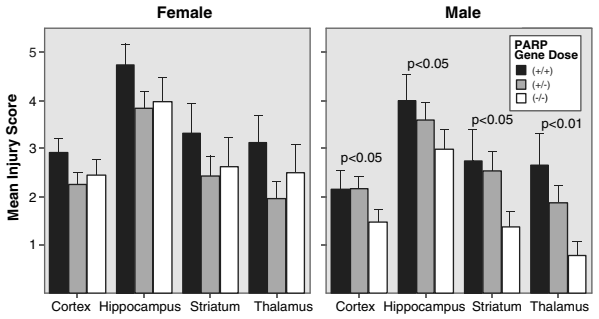

Fig. 4 Brain injury in *parp* gene-deficient mice was markedly reduced in all brain regions studied in males, whereas no significant differences were detected in females. Brain injury score for male and female *parp* (*wt, +/–, –/–)* genotypes are presented (mean ± SEM) for the cerebral cortex, striatum, hippocampus and thalamus. Levels of significance were determined by non-parametric regression analysis. The number of mice in each group is the same as in Fig. 3.

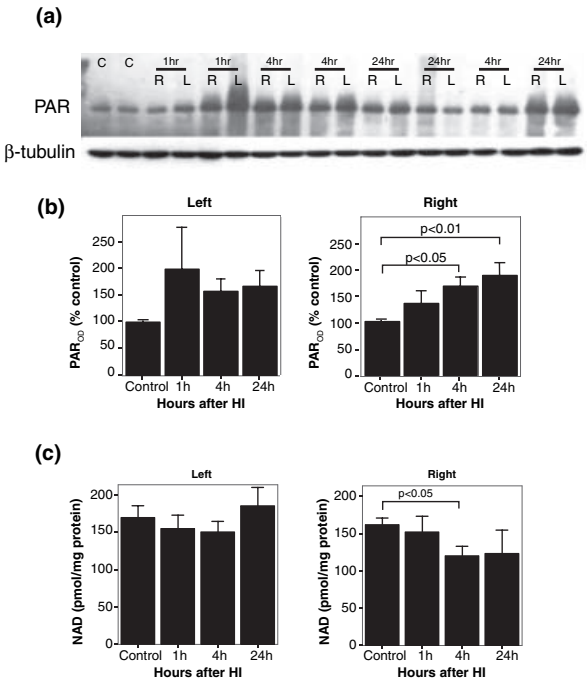

Fig. 5 Poly ADP-ribose (PAR) accumulation and reduction in NAD^+ after hypoxia-ischemia (HI) in the brain of 8-day-old mice. (a) One of three western blots examining the time course of PAR accumulation after hypoxia-ischemia in immature mice. (b) Quantitative densitometric analysis of all three western blots (mean ± SEM), with the total number of animals at each survival time indicated. PAR accumulation is significantly increased in the right, hypoxic-ischemic hemisphere 4 and 24 h after hypoxia-ischemia, compared with control (ANOVA, Fisher's PLSD test). Differences in the left hemisphere, which is subjected to hypoxia but not ischemia, are not significant. (c) The brain tissue levels of NAD^+ decreased at 4 and 24 h after hypoxia-ischemia in immature mice. The NAD^+ content (pmol/mg protein; expressed as mean ± SEM) was lower ($p < 0.05$) than control at 4 h of recovery (ANOVA and Fisher's PLSD test), and significantly lower compared with the contralateral hemisphere (Student's *t*-test) 24 h after hypoxia-ischemia. $n = 5$–7 animals at each time point [Poly ADP-ribose (PAR); R, right hemisphere; L, left hemisphere].

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, speculatively, the degree of mitochondrial impairment and ability to maintain the production of NAD^+ may depend on gender. Indeed, the time course of PARP-1 activation parallels the phase of mitochondrial impairment and excessive NMDA receptor activation after hypoxia-ischemia (McDonald and Johnston 1990; Gilland and Hagberg 1996; Gilland *et al*. 1998a) and precedes secondary energy failure and brain injury in this model (Blumberg *et al*. 1997; Gilland *et al*. 1998b). Future studies should investigate gender differences with respect to the mitochondrial response to HI.

NMDA-induced PARP-1-dependent neuronal injury *in vitro* has also been shown to rely on translocation of apoptosis inducing factor (AIF) from mitochondria to the nucleus (Yu *et al*. 2002). AIF has been shown to trigger cytochrome *c* release and caspase activation, but its cytotoxic

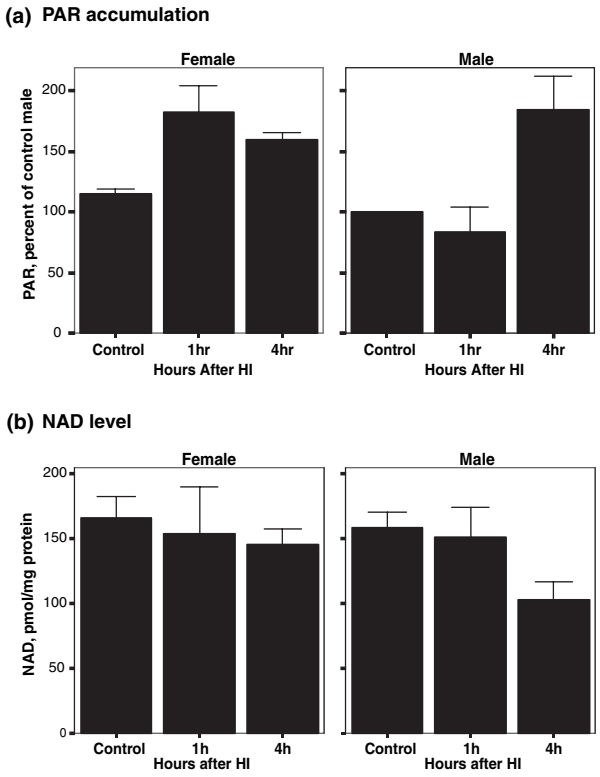

Fig. 6 Poly ADP-ribose (PAR) accumulation (a) and NAD^+ content (b) during control and after hypoxia-ischemia (HI) in the brain of 8-day-old female (left panel) and male (right panel) mice. (a) Quantitative densitometric analysis of western blots (mean ± SEM), demonstrate a significantly increased PAR accumulation in both female and male mice compared with control (ANOVA, Fisher's PLSD test). (b) The brain tissue levels of NAD^+ (pmol/mg protein; expressed as mean ± SEM) decreased with time 0–4 h after hypoxia-ischemia in male, but not in female, mice (regression analysis; $\beta = 0.77$, $p < 0.05$). Poly ADP-ribose (PAR).

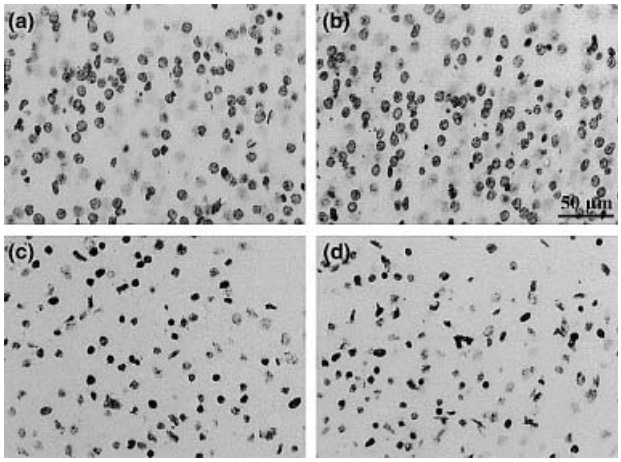

Fig. 7 PAR immunohistochemistry demonstrating increased immunoreactivity in the cerebral cortex 4 h after hypoxia-ischemia. Weak nuclear PAR immunostaining was found in the control brain of 8-day-old male (a) and female (b) rats. After 4 h of recovery, PAR immunoreactivity was moderately stronger in the hypoxic-ischemic hemisphere both in males (c) and females (d). Scale bar: 50 μm.

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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