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Deleterious Activation of Poly(ADP-Ribose)Polymerase-1 in Brain after *In Vivo* Oxidative Stress

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Oxidative stress has been shown to be implicated in the pathogenesis of central nervous system injuries such as cerebral ischemia and trauma, and chronic neurodegenerative diseases. *In vitro* studies show that oxidative stress, particularly peroxynitrite, could trigger DNA strand breaks, which lead to the activation of repairing enzymes including Poly(ADP-ribose) Polymerase-1 (PARP-1). As excessive activation of this enzyme induces cell death, we examined whether such a cascade also occurs *in vivo* in a model of oxidative stress in rat brain. For this purpose, the mitochondrial toxin malonate, which promotes free radical production, was infused into the left striatum of rats. Immunohistochemistry showed that 3-nitrotyrosine, an indicator of nitrosative stress, and poly(ADP-ribose), a marker of poly(ADP-ribose)polymerase-1 activation, were present as early as 1 h after malonate, and that they persisted for 24 h. The PARP inhibitor, 3-aminobenzamide, significantly reduced the lesion and inhibited PARP-1 activation induced by malonate. These results demonstrate that oxidative stress induced *in vivo* in the central nervous system leads to the activation of poly(ADP-ribose)polymerase-1, which contributes to neuronal cell death.

Keywords: 3-Nitrotyrosine; Brain; Cell death; Oxidative stress; Peroxynitrite; Poly(ADP-ribose)polymerase-1

INTRODUCTION

Oxidative stress is implicated in the pathophysiology of acute and chronic neurodegenerative diseases.[1–4] Indeed, numerous antioxidant therapies have shown neuroprotection in experimental models of cerebral ischemia[5] and trauma,[1] Alzheimer's[6] and Parkinson's diseases.[7] After brain injury, the production of reactive oxygen species (ROS) may increase, leading to tissue damage via several different molecular pathways. Free radicals can cause damage to vital cellular components such as lipids, proteins and DNA, leading to subsequent cell death.[1] Accordingly, in cultured cells exposed to ROS, including hydrogen peroxide, peroxynitrite or superoxide anions, DNA strand breaks are increased.[8] In turn, DNA strand breakage activates poly(ADP-ribose) polymerase-1 (PARP-1), a constitutive nuclear enzyme which is implicated in the DNA repair process,[9] genomic stability[10] and apoptosis.[11] Poly(ADP-ribose) polymerase-1 (EC 2.4.2.30), also called poly(ADP-ribose) synthase or poly(ADP-ribose) transferase, is an abundant nuclear enzyme present in eukaryotes. Activated PARP-1 catalyses the addition of long branched chains of poly(ADP-ribose) from its substrate nicotinamide adenine dinucleotide (NAD) to a set of nuclear proteins including DNA polymerases I and II, Ca^{2+}–Mg^{2+}-endonuclease, histones, several chromatin-binding proteins and PARP-1 itself.[9] PARP-1 activation leads to NAD depletion, resulting in a loss of ATP as it is used to synthetize new NAD, and finally to cell death.[12] In cultured cells exposed to hydrogen peroxide or peroxynitrite, DNA strand breaks are increased, PARP-1 is activated and energy resources are depleted as indicated by marked drops in NAD and ATP concentrations. These cellular disturbances are prevented by PARP inhibitors.[13–17] Similarly, *in vivo* studies on models of local and systemic inflammation demonstrate

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peroxynitrite production and show a protective effect with PARP inhibitors.[18,19]

Reading the central nervous system (CNS), *in vitro* studies demonstrate PARP-1 activation following oxidative stress. Accordingly, it has been shown that in C6 astrocytoma cells[20] and human glioma cells[17] exposed to peroxynitrite or hydrogen peroxide, PARP-1 is activated and subsequent cell death is induced, and this cascade can be prevented by PARP inhibitors. Additionally, PARP inhibitors have been shown to prevent neuronal cell injury from direct nitric oxide[21] and nitric oxide donors[22] application. It has also been demonstrated that PARP inhibitors attenuate neuronal injury induced by oxygen–glucose deprivation in cortical cell cultures[23,24] and neonatal rat brain slices.[25] Furthermore, neuroprotective effects have been described with PARP inhibitors, suggesting a deleterious role of PARP-1 activation in cerebral ischemia,[26–29] traumatic brain injury,[30–32] MPTP-induced toxicity[33] and experimental allergic encephalomyelitis.[34,35] However, to our knowledge, there is no *in vivo* evidence showing a direct relationship between cerebral oxidative stress, ROS production and PARP-1 activation. In this context, the aim of the present study was to investigate the oxidative stress-PARP-1 pathway in the brain. We evaluated whether *in vivo* oxidative stress in the brain induces peroxynitrite production and PARP-1 activation and, whether such PARP-1 activation contributes to the neuronal lesion. For this purpose, malonate, a mitochondrial toxin, was used to induce an *in vivo* oxidative stress. This compound inhibits the succinate dehydrogenase enzyme in both the tricarboxylic acid circle and respiratory chain, leading to an impairment of the mitochondrial respiratory chain and an increase in free radical production.[36,37]

MATERIALS AND METHODS

Animal care complied with the French regulations on the protection of animals used for experimental and other scientific purposes (D2001-486), as well as with the European Community regulations (Official Journal of European Community L358 12/18/1986).

Materials

Male Sprague–Dawley rats were supplied by Iffa-Credo (L'Arbresle, France). Bovine serum albumin, diaminobenzidine, hydrogen peroxide, gelatin, Triton X-100, sucrose and 3-aminobenzamide were purchased from Sigma Chemicals Corporation (Saint Quentin Fallavier, France). Rabbit anti-poly(ADP-ribose) antibody (SA 276) came from Biomol (TEBU, Le Perray, France), rabbit anti-3-nitrotyrosine antibody (06-284) from Upstate Biotechnology (Euromedex, Mundolsheim, France), donkey anti-rabbit biotinylated antibody (RPN 1004) from Amersham (Orsay, France). Swine serum (X-0901) and streptavidin–biotin peroxidase complex (streptABC complex HRP kit) were obtained from Dako (Trappes, France).

Surgical Procedure

Male Sprague–Dawley rats (weighing 300–330 g) were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and placed on a stereotaxic frame. During surgery, animals were positioned on a heating blanket (Harvard, UK) to maintain body normothermia (37.5 ± 0.5°C).

The scalp was incised and a craniotomy was made at the following coordinates: 3.5 mm lateral to the bregma, 0 mm posterior to the bregma.[38] A cannula (Sofijet 30G) was placed at a depth of 7 mm below the surface of the skull. Malonate was dissolved in distillated water and pH was adjusted to 7.4 Intrastriatal injections of malonate (3 μmoles) were performed via the cannula using a pump (Infors AG HT type Precidor). Infusions were carried out over 5 min and the injection cannula was left in place for a further 5 min. The scalp was sutured and the animal was returned to its home cage in a room warmed to 26–28°C to recover from the anesthesia. Thereafter, rats were group-housed under temperature- and light-controlled conditions with food and water *ad libitum*. Sham-operated rats underwent the same surgery and were given an intrastriatal injection of malonate vehicle (distilled water adjusted to pH 7.4)

Preparation of Brain Tissue for Immunohistochemistry

Rates were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.) and perfused transcardially with 200 ml of heparinized saline followed by 500 ml of phosphate-buffered saline (0.1 M, pH 7.4) (PBS) containing 4% paraformaldehyde. The brains were then removed, kept for 1 h in the same fixative solution, and placed in two successive 10% sucrose solutions, each for 24 h. Brains were rapidly frozen in isopentane (−40°C) and stored at −40°C until used. Serial coronal sections were cut (20 μm) on a cryostat (−18°C, Jung CM 3000, Leica), and collected on gelatin-coated slides, and processed for immunohistochemistry.

Immunohistochemistry of 3-nitrotyrosine and Poly(ADP-ribose)

Sections were incubated for 5 h at room temperature with the primary antibody: rabbit anti-3-nitrotyrosine antibody (diluted 1:100) in PBS containing 5%

swine serum or rabbit anti-poly (ADP-ribose) antibody (diluted 1:100) in PBS containing 0.3% gelatin and 0.25% Triton X-100 overnight at room temperature.

Specific labelling was detected by incubating the sections for 2 h with the secondary antibody (donkey anti-rabbit biotinylated antibody, 1:200 dilution) and amplified with streptavidin–biotin peroxidase complex. Diaminobenzidine was used as chromogen. All sections were incubated in 3% hydrogen peroxide and 10% methanol in PBS before the primary antibody was added in order to block non-specific peroxidase activity. As negative controls, alternative sections were incubated without the primary antibody.

Histology

Rate were anaesthetized with sodium pentobarbitone and killed by decapitation. Their brains were promptly removed, frozen in isopentane and stored at −40°C. Serial coronal sections were cut (50 μm thick) in a cryostat (−15°C) at 500 μm intervals, beginning at the level 11.2 mm anterior to the interaural line which corresponds to the beginning of the striatum. After staining with cresyl violet the infarction appeared to be well demarcated: lesioned areas were unstained (white tissue) and easily contrasted with areas of viable tissue, which stained violet. The lesion areas were measured with an image analyser (IMSTAR, Paris, France) and the distances between respective coronal sections were used to calculate the lesion volume. The lesion volume (in mm^3) was calculated by integrating the necrotic areas, corrected for oedema by the Golanov and Reis method.[39]

Experimental Protocols

Experiment 1: Kinetics of Immunohistochemistry of 3-nitrotyrosine and Poly(ADP-ribose) after Malonate

The temporal and spatial changes in the production of peroxynitrite and/or other nitrogen species, and the activation of PARP-1, were assessed by immunohistochemistry of 3-nitrotyrosine and poly(ADP-ribose) on brains removed 1, 2, 4 and 24 h after malonate injection ($n = 4$ for each time point). Parallel immunohistochemical examinations of 3-nitrotyrosine and poly(ADP-ribose) in brains from non-operated rats ($n = 4$) and sham-operated rats ($n = 4$ for each time point) were used as controls.

Experiment 2: Effect of 3-aminobenzamide on Striatal Lesion after Malonate

The role of PARP-1 activation on the neuronal lesion induced by *in vivo* oxidative stress was assessed by evaluating the effect of a commonly used PARP inhibitor, 3-aminobenzamide (3-AB),[40] on the striatal lesion caused by malonate injection.

Rats were given 3-AB (54 and 162 μg) or its vehicle (PBS), intracerebroventricularly (i.c.v.) 30 min before the infusion of malonate. The rats were killed 4 h after malonate infusion and the striatal lesion determined. For reasons of solubility, the low dose (54 μg) was dissolved in 20 μl PBS and the high dose (162 μg) in 60 μl PBS.

Experiment 3: Effect of 3-aminobenzamide on PARP-1 Activation after Malonate

Rats were given 3-AB (54 μg) or its vehicle (PBS), i.c.v. 30 min before malonate infusion. Rats were anesthetized with sodium pentobarbitone and killed by decapitation 4 h after malonate administration. They were perfused transcardially as described above. The effect of 3-AB on PARP-1 activation caused by *in vivo* oxidative stress was determined by immunohistochemistry of poly(ADP-ribose).

Statistical Analysis

Data are mean ± SEM. Differences in lesion volume were evaluated by a Mann–Whitney U-test. A P value of 0.05 was considered to be the threshold for a significant difference.

RESULTS

Experiment 1: Kinetics of Immunohistochemistry of 3-nitrotyrosine and Poly(ADP-ribose) after Malonate

One hour after malonate infusion, the left striatum exhibited strong immunostaining for 3-nitrotyrosine. Large stained areas were present at 2 h and persisted until 24 h after malonate (Fig. 1). Some poly(ADP-ribose)-positive cells were also found in the striatum 1 and 2 h after malonate infusion. By contrast, there was considerable poly(ADP-ribose) immunoreactivity at 4 h, and this staining appears to be less important 24 h after malonate (Fig. 2).

No staining was detected in sections from non-operated rats or in sections from sham-operated rats at any time (Figs. 1 and 2), or in sections from rats receiving malonate when the primary antibodies were omitted (not shown).

Experiment 2: Effect of 3-aminobenzamide on Striatal Lesion after Malonate

In 20 and 60 μl vehicle-treated rats, malonate induced the same striatal lesion (27 ± 1.4 and 23 ± 3.1 mm^3, respectively) (Fig. 3). In rats treated

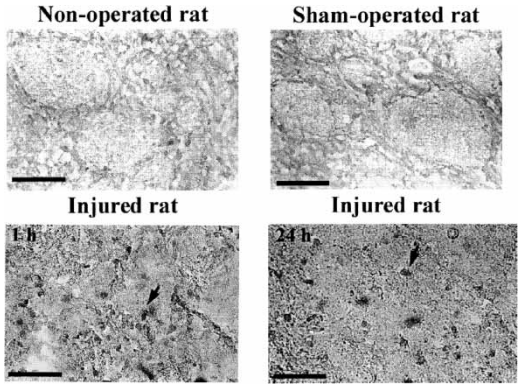

FIGURE 1 Time course of peroxynitrite and/or other nitrosylating compound production after *in vivo* cerebral oxidative stress. Representative photomicrographs of 3-nitrotyrosine immunoreactivity in the left striatum of non-operated, sham-operated and injured rats 1 and 24 h after malonate. Scale bar represents 80 μm.

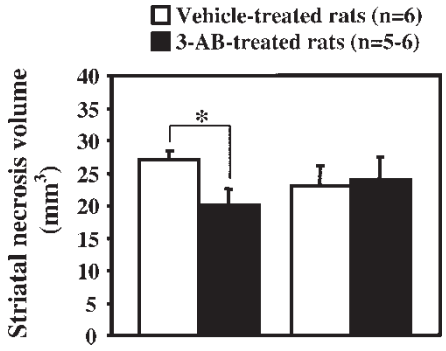

FIGURE 3 Effect of 3-AB on striatal necrosis volume evaluated 4 h after malonate infusion. Rats were given 3-AB (54 and 162 μg) or its vehicle (respectively 20 and 60 μl) i.c.v. 30 min before malonate infusion. Values are shown as mean ± SEM of measures carried out in 5–6 rats/group. $^*P < 0.05$ compared with 20 μl vehicle-treated rats.

with the low dose of 3-AB (54 μg), the lesion was significantly reduced by 26% (20 ± 2.5 vs 27 ± 1.4 mm^3 in vehicle-treated rats, $p < 0.05$). At 162 μg, 3-AB was devoid of any effect on the striatal lesion (24 ± 3.5 vs 23 ± 3.1 mm^3 in vehicle-treated rats).

Experiment 3: Effect of 3-aminobenzamide on PARP-1 Activation after Malonate

As in experiment 1, striatal infusion of malonate resulted in considerable immunostaining for poly(ADP-ribose) in the left striatum (Fig. 4). Treatment with 3-AB (54 μg) markedly reduced the immunoreactivity of (ADP-ribose) polymers 4 h after malonate infusion.

DISCUSSION

The present study demonstrated that, *in vivo*, a cerebral oxidative stress induced by the infusion of malonate, a mitochondrial toxin, leads to the production of peroxynitrite and/or other nitrosylating agents, and to the activation of PARP-1. The PARP inhibitor, 3-aminobenzamide, reduces the striatal lesion with a decrease in the formation of (ADP-ribose) polymers caused by malonate injection, demonstrating PARP-1 inhibition. These results show that oxidative stress induced *in vivo* in the CNS can cause the activation of PARP-1, which contributes to neuronal cell death.

In the current study, intrastriatal malonate injection was used to induce *in vivo* a cerebral oxidative stress, as previously described by Schulz *et al.*[36] Our data provide evidence that such oxidative stress induces peroxynitrite production, as demonstrated by 3-nitrotyrosine immunoreactivity, as early as 1 h

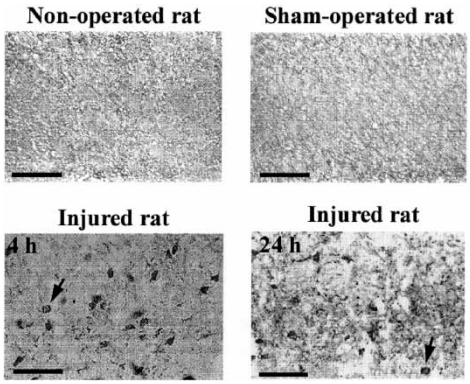

FIGURE 2 Time course of PARP-1 activation after *in vivo* cerebral oxidative stress. Representative photomicrographs of (ADP-ribose) polymer immunoreactivity in the left striatum of non-operated, sham-operated and injured rats 4 and 24 h after malonate. Scale bar represents 80 μm.

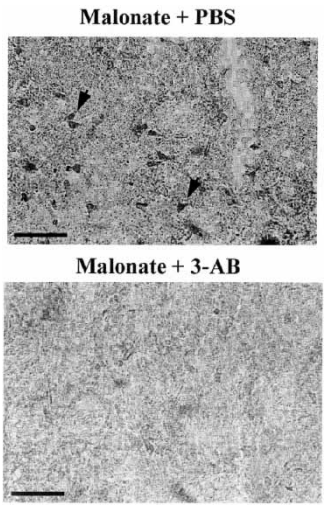

FIGURE 4 Effect of 3-AB on PARP-1 activation 4 h after malonate infusion. Rats were given 3-AB (54 μg) or its vehicle (20 μl) i.c.v. 30 min before malonate infusion. Representative photomicrographs of coronal sections of (ADP-ribose) polymer immunoreactivity in the left striatum of vehicle-and 3-AB-treated rats. Scale bar represents 60 μm.

and up to 24 h after malonate. Detection of 3-nitrotyrosine by immunohistochemistry was initially proposed as a relatively specific indirect way of detecting peroxynitrite.[41] However, recent evidence indicates that other reactions can also lead to tyrosine nitration, including one catalyzed by myeloperoxidase.[42] Therefore, increased nitrotyrosine staining is now considered to be an indicator of nitrosative stress rather than a specific marker of peroxynitrite.

We followed the time course of PARP-1 activation by assessing poly(ADP-ribose) formation by immunostaining since poly(ADP-ribosyl)ation is commonly used to indicate PARP-1 activity.[43] New enzymes, other than PARP-1, that can catalyze the formation of ADP-ribose polymers, have recently been identified, these include PARP-2,[44] short PARP-1 (sPARP-1),[45] VPARP,[46] and tankyrase 1 and 2.[47,48] Although limited data are available on the overall contribution of PARP-like enzymes to poly(ADP-ribosyl)ation *in vivo*, analysis of PARP-1 deficient mice indicates that poly(ADP-ribosyl)ation in the brain almost exclusively reflects PARP-1.[49] *In vivo* cerebral oxidative stress induced by malonate intrastriatal infusion activates PARP-1, as demonstrated by poly(ADP)-ribose immunostaining. The staining was present at 1 h and persisted until 24 h after malonate. Thus, our data show *in vivo* that cerebral oxidative stress induces reactive nitrogen species production associated with PARP-1 activation. These data obtained from a cerebral model are in accordance with data from experimental studies on peripheral cells. Indeed, Chatterjee *et al*.[50] have demonstrated that such cascade oxidative stress-PARP-1 activation also occurs in renal proximal tubular cells. Moreover, it has been demonstrated that ROS such as hydrogen peroxide and peroxynitrite lead to an increase in enzymatic activity of PARP-1 in human cardiomyoblasts and rat cardiomyocytes.[51,52]

Several studies have demonstrated that peroxynitrite are potent triggers of DNA strand breaks, with subsequent activation of PARP-1 leading to an energetic failure estimated by a severe loss of NAD and ATP cellular content.[11,12] The concept of PARP-mediated cell death following excessive DNA damage is supported by a number of *in vitro* studies showing that cell death induced by oxidative stress is prevented by PARP inhibitors.[40] In this respect, we determined *in vivo* the role of PARP-1 activation in cell death induced by oxidative stress. We studied the effect of 3-aminobenzamide (3-AB), a widely used PARP inhibitor,[40] on striatal lesion at 4 h after malonate, since poly(ADP-ribose) immunoreactivity is important at this time point. We showed that the lesion was significantly reduced by 26% in rats treated with a low dose of 3-AB (54 μg) while the high dose was devoid of effect. The neuroprotection observed with 3-AB suggests a deleterious involvement of PARP-1 in the striatal lesion induced by oxidative stress. This is consistent with *in vitro* data showing that PARP inhibitors attenuated neuronal injury induced by nitric oxide,[21,22] oxygen–glucose deprivation[23–25] and ROS.[16,17,20] Moreover, it has been previously shown that NOS inhibitors or neuronal NOS gene deletion induces neuroprotection in the same model of oxidative stress.[53,54] These findings strengthen the hypothesis that *in vivo* oxidative stress elicited by malonate injection induces some NO-derived reactive species (e.g. peroxynitrite, or possible nitroxyl anion), with consequent DNA injury which, in turn triggers PARP-1 activation. It is not clear why the high dose of 3-AB is not neuroprotective in our model, but similar results have been observed with different PARP inhibitors, including 3-AB, in proximal tubular cells exposed to hydrogen peroxide,[50] traumatic brain injury,[30,32] and cerebral ischemia.[55] It has been proposed that the absence of protective effect of 3-AB was likely due to separate metabolic actions that prevail at higher concentrations of the PARP inhibitor, including inhibition of protein, DNA and RNA synthesis and inhibition of cytochrome P-450.[56] The beneficial effect of 3-AB may be partly due to oxidant and free radical scavenging properties. However, there is evidence that several PARP inhibitors, including 3-AB, do not scavenge reactive oxygen species.[57–59]

Therefore, having found that 3-AB protects against the malonate-induced striatal lesion, we investigated whether this neuroprotection is associated with a decrease in the activation of PARP-1. Treatment with 3-AB caused a marked drop in PARP-1 activation consecutive to malonate infusion since there was much less poly(ADP-ribose) immunostaining in the striatum of 3-AB-treated rats than in those given 3-AB vehicle alone. Thus, the neuroprotection due to 3-AB appears to be directly related to the inhibition of PARP-1. However, our data should be confirmed by using PARP deficient mice and more selective and potent PARP inhibitors. In addition, measurement of PARP activity, DNA strand breaks, nitrotyrosine and superoxide generation, as well as NAD and ATP contents would strengthen our data. In this respect, nitrotyrosine and superoxide generation,[53] and a significant reduction in ATP levels[60,61] have been demonstrated after malonate injection. These data strongly support the conclusion that *in vivo* oxidative stress elicited by malonate injection induces nitrotyrosine and superoxide generation, which mediates PARP-1 activation leading to energetic depletion.

The oxidative stress-PARP-1 pathway has profound implications in neuronal death in acute and chronic disorders of the CNS. Several authors have

demonstrated the protective effect of the inactivation of PARP-1, either pharmacologically or using genetically engineered mice lacking PARP-1, in various experimental models of acute neurodegenerative diseases including cerebral ischemia[26–29,43] and traumatic brain injury.[30–32,62] Protection by PARP-1 inhibition is not restricted to acute neurological diseases. Thus, the mechanisms described here may have relevance in chronic neurological diseases (Parkinson's, Alzheimer's and Huntington's diseases) in which oxidative stress plays an important role.[63] Indeed, deleterious PARP-1 activation has been shown in a model of MPTP-induced parkinsonism.[64,65] Very interestingly, recent *post-mortem* studies in humans have shown PARP-1 overactivity, as demonstrated by elevated levels of (ADP-ribose) polymers in the brain, in cases of stroke[66] and Alzheimer's disease.[67]

In conclusion, this study has a two-fold importance: it demonstrates, to our knowledge for the first time, that cerebral oxidative stress *in vivo* leads to reactive nitrogen species production associated with PARP-1 activation, and it shows that this PARP-1 activation is a critical factor mediating neuronal death. Such an oxidative stress-PARP-1 pathway in the brain emphasizes the usefulness of PARP inhibitors as a therapeutic approach to cerebral damage associated with free radical production.

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