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Short communication

Reduction of tyrosine nitration after N^{ω}-nitro-L-arginine-methylester treatment of mice with traumatic brain injury

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Abstract

Oxygen free radicals and nitric oxide (NO) have been proposed to be involved in the cascade of injury elicited by traumatic brain injury. However, the mechanism(s) of injury remain to be explored. Since superoxide generation is triggered by traumatic brain injury, the cytotoxic peroxynitrite could be formed, but it is not known if this actually occurs. Dot blot and immunohistochemistry studies were performed to quantify tyrosine nitration and identify cell types in which such reactions occur in the brain of mice submitted to traumatic brain injury. Nitrotyrosine formation increased from 4 to 24 h after traumatic brain injury and was primarily observed in degenerating neurons, in areas corresponding to the sites of direct impact (frontal cortex) and diffuse impact (frontoparietal cortex and ventromedial hypothalamic nucleus). Furthermore, N^{ω}-nitro-L-arginine-methylester (L-NAME), a NO-synthase inhibitor which has previously been shown to promote neurological recovery in traumatic brain injury, reduced nitrotyrosine formation and the number of nitrotyrosine-positive neurons. These results indicate that traumatic brain injury induces peroxynitrite formation which may contribute to cell damage.

Keywords: Traumatic brain injury; Nitric oxide (NO); Nitrotyrosine; Peroxynitrite; Mouse

1. Introduction

There is increasing evidence that supports a role of oxygen radicals in the pathophysiology of acute central nervous system injury and ischemia (Hall and Braughler, 1989; Hall et al., 1993). Moreover, free radical scavengers have been reported to attenuate post-traumatic pathophysiology and/or to promote survival and recovery in experimental head injury (Hall, 1985; Hall et al., 1988). In a previous study, we reported that α-phenyl-*tert*-butylnitrone and melatonin, two free radical scavengers, decrease the neurological deficit induced by traumatic brain injury in mice (Mésenge et al., 1998). Nitric oxide (NO) is also involved in traumatic brain injury, because N^{ω}-nitro-L-arginine-methylester (L-NAME), a non-selective nitric oxide synthase inhibitor, was shown to reduce the consequences of traumatic brain injury (Mésenge et al., 1996). NO-related tissue injury may be largely due to peroxynitrite ($ONOO^-$), generated by the near diffusion-limited reaction between NO and superoxide free radicals (Beckman, 1991; Huie and Padjama, 1993), and peroxynitrite is cytotoxic via its reaction with a variety of molecular targets (Stamler, 1994). One of the actions of peroxynitrite is to nitrate tyrosine residues, leading to protein nitration. Recently, immunohistochemical studies with anti-nitrotyrosine antibody have revealed that nitration of tyrosine residues takes place in various tissues and organs under pathological conditions, for example in postischemic heart tissue (Wang and Zweier, 1996), the brain after carbon monoxide poisoning (Ischiropoulos et al., 1996), the brain of patients with Alzheimer's disease (Smith et al., 1997) or amyotrophic lateral sclerosis (Beal et al., 1997). The aim of the study was, using a similar approach, to investigate peroxynitrite production after traumatic brain injury and whether L-NAME treatment was able to reduce this production.

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2. Materials and methods

All experiments were conducted strictly according to NIH recommendations and French Department of Agriculture guidelines (licence no. 01352).

2.1. Closed head injury model

Male Swiss mice (Charles River, France), weighing 25 to 30 g, were given water and food ad libitum before the experiment. The mouse head injury is the model described by Hall (Hall, 1985; Hall et al., 1993) and modified by ourselves (Mésenge et al., 1996). Briefly, trauma was induced by a 50-g weight falling freely from 22-cm height along a stainless steel string. Mice were killed by decapitation at 4 and 24 h recovery ($n = 6$ and $n = 8$, respectively). Three mice of the two groups were treated with L-NAME (3 mg kg^{-1}, i.p.), 5 min after traumatic brain injury, whereas the others were treated with its vehicle (distilled water).

2.2. Quantification of nitrotyrosine immunoreactivity

Sample tissues, obtained with a punch (8-mm diameter and 3-mm thick) from in front of the impact site (frontal cortex), were freshly excised and homogenised at 4°C in 50-mM Tris–HCl (pH 7.4), 1-mM EDTA and 1-mM phenylmethyl–sulfonyl fluoride, and then centrifuged at $15\,000 \times g$ for 15 min to remove insoluble material. The supernatant was retained. Protein concentration was determined by Lowry's method (Lowry et al., 1951). Samples (10 μg protein each) were analysed by dot blots on nitrocellulose membranes. After blockage of non-specific binding sites with 3% dried fat-milk in 20-mM Tris (pH 7.4, 150-mM NaCl), membranes were processed with the affinity-purified rabbit polyclonal anti-nitrotyrosine antibodies (Ye et al., 1996), from Upstate Biotechnology (Euromedex, Souffelweyersheim, France). Membranes were incubated with the first antibody (dilution 1:100) for 1 h at room temperature, washed, and then incubated with horseradish peroxidase-labelled second antibody for 45 min. Specific proteins were detected on X-ray films using enhanced chemiluminescence (ECL; Amersham).

As previously described (Coereli et al., 1998), quantification was performed with densitometric measurement software for image analysis (Samba 2005/Alcatel TITN Answare, Grenoble, France). Data are expressed as means ± S.D. (optical density in arbitrary unit). Statistical analysis was performed by using the Mann–Whitney *U*-test.

2.3. Immunocytochemistry

In another set of experiments, traumatic injured mice ($n = 3$, at 24 h) were perfused via the ascending aorta under deep anaesthesia with warm heparinized saline followed by phosphate-buffered solution (PB 0.12 M, pH 7.4) containing 4% *para*-formaldehyde. Brains were then removed, post-fixed for 1 h in the same fixative and placed in 0.1-M PBS containing 10% sucrose for 2 days. Brains were rapidly frozen in isopentane (−40°C) and subsequently stored at −70°C until used. Serial coronal cryostat sections (20 μm) were collected on silanated slides. Sections were incubated overnight at 4°C with the first antibody (dilution 1:100) in PBS containing 2% bovine serum albumin and 0.2% Triton X-100. The second anti-rabbit IgG biotinylated antibodies (Dako, 1:200 dilution) were visualised with avidin–biotin peroxidase (Elite ABC kit, Vectastain Vector, Byosis, France) using diaminobenzidine. Non-specific peroxidase activity was prevented by incubating the sections in 2% hydrogen peroxide in 10% methanol before the primary antibody was added. As previously described (Ye et al., 1996), nitrotyrosine was reduced in situ by the addition of 10-mM sodium hydrosulfite (pH 9) for 15 min and then sections were processed for immunohistochemistry. Adjacent coronal sections were stained with Cresyl violet. Uninjured mice were used as control.

3. Results

In the control mice, no significant nitrotyrosine formation was detected. Nitrotyrosine formation increased from 4 h (46 ± 21.0 arbitrary unit) to 24 h (161 ± 17.5 arbitrary unit) following traumatic brain injury, as shown in Fig. 1. Samples from mice treated with L-NAME (3 mg kg^{-1}) showed a marked and significant reduction in nitrotyrosine formation at both 4 and 24 h recovery (17 ± 3.5 and 82 ± 4.0 arbitrary unit, respectively).

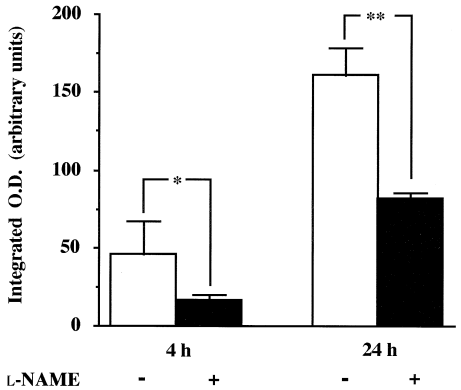

Fig. 1. Effect of L-NAME on nitrotyrosine detection at 4 and 24 h after traumatic brain injury in mice. Nitrotyrosine was detected in sample tissues by dot blots (see Section 2). Results are represented as histograms of densitometric measurements (optical density, O.D.). Data are expressed as means ± S.D. Note that L-NAME significantly reduced nitrotyrosine formation at 4 and 24 h. Treated mice (filled bars) vs. untreated mice (open bars). * $P < 0.05$; ** $P < 0.01$.

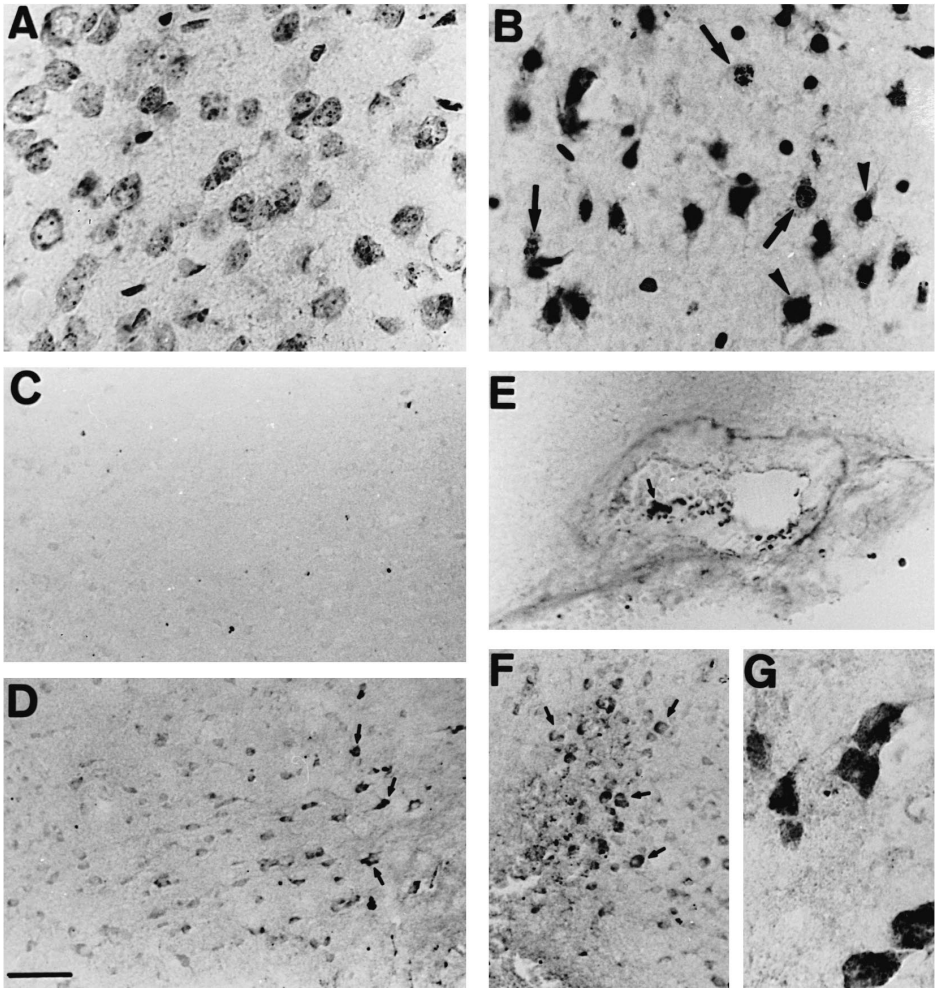

Fig. 2. Representative Cresyl violet-stained coronal sections and immunohistology of the presence of nitrotyrosine in control mice and mice 24 h after brain trauma. Cresyl violet in control mice (A) and in mice 24 h after trauma (B) in the cortex. Note the presence of pale-stained neuropil, pyknotic (arrowhead) and karyorrhectic (arrows) nuclei following trauma. The $ONOO^-$ specific nitration product was visualised using a specific affinity purified nitrotyrosine antibody as primary antibody, biotinylated secondary antibody, and peroxidase-conjugated tertiary antibody. C: Control cortex. D–G: nitrotyrosine-positive cells in the frontoparietal cortex (D), arachnoid vessel (E) and ventromedial hypothalamic nucleus (F) at 24 h after traumatic brain injury. Note nitrotyrosine immunoreactivity in the cytoplasm and dendritic processes of cortical neurons (G). Scale bar represents 10 μm (A, B) 20 μm (E), 40 μm (C, D, F) and 15 μm (G).

Examination of Cresyl violet-stained sections showed that the cytoarchitecture and cell morphology of the cortex from control mice were normal (Fig. 2A). In contrast, the brains of all mice with traumatic brain injury showed neurons containing pyknotic or karyorrhectic nuclei (Fig. 2B) in dorsal and ventral brain areas, corresponding to the site of direct impact (frontal cortex) and the site of diffuse impact (frontoparietal cortex and ventromedial hypothalamic nucleus). No nitrotyrosine immunoreactivity was observed in sections from control mice (Fig. 2C) or in sections from mice with traumatic brain injury when the primary antibody was omitted (not shown). In contrast, intense nitrotyrosine staining was observed in cells in the frontoparietal cortex (Fig. 2D) and ventromedial hypothalamic nucleus (Fig. 2F). The staining was present in the cytoplasm and dendritic processes of neurons (Fig. 2G). Little, if any, nitrotyrosine immunoreactivity was observed in vessels surrounding the lesioned areas (Fig. 2E). Nitrotyrosine immunoreactivity was not observed in non-lesioned brain areas. In addition, nitrotyrosine immunostaining was markedly reduced in L-NAME-treated mice with traumatic brain injury (not shown).

4. Discussion

In the present study, we used immunoreactivity for nitrotyrosine, a product of the nitration of tyrosine residues by peroxynitrite, as a marker for peroxynitrite formation and more generally for reactive nitrogen species (Van der Vliet et al., 1997). The formation of peroxynitrite cannot be determined directly due to its short half-life, but the

detection of nitrotyrosine provides evidence of its formation (Beckman et al., 1994). Moreover, nitrotytrosine has been documented as an indirect indicator of peroxynitrite-induced damage (Ischiropoulos et al., 1996; Vilet et al., 1994) and has been used as peroxynitrite marker in several injury models, i.e., cerebral ischemia (Coereli et al., 1998; Forman et al., 1998; Fukuyama et al., 1998).

Our data show that nitrotyrosine was formed in degenerating neurons at 4 and 24 h after traumatic brain injury, suggesting that peroxynitrite is produced in the brain after trauma. The immunohistochemical studies indicated that nitrotyrosine is predominant in neurons present in lesioned areas at the sites of direct and diffuse impact. Increased nitration of key tyrosine residues in motor neurons has also been described in amyotrophic lateral sclerosis (Beckman et al., 1993; Beal et al., 1997) and peroxynitrite production has been demonstrated in cerebral ischemia (Coereli et al., 1998; Forman et al., 1998; Fukuyama et al., 1998). In addition, our results show that nitrotyrosine immunoreactivity was inhibited by treatment with L-NAME, a NO-synthase inhibitor. These results suggest that the generation of peroxynitrite is dependent on the production of NO. Consistent with this, it has been reported that nitrotyrosine immunostaining occurs in both neurons and the surrounding neuropil following excitotoxicity but not in neuronal NO synthase knock-out mice (nNOS $-/-$) (Ayata et al., 1997).

It can be postulated that NO can combine with superoxide free radicals to form peroxynitrite (Beckman, 1991; Huie and Padjama, 1993). Previous data from our group indicate that NO and reactive oxygen species are produced following traumatic brain injury, as neurological recovery was observed after treatment with L-NAME (Mésenge et al., 1996) and with α-phenyl-*tert*-butyl-nitrone and melatonin, two free radical scavengers (Mésenge et al., 1998). NO and superoxide may damage cells (Dawson et al., 1993). However, peroxynitrite formed from NO and superoxide is considered to be the most damaging of the reactive oxygen species (Beckman et al., 1990). Taken together, these data suggest that NO and superoxide free radicals are produced in traumatic brain injury, leading to peroxynitrite formation which contributes to neuronal damage and impairs neuronal function.

The cytotoxic effects of peroxynitrite could be related to lipid peroxidation, protein nitration, DNA damage and apoptosis, and energy depletion attributable to poly(ADP-ribose) synthetase activation (for review, see Beckman et al., 1996). Interestingly, poly(ADP-ribose) synthetase inhibitors have been shown to be neuroprotective in a model of traumatic neuronal injury (Wallis et al., 1996).

In conclusion, our study strongly suggests that reactions mediated by the $NO/ONOO^-$ pathway contribute to traumatic brain injury. Inhibition of NO synthase may prevent activation of this deleterious pathway by reducing NO production and subsequent cytotoxic peroxynitrite generation.

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