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BRAIN RESEARCH

Research Report

Diphenyleneiodonium and dimethylsulfoxide for treatment of reperfusion injury in cerebral ischemia of the rat

Simon Nagel[a,*], *Just Genius*[b], *Sabine Heiland*[c], *Solveig Horstmann*[a], *Humphrey Gardner*[d], *Simone Wagner*[a]

[a]*Department of Neurology University of Heidelberg, Medical School, Heidelberg, Germany*
[b]*Department of Psychiatry Ludwig–Maximilians University of Munich, Medical School, Germany*
[c]*Department of Neuroradiology University of Heidelberg, Medical School, Heidelberg, Germany*
[d]*Novartis Institutes for Biomedical Research, MA 02139, USA*

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ABSTRACT

Diphenyleneiodonium (DPI) is an inhibitor of the free radical producing NAD(P)H-oxidase. We tested whether DPI shows neuroprotective properties after focal cerebral ischemia and we used dimethylsulfoxide (DMSO), a nonspecific free radical scavenger, as a solvent. In male Wistar rats middle cerebral artery occlusion (1.5 h) and subsequent reperfusion (48 h) (MCAO/R) was induced with the filament model. Immediately after reperfusion the animals received either 0.25 ml normal saline, DMSO, or a combination of DMSO and DPI; each group consisted of 10 animals. MRI was performed at different times after reperfusion. Gelatine zymography of brain tissue for MMP-2 and MMP-9 was performed. The infarct sizes and BBB damage showed a significant difference between controls and the DPI/DMSO group for almost all points in time in all sequences. The activity of MMP-2 and MMP-9 was significantly reduced by DPI/DMSO but not by DMSO alone. DMSO treatment alone resulted in a protective effect with reduced lesion sizes measured by MRI at selected points of time, consistent with its known free radical scavenger effect. The combination of DMSO with DPI partly augmented this effect, presumably due to the additional inhibition of MMP-2 and MMP-9 by DPI. Moreover, the neurological outcome in both therapeutic groups was improved compared to controls with a significant difference between the therapeutic groups in favour of DPI and DMSO. The combination of DPI and DMSO reduced the activity of MMP-2 and MMP-9, attenuated the postischemic blood–brain barrier damage and improved neurological outcome. This was most likely due to reduced oxidative stress.

1. Introduction

Several pathophysiological phenomena contribute to reperfusion injury, consisting of secondary release of excitatory amino acids and nitric oxide or formation of reactive oxygen species (ROS), that can enlarge the initial ischemic damage. These phenomena furthermore include upregulation of adhesion molecules and inflammatory cytokines which mediate the invasion and activation of leucocytes, and consecutively increase metalloproteinases (MMPs) which contribute to

* *Corresponding author*. Fax: +1 49 6221 56 5461.
E-mail address: simon_nagel@med.uni-heidelberg.de (S. Nagel).

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structural damage (del Zoppo et al., 2000; Jean et al., 1998; Maxwell and Lip, 1997; Winquist and Kerr, 1997). These mechanisms are partly interdependent and form cascades. ROS, like superoxide or peroxynitrite, play a pivotal role in the pathophysiology of ischemia when they appear at toxicologically relevant or unphysiologically high concentrations (Becker et al., 1999; Hall and Braughler, 1993; Johnson and Weinberg, 1993). Consistent with this, scavengers of ROS and inhibitors of lipid peroxidation have shown neuroprotective effects after ischemia and reperfusion (Chan, 2001; Kontos, 2001). One major source of oxidative stress and ROS are leucocytes that migrate into the damaged cerebral tissue. They produce ROS mainly through the action of NAD(P)H-oxidase and other enzymes. We have evidence that the lack of the NAD(P)H-oxidase may lead to a significant reduction in ROS mediated neurotoxicity (Walder et al., 1997). Diphenyleneiodonium (DPI) is a potent inhibitor of the NAD(P)H-oxidase which specifically and irreversibly binds to the flavicenter of the enzyme (Hampton and Winterbourn, 1995; Wang and Pang, 1993). It has shown its selective inhibitory properties in several studies (Dodd-O and Pearse, 1998; Doussiere et al., 1998). Moreover, it is also able to interact with other enzymes potentially involved in the pathophysiology of ischemia–reperfusion injury, i.e., nitric-oxide synthase, xanthine oxidase and NADH cytochrome P450 oxidoreductase (Doussiere and Vignais, 1992; Stuehr et al., 1991; Tew, 1993). DMSO is a simple organic compound, is highly distributive in the CNS, solubilizes many lipophilic substrates and moreover has antioxidative, cell membrane stabilizing and antiinflammatory properties (Kolb et al., 1967). It has already been used as a nonspecific free radical scavenger in studies of cerebral ischemia in rats and humans and proved to possess beneficial properties (Karaca et al., 2001; Shimizu et al., 1997). It is known that MMP-2 and MMP-9 are increased in cerebral ischemia cleaving extracellular matrix molecules which leads to BBB breakdown (Rosenberg et al., 1998; Wagner et al., 2003). Besides other activation pathways MMPs might also be increased by oxidative stress. The recent findings of Gursoy–Ozdemir et al. suggest that superoxide and peroxynitrite formation in and around microvessels after ischemia and reperfusion contribute to loss of selective BBB permeability by activation of MMP-9 (Gursoy-Ozdemir et al., 2004).

We therefore tested the hypothesis whether DPI protects the postischemic BBB and reduces the activity of MMPs by the postulated reduced production of free radicals. As DPI is not soluble in hydrophilic substances we used DMSO as a carrier. We quantitatively monitored the evolution of ischemic lesions and the BBB breakdown with MRI and performed quantitative zymography.

2. Results

2.1. *Dose finding for DPI*

In our in vitro dose-finding experiment NAD(P)H-oxidase induced superoxide generation was dose-dependently decreased following addition of DPI. Approaching a concentration of about 100 μM DPI this inhibitory effect was saturated (possibly due to alternative sources of superoxide, non-responsive to DPI). The results of the in vitro lucigenin assays are presented in Fig. 1. For further analysis of the in vivo capabilities of DPI in attenuating stroke damage we chose a dose in a range where DPI had a maximal inhibitory effect on the superoxide generation in vitro without affecting cardiorespiratory parameters or showing overt acute toxicity in vivo. The dose chosen, a bolus injection of 0.25 ml of a 50 mM DPI solution, would be expected to result in an effective peak blood concentration of about 0.7 mM and an estimated tissue concentration of 50–100 μM after complete distribution assuming a mean body weight of 275 g. Throughout the entire phase of the experiments including the period immediately following the bolus injection no significant alterations of mean blood pressure or respiratory parameters occurred (see Table 1) (Li and Trush, 1993).

2.2. *MRI studies*

All MR images displayed a progressively increasing lesion over time in all experimental groups whereas sham animals showed no ischemic signs in all sequences (Fig. 2A). The combination of DPI/DMSO reduced lesion size, measured as hemispheric lesion ratio (HLR) (Nagel et al., 2004), at all points in time compared to the control group ($p<0.05$). After 24 h of reperfusion DMSO alone significantly reduced the HLR in all sequences as well as after 48 h in pcT1WI ($p<0.05$). A significant additive neuroprotective effect of the combination of DPI/DMSO over DMSO alone could only be observed at 4 h in DWI and T2WI ($p<0.05$). Beyond 4 h the difference was not significant any more in these sequences; however the lesion size of BBB breakdown measured in postcontrast T1WI at 48 h was significantly reduced again by DPI/DMSO compared to DMSO ($p<0.05$). The HLR of all groups can be beheld in Fig. 2B as bar graphs and in Table 2 as numbers.

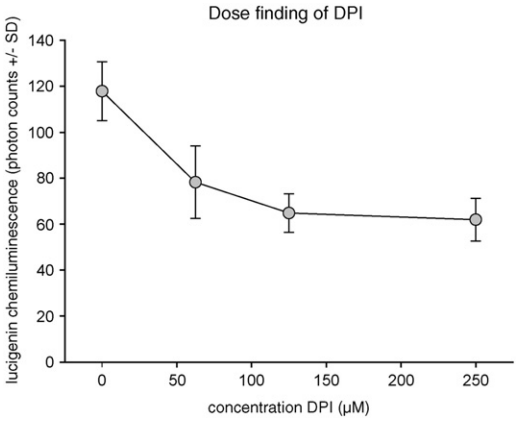

Fig. 1 – Inhibition of fMLP-triggered superoxide generation in isolated blood leucocytes by DPI. Freshly isolated rat blood leucocytes were stimulated with 100 nM of the chemotactic peptide fMLP. The maximal superoxide generation during the first 30 min was determined following 10 min of preincubation with different concentrations of DPI. Each data point represents the mean ± SD of 6 independent measurements.