Acta Neurochir (2003) 145: 673–677
DOI 10.1007/s00701-003-0052-z

Acta Neurochirurgica
Printed in Austria

Experimental Research

The attenuation of vasospasm by using a SOD mimetic after experimental subarachnoidal haemorrhage in rats

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Published online July 25, 2003

Summary

Background. Delayed cerebral vasoconstriction and brain ischemia, are critical problems in the management of a patient affected by rupture of an intracranial aneurysm. Overexpression of Cu–Zn superoxide dismutase (Cu–Zn SOD) can reduce the extent of cerebral vasospasm. We, therefore investigated if vasospasm, can be prevented by a novel, stable, and cell permeable SOD mimetic, MnTBAP [Mn(III) tetrakis (4-benzoic acid) porphyrin] which permeates the biological membranes and scavenges superoxide anions and peroxynitrite.

Methods. 28 rats (225–250 g) were divided equally into four groups: group 1: control; group 2: SAH only; group 3: SAH plus placebo; and group 4: SAH plus MnTBAP. We used a double haemorrhage method to produce SAH. Starting six hours after SAH, 5 mg/kg MnTBAP (Calbiochem, Darmstadt-Germany; Cat. No 475870)) or an equal volume of 0.9% saline (37 °C) was administered by intraperitoneal injection twice daily for 5 days to groups 4 and 3 respectively. MnTBAP or 0.9% saline injections were continued up to fifth day after SAH and rats were sacrificied on the fifth day. Brain sections at the level of the pons were examined by light microscopy. Planimetric measurements were made for the cross-sectional areas of the lumen and the vessel wall (intima plus media) of the basilar artery by a micrometer.

Finding. Administration of MnTBAP significantly attenuated the vasoconstriction of the basilar artery in group 4 compared with the groups 2 and 3 ($p < 0.001$).

Interpretation. These results suggest that this SOD mimetic (MnTBAP) attenuates delayed cerebral vasoconstriction following experimental SAH and that superoxide anions have a role in the pathogenesis of vasospasm after SAH.

Keywords: Rat; subarachnoid haemorrhage; vasospasm; SOD mimetic.

Introduction

Cerebral vasospasm remains a devastating medical complication of aneurysmal SAH. It is associated with high morbidity and mortality rates, even after the aneurysm has been secured surgically or radiologically. A great deal of experimental and clinical research has been conducted in an effort to find ways to prevent this complication.

Cerebral vasospasm after SAH results from an altering in the balance of vasoconstricting and vasodilating influences to the arterial endothelium in favor of vasoconstriction [11, 13, 22, 35]. Amongs the mechanisms responsible may be scavenging of NO by O_2^- and activation of vasocontricting substances by peroxynitrite and other oxidants by SAH [16, 20, 21, 23, 24, 30, 32, 36].

There is an excessive production of reactive oxygen and nitrogen species (ROS and RNS), especially O_2^- and NO, after SAH [3, 9, 26, 29, 34], and O_2^- rapidly reacts with NO under oxidative stress. Interactions between NO and O_2^- can produce cytotoxic molecules such as peroxynitrite and hydroxyl radicals [1, 5, 6, 9, 10, 12, 15]. Although endogenous intracellular enzymatic antioxidants such as SOD prevent the interaction between NO and O_2^-, these mechanisms are overwhelmed in SAH as a result of excessive production of O_2^-, NO, and peroxynitrite [1, 6, 12]. The reaction between NO and O_2^-, leads to reduction in NO levels and its bioactivity so that vasoconstricting agents are activated [2, 3, 16, 17, 30, 35–37]. Subsequently, endothelium-dependent relaxation is impaired and vasospasm occurs.

A study in mice, showed that overexpression of Cu–Zn SOD is a protection against vasospasm following

SAH [15]. Nevertheless neither intracisternal nor intrathecal-administration of SOD ameliorated vasospasm [8, 18]. This failure may reflect the inability of SOD to cross biological membranes [4]. Likewise systemically administrated SOD is unable to cross the blood-brain barrier [27]. Because of the problems of delivery of SOD, we have used a novel, stable and cell permeable SOD mimetic, MnTBAP [7, 28] which permeates biological membranes and scavenges superoxide anions and peroxynitrite. We report findings of an experimental model of SAH in the rat.

Methods and material

Experiments were performed on 15 week-old 28 male Wistar rats ranging in weight from 225 to 250 g (a mean weight of 235 g) obtained from Inonu University Animal Research Laboratory. Rats were divided into four groups: group 1: control (no SAH); group 2: SAH only; group 3: SAH plus intraperitoneal saline (0.9% NaCl), and group 4: SAH plus intraperitoneal SOD mimetic.

Experimental model of SAH and study protocol

One day before surgery, rats were fasted and pretreated with an antibiotic, enrofloxacin (Baytril, 2.27 mg/kg sc; Bayer). The rats in groups 2, 3, and 4 were anesthetized with intraperitoneal ketamine (60 mg/kg) and xylazine (6 mg/kg) and placed on a heated surgical table to maintain the body temperature of the animal at 37 °C. Anesthesia was maintained by repeated injections of ketamine as needed. Under sterile conditions and using a surgical microscope, a midline skin incision was made from middle of the calvarium to the lower cervical spine. The occipital bone was cleared of muscular attachments by sharp dissection. The atlanto-occipital membrane was dissected under a surgical microscope and a 27-gauge needle was inserted through the dura and the arachnoid membrane into the cisterna magna. A blood sample (0.3 ml) was drawn from the tail vein into a heparinized syringe and then injected into the cisterna magna over a 10-minute period. The needle was then withdrawn, the dural opening was plugged with an absorbable sponge, and the wound was sutured. The rats were given 5 ml of normal saline (37 °C) subcutaneously to prevent dehydration before recovery from anesthesia.

Throughout period of observation, the rats were allowed access to food and water ad libitum.

The SOD mimetic MnTBAP (5 mg/kg in 1 ml saline) or placebo (1 ml) was administered by intraperitoneal injection twice daily for 5 days to groups 4 and 3, respectively, starting 6 hours after SAH.

The rats in groups 2, 3, and 4 were re-anesthetized at 48 hours after the initial intracisternal blood injection and then 0.3 ml blood drawn from tail vein was re-injected into the cisterna magna. Intraperitoneal SOD mimetic, MnTBAP or saline injections (37 °C) were continued up to fifth day after SAH. Control rats were sacrificed as described below for determination of the baseline of the basilar artery diameter.

Sample collection and sacrifice of rats

On the fifth day after first application of blood or saline, the animals were re-anesthetized as described above and the ascending aorta was cannulated retrogradely through a thoracotomy. The craniocervical circulation was perfused with 200 ml of heparinized iso-osmotic phosphate buffer saline (0.1 M, pH 7.4) at a physiological mean arterial pressure (80–90 mm Hg) via a peristaltic pump (May/PRS9508/991129-1). The perfusion was followed by 200 ml of 0.1 M phosphate buffer saline containing 4% paraformaldehyde at a physiological mean arterial pressure as above. the rats in the control group were sacrificied without procedure of SAH.

A block of tissue was taken by cutting the brain stem above and below the pons. The sample was subdivided into two segments at the level of middle pons and then these were embedded in liquid paraffin and sectioned at 6 μm thickness (three section in each block), mounted on glass slides, and stained with hematoxylin and eosin. Sectioned-slices were examined by light microscopy and photographed. Planimetric measurements were performed under light microscopy by a micrometer (Olympus BX 50) in order to measure the cross-sectional areas of the lumen and the vessel wall (intima plus media). Final data represented the mean of sections of two blocks from each animal.

Data analysis

Data were expressed as mean ± SEM. Statistical differences between the control and SAH only, and SAH plus intraperitoneal saline (0.9% NaCl), and SAH plus intraperitoneal SOD mimetic were compared by student-t test. A value of p of less than 0.05 was considered statistically significant.

Results

Three rats died in group 2 (SAH only), and two rats died in group 3 (SAH plus saline) following the loss of consciousness without focal neurological deficits between days 3 and 5 after SAH. In group 4 (SAH plus MnTBAP), none of the rats died after SAH. In none of the surviving rats, was any neurological deficit observed.

Representing photographs of the light microscopic appearances of group 2 (SAH only) and, group 3 (SAH plus saline) are shown in Figs. 2 and 3. In groups 1, 2, 3 and 4; the diameters of basilar artery lumens were found to be 290 ± 1.4, 77 ± 10.3, 78 ± 10.2, and 254 ± 1.6 micro meters (μm), respectively. There were marked narrowings in the lumens of basilar arteries in

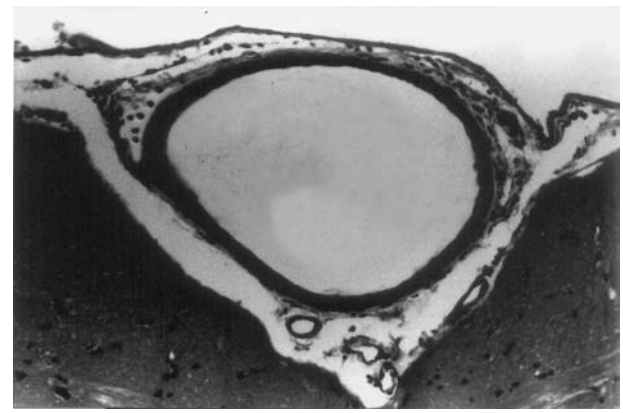

Fig. 1. Representing photograph of light microscopic appearance of a cross-sectional area of the basilar artery in control group (group 1) (H&E ×66)

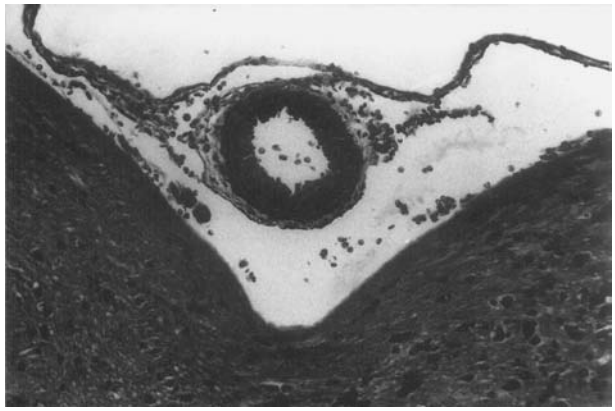

Fig. 2. Representing photograph of light microscopic appearance of a cross-sectional area of the basilar artery in SAH group (group 2) (H&E ×66). A significant degree of reduction in the luminal diameter and increase in wall thickness is seen

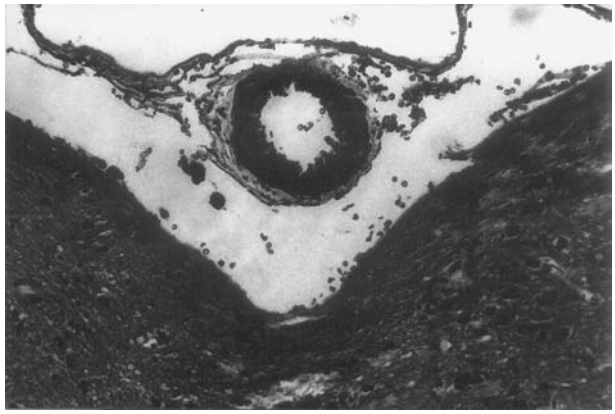

Fig. 3. Representing photograph of light microscopic appearance of a cross-sectional area of the basilar artery in SAH plus placebo group (group 3) (H&E ×66). A significant degree of reduction in the luminal diameter and increase in wall thickness is seen

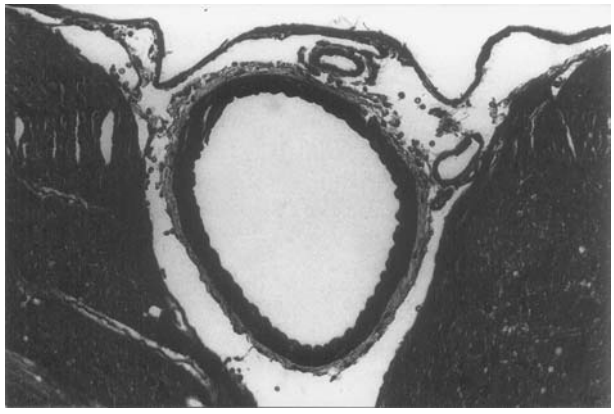

Fig. 4. Representing photograph of light microscopic appearance of a cross-sectional area of the basilar artery in SAH plus MnTBAP group (group 4) (H&E ×66). A minimal degree of reduction in the luminal diameter and increase in wall thickness is seen

Table 1. *The comparison of internal diameters and wall thicknesses of the basilar arteries of group 1. and group 2*

Group	The diameter of basilar artery lumen (μm)	Basilar artery wall thickness (μm)
Group 1 (control)	290 ± 1.4	25.5 ± 1.1
Group 2 (only SAH)	77 ± 10.3	51.3 ± 1.2
P*	$p < 0.001$	$p < 0.001$

* Student-t test.

Table 2. *The comparison of internal diameters and wall thicknesses of the basilar arteries of group 3 and group 4*

Group	The diameter of basilar artery lumen (μm)	Basilar artery wall thickness (μm)
Group 3 (SAH + saline)	78 ± 10.2	51 ± 1.3
Group 4 (SAH + MnTBAP)	254 ± 1.6	37.8 ± 1.2
p*	$p < 0.001$	$p < 0.001$

* Student-t test.

groups 2 (SAH only) compared to the control group (Table 1) ($p < 0.001$). The thicknesses of the basilar artery walls in groups 1, 2, 3 and 4 were 25.5 ± 1.1, 51.3 ± 1.2, 51.0 ± 1.3, and 37.8 ± 1.2μm, respectively. The thickness of the basilar artery walls increased in groups 2 (SAH only) compared to control group ($p < 0.001$).

Thickening of the arterial wall involved the tunica media and adventitia. No additional histological change was noted in arterial walls in these groups.

Thickening of the arterial wall and narrowing of the arterial lumen were also minimal in group 4 (SAH plus MnTBAP). the changes in the internal diameter and wall thickness of group 4 were statistically significant when compared to group 3 (SAH plus saline) (Table 2) ($p < 0.001$).

Discussion

Cerebral vasospasm after SAH has the features of a typical free radical-mediated disease [19]. It has been thought that the reactions between O_2^- and NO may contribute to vasospasm after SAH by reducing bioactivity of basal NO and leading to the production of free radicals [9, 10, 15].

It is generally considered that excess NO is generated by iNOS and superoxide anion is produced by autoxidation of oxyhemoglobin (oxyHb) to met-Hb and by the infiltrating inflammatory cells after the insult of SAH

[21, 25, 33]. Within the subarachnoid space, electron dense fine granules that indicate O_2^- anions preferentially located around erythrocytes and secondarily around the macrophages and neutrophils infiltrate. There is expression of iNOS by these infiltrated inflammatory cells, endothelial cells, vascular smooth-muscle cells, and adventitial cells [21, 34]. In addition; hemin, a breakdown product of hemoglobin, stimulates the expression of iNOS in vascular smooth muscle in the rat [29, 31]. O_2^- avidly binds NO so that the presence of O_2^- may contribute to vasospasm after SAH by reducing the bioactivity of basal NO and second messenger cGMP in vascular muscle. Interactions between NO and O_2^- can produce highly cytotoxic molecules such as peroxynitrite and hydroxyl radicals [8–10, 15]. As a protective mechanism, an antioxidant enzyme and O_2^- scavenger, SOD, can limit the reactions between O_2^- and NO but, this process is overwhelmed in SAH because NO concentrations increase above the tissue levels of SOD [1, 6, 12].

A study in transgenic mice, showed that overexpression of CuZn-superoxide dismutase ameliorates vasospasm [15]. Intracisternal or intrathecal-administering of SOD, does not ameliorate vasospasm after SAH [14]. This failure is probably because of the inability of SOD to cross biological membranes [4], and to cross the blood-brain barrier [27]. The problems of delivery of SOD may be avoided by the systemic administration of a novel, stable, and cell permeable SOD mimetic, MnTBAP [Mn(III) tetrakis (4-benzoic acid) porphyrin] [7, 28] which we used. This penetrates biological membranes and scavenges superoxide anions and peroxynitrite. However, it is not yet known if MnTBAP is able to the cross blood-brain barrier.

Our aim was to inhibit the reaction between O_2^- and NO, thus to prevent scavenging of NO by O_2^- and inhibit the cascade of free radical production, and subsequently, to prevent the development of vasospasm after SAH using a systemically administered SOD mimetic.

In our study, experimental SAH elicited vasospasm in all animals of group 2 (SAH only) and group 3 (SAH plus saline). The narrowing in basilar artery lumen was 223% more in group 2 (SAH only) than in group 1 (control) ($p < 0.001$) and 195% more in group 3 (SAH plus saline) than in group 4 (SAH plus MnTBAP) ($p < 0.001$). In addition, the thickening in the basilar artery wall was found to be 200% more in group 2 than the control group ($p < 0.001$) and 134% more in group 3 than group 4 (SAH plus MnTBAP) ($p < 0.001$). In animals of group 4 which were treated with MnTBAP, narrowing in arterial lumen and thickening in arterial wall were markedly attenuated as compared to groups 2 and 3. Although the changes of internal diameter and wall thickness of SAH plus MnTBAP group were not statistically significant compared to group 1 (control), they were statistically significant compared to group 3 (SAH plus saline), (Table 2) ($p < 0.001$).

In conclusion, our results suggest that this SOD mimetic (MnTBAP) can attenuate the delayed cerebral vasoconstriction following experimental SAH indicating the role of superoxide anions in the pathogenesis of vasospasm after SAH. We suggest that further studies are needed to clarify the issue.

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