Short cerebral ischemia and subsequent reperfusion and treatment with stobadine

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Abstract. Lipid peroxidation and activities of antioxidative enzymes were studied in the brain cortex after short (15 min) cerebral ischemia and reperfusion (10 min) in rats. Conjugated dienes (CD) and thiobarbituric acid-reactive substances (TBARS) were significantly elevated in the group of rats with ischemia followed by reperfusion in comparison to the ischemic animals. Superoxide dismutase (SOD) activity significantly increased in the group of animals with ischemia and reperfusion. No significant changes in the activities of glutathione peroxidase (GP) were observed. Stobadine administered before ischemia or before reperfusion decreased the level of TBARS. Stobadine probably prevents malondialdehyde (MDA) formation from hydroperoxide or might elevate the activity of aldehyde dehydrogenase. In contradiction to the findings after long-lasting (4 h) ischemia and subsequent reperfusion [1], no decrease in the concentration of CD or in the activity of SOD or GP was found.
Key words. Cerebral reperfusion injury; oxygen radicals; lipid peroxidation; antioxidative enzymes; stobadine.

Long-lasting (4 h) ischemia, induced by ligation of both common carotid arteries, followed by reperfusion, caused intensive oxygen radical generation and subsequent lipid peroxidation [2]. Stobadine (ST), a novel drug of a pyridoindole structure [3], was said to exhibit a protective effect against brain injury after 4-h ischemia followed by reperfusion [1]. A key antioxidant activity of stobadine may be exerted by 1) trapping lipid radicals, as the production of conjugated dienes in liposomes exposed to air and elevated temperature was inhibited [4], 2) trapping $^{\cdot}OH$ radicals, measured by ESR method [5], and 3) quenching singlet oxygen $^1O_2$ [6].

The relatively inert superoxide radical $^{\cdot}O_2^-$ is generated in the early stages of reperfusion and is a precursor of other reactive oxygen species [7]. ST proved not to be a superoxide radical scavenger, yet was suggested to prevent superoxide radical generation [1]. ST, now in the third phase of clinical testing, is a prospective drug for its cardioprotective and antihypoxic properties. From the point of clinical practice (stobadine could be used as an agent for high risk geriatric patients) as well as with regard to a better understanding of the mechanism of ST's protective action in reoxygenation injury, it was necessary to establish whether stobadine was also effective when administered before ischemia. Pharmacokinetic studies showed that 4 h after i.v. injection only a minimal level of ST was found in the brain [8]. Thus we tried to use shorter ischemia (15 min) instead of 4-h ischemia in this study. Moderate brain hypoxia [9] or short term hypoxia of hepatocytes need not always be accompanied by oxygen radical formation during reoxygenation [10]. Thus we first tested whether our model of short ischemia was sufficient to cause cerebral injury induced by oxygen radicals.

Materials and methods

Male normotensive rats of inbred Wistar strain (400–450 g) were used. Brain ischemia and reperfusion were induced by occlusion of the common carotid arteries according to the method of Itoh et al. [11], but instead of 4 h, only 15-min ischemia was performed. One group of animals was killed immediately after 15-min ischemia; the second group was sacrificed after 10-min reperfusion. A control group was subjected to the surgical procedure without carotid artery occlusion. One experimental group was treated with 2 mg/kg of stobadine administered intravenously before occlusion of the carotid artery, the second group was treated with the same amount of stobadine immediately before reperfusion was started. The animals were killed 10 min after reperfusion had been started. Immediately after decapitation the brains were removed and the brain cortexes frozen in liquid nitrogen. The number of animals in the individual groups ranged from 5 to 15.

Conjugated dienes were determined in the total lipid phase of brain cortexes. Total lipids were isolated, and purified, and CD were determined according to Kogure et al. [12]. TBARS were determined according to Scherer and Deamer [13] as modified previously [2]. The concentrations of CD and TBARS were expressed per mg of protein homogenate. Protein was determined by the method of Lowry et al. [14].

Antioxidative enzymes were assayed according to Das et al. [15] in 65,000 × g supernatant fractions of brain cortex samples. Clear supernatant fluid was assayed for both enzyme activities. The activity of superoxide dismutase was determined by its inhibitory action on the superoxide-dependent reduction of ferricytochrome by xanthine-xanthine oxidase, according to McCord and Fridovich [16]. The enzyme unit was defined as the amount of SOD necessary for inhibition of the reaction rate of reduction cytochrome c by 50%.

The assay for glutathione peroxidase was based on the coupling of the enzyme to NADPH via glutathione reductase, and the rate of NADPH oxidation was measured spectrophotometrically at 340 nm according to Das et al. [15]. The reaction mixture in the total volume of 1 ml contained 0.25 mM reduced glutathione, 0.2 mM

cumene hydroperoxide, 0.12 mM NADPH, 1 unit of glutathione reductase (GR), 0.09 mM EDTA, 50 mM tris-HCl buffer, pH 7.6. Specific activity was expressed as nM NADPH oxidized per mg protein.

Results and discussion

Short cerebral ischemia of rats induced a significant decrease of TBARS concentration in comparison to the control sham-operated animals where no carotid ligation was performed (table 1). This decrease could be caused by limited oxygen access, as hypoxia usually decreases the formation of reactive oxygen species [10]. But no significant decrease was observed in the level of conjugated dienes. Thus the decrease of TBARS may also reflect MDA decomposition. In vivo, the tissue MDA levels probably reflect the balance between MDA formation and catabolism [17]. From the increased level of CD and TBARS (table 1) and the elevated activity of SOD after reperfusion in comparison with the group where only ischemia was performed (table 2), we can conclude that oxygen radicals are involved in this model of short cerebral ischemia and reperfusion. The activity of GP was not significantly influenced in our present experiments (table 2). Stobadine administered before ischemia or before reperfusion decreased the level of TBARS. But no effect of stobadine was observed on the concentration of CD, or activities of SOD or GP (tables 1 and 2). Not every reoxygenation injury is caused by oxygen radicals: cells can die from hypoxia, or can be destroyed upon reoxygenation presumably by an increase in cellular ATP content [10]. But successful use of antioxidant and free radical scavengers in the treatment of brain ischemia provides indirect evidence for the contribution of oxygen radicals and lipid peroxidation in brain injury under these conditions [11, 18–21]. Korthuis and Granger [22] summarized the evidence on the involvement of oxygen radicals in ischemia-reperfusion injury in many organs, including the brain. Infusion of an oxygen radical-generating system into the brain was found to be associated with changes in permeability of the blood-brain barrier, cellular injury and edema [22]. In our last paper on brain ischemia (4 h) and reperfusion, as well as lipid peroxidation we also studied the survival and behavior (seizures) of animals. In these experiments, both the main lipid antioxidant, vitamin E, and stobadine significantly decreased lipid peroxidation and simultaneously significantly improved the survival of animals [1]. Moreover, no seizures were observed in rats treated with vitamin E or stobadine. All these results indicate that oxygen radicals can contribute to the brain damage caused by ischemia-reperfusion.

Table 1. Lipid peroxidation in cerebral cortex in ischemic, ischemic-reperfused, and stobadine-treated animals

	Control (Pre-ischemia)	Ischemia (15 min)	Reperfusion (10 min) following ischemia	ST admin. before ischemia	ST administered before reperfusion
CD (nmol/mg protein)	29.5 ± 2.22	28.56 ± 1.93	35.77 ± 1.41	36.06 ± 1.23	33.56 ± 1.18
		$p < 0.001$	$p < 0.001$		
TBARS (nmol/mg protein)	0.543 ± 0.033	0.488 ± 0.023	0.667 ± 0.035	0.538 ± 0.04	0.516 ± 0.05
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	

All values are the mean ± SD. The number of samples ranged between 10 and 15.

Table 2. Antioxidative enzymes in cerebral cortex in ischemic, ischemic-reperfused, and stobadine-treated animals

	Control (pre-ischemia)	Ischemia (15 min)	Reperfusion (10 min) following ischemia	ST admin. before ischemia	ST administered before reperfusion
SOD (U/mg prot.)	9.83 ± 2.42	9.27 ± 1.20	12.08 ± 2.21	11.2 ± 0.66	12.15 ± 1.01
		$p < 0.05$			
GP (U/mg prot.)	33.7 ± 3.1	31.3 ± 1.8	29.1 ± 2.8	27.5 ± 1.4	30.5 ± 0.7

All values are the mean ± SD. The number of samples ranged between 5 and 8.

The decreased level of TBARS in short ischemia followed by reperfusion and treatment with stobadine when levels of conjugated dienes were unchanged, can be explained by several possible mechanisms: 1) stobadine prevents MDA formation from hydroperoxides, 2) stobadine might inhibit cyclooxygenase activity, since MDA is formed not only by lipid peroxidation but also enzymatically as a product of the cyclooxygenase reaction in prostaglandin metabolism [23], 3) ST might elevate the activity of aldehyde dehydrogenase which rapidly oxidizes MDA in mitochondria. It was indicated that ST did not inhibit significantly the formation of MDA by cyclooxygenase reaction from prostaglandin metabolism (G. Petrikova, unpublished results, 1990). We can conclude that short-term ischemia (15 min) followed by reperfusion is sufficient for the generation of oxygen radicals and development of lipid peroxidation. ST in reperfusion following short brain ischemia does not prevent oxygen radical formation and has no effect on the early stages of lipid peroxidation. ST decreased only the level of TBARS, probably by supporting MDA decomposition in mitochondria or by preventing MDA formation from hydroperoxide. It is known that MDA, which is one of the end products of lipid peroxidation, is extremely reactive and dangerous to living organisms. It is able to react with amino groups of proteins or phospholipids and with nitrogen bases of nucleic acids as well as with SH groups [24–27]. In the light of these interactions, MDA, as a product of lipid peroxidation, can be assumed to be able to cause brain injury and thus stobadine, by decreasing the level of MDA, is able to reduce the brain damage caused by ischemia-reperfusion.

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