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Spermine protects *in vivo* the antioxidant enzymes in transiently hypoperfused rat brain

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Abstract. The antioxidant enzymatic system in brain hypoperfusion/reperfusion model in rats after spermine administration was evaluated. Incomplete cerebral ischemia/reperfusion induced by temporal occlusion of common carotid arteries caused a decrease in the activities of superoxide dismutase and glutathione reductase as well as total and free sulfhydryl groups, while thiobarbituric acid-reactive substances became elevated. Administration of spermine after the reperfusion led to restoring all above parameters to normal values. Protective effect of spermine in transiently hypoperfused and subsequently reperfused rat brain is briefly discussed.

Key words: spermine, hypoperfusion/reperfusion, brain, lipid peroxidation, sulfhydryl groups

INTRODUCTION

The classical role of the polyamines in cell growth proliferation (Tadolini et al. 1988) has been extended to brain development (Slotkin and Bartholome 1986) and metabolism (Seiler and Bolkenius 1985), and lately to a variety of regulatory functions in the central nervous system (Williams et al. 1991, Scott et al. 1993). At present, it has been suggested that spermine can influence an endogenous neuroprotective mechanism by limiting excessive calcium entry (DiScenna et al. 1994). Lovaas and Cartin (1991) and Matkovics et al. (1993) found that spermine *in vitro* possesses antioxidant and antiinflammatory properties, such as suppressing local vascular permeability and inhibiting the release of inflammatory mediators (Theoharides 1980). A more recent study in adult gerbils demonstrated that spermine treatment can protect forebrain neurones from degeneration after ischemia (Gilad and Gilad 1991a,b). The effect of spermine *in vivo* on some antioxidant enzyme activities in transiently hypoperfused and subsequently reperfused rat brain has not been studied. It is well known that ischemia and reperfusion are linked to the generation of free radicals which place the cells under oxidative stress with consequences outside the site of the stress. Free radicals contribute to various disorders including trauma and various neurodegenerative diseases (Halliwell 1992).

Free radicals are formed during the normal life of a cell. They result from the single-electron reduction of molecular oxygen. These chemical species have unpaired electrons and so they are highly reactive and very short lived (10 s) (Halliwell et al. 1992). Oxygenated free radicals can react with polyunsaturated fatty acids in membrane phospholipids to form lipid peroxides by enzymatic or non-enzymatic routes (Emerit et al. 1991). Under normal conditions, the production of these peroxides is controlled by protective physiological systems such as cytosolic enzymes and other antioxidants. These systems of protection may often be insufficient, in particular during ischemia/reperfusion.

Hypoperfusion/reperfusion *via* carotid ligature is regarded as an acceptable experimental model to study the effect of various substances on biological targets under limited blood supply. Among others, this model may be useful for testing the efficacy of spermine in reducing disturbances at all levels, from the cell *via* the biomembrane up to the metabolic process involved.

The authors are aware that the above model of cerebral hypoperfusion/reperfusion in rats cannot be compared to acute, complete ischemia. In most vertebrates there exists a collateral circulation between vertebral and carotid arteries prior to their entry to the brain. Thus, this model induced by clamping of carotid arteries is mild and incomplete.

Our purpose was to assess the *in vivo* effects of spermine on the antioxidant enzymes: superoxide dismutase (SOD), glutathione reductase (GSH-R), catalase (CAT) and on free and total sulfhydryl groups (SH) in the hypoperfusion/reperfusion-induced brain damage in rats. The concentration of thiobarbituric acid - reactive substances (TBA-rs) as an indicator of lipid peroxidative processes in this tissue was also evaluated.

METHODS

Spermine used in these studies was produced by Sigma (USA); thiobarbituric acid (TBA) was obtained from International Enzymes (Windsor UK); 5,5'-dithiobis-2-nitro-benzoic acid (DTNB), glutathione reductase (GSH-R) and 1,1,3,3-tetraethoxypropane, from Sigma (St. Louis, USA); nicotinamide-adenine dinucleotidephosphate reduced sodium salt (NADPH), from Sigma Chemie GmbH (Germany); superoxide dismutase (SOD) from bovine erythrocyte, from Fluka Chemie AG, (Busch, Switzerland); and glutathione, reduced and oxidized, from Mann Research Laboratories (New York, USA). All other chemicals were from Polfa Chemicals (Gliwice, Poland) and were of the highest quality available. De-ionized water was used throughout.

Male Wistar rats (body weight 180-220 g) were used for the experiment. All rats were anaesthetized

with pentobarbital (i.p., 50 mg/kg body weight), intubated and placed in a supine position. The rats were maintened at approx 37°C under a light bulb. A small median incision was made in the neck and both carotid arteries were separated from vagal nerves, then exposed bilaterally and occluded by applying a atraumatic microclip for 30 min (group 1). Subsequently, the rats were killed by decapitation. In the reperfusion group, the clips were removed after ischemia and reperfusion was allowed to take place for 60 min, and 15 min later the animals were killed (group 2). The third group of animals was treated with 5 mg/kg i.v. of spermine directly after ligature and after 60 min reperfusion. Fifteen minutes later the rats were killed. The vehicle group animals was treated like those of group 3, using the same volume of the vehicle (physiological saline) 15 min later the animals were killed (group 4). Each group consisted of 12 rats.

Following decapitation, the brain was removed and washed in cooled 0.15 M NaCl, kept on ice and subsequently blotted on filter paper, then weighed and homogenized for 2 min using a glass-teflon homogenizer in 9 volumes of cold 0.25 M sucrose. Homogenization procedures were performed as quickly as possible under completely standardized conditions. The homogenates were centrifuged at 6,000 g for 10 min at 4°C and the supernatant was kept on ice until assayed. Protein was determined in diluted aliquots of the tissue homogenates by the method of Lowry et al. (1951).

Cu,Zn-SOD activity was measured after re-homogenization of the initial supernatant and after centrifugation at 30,000 g for 30 min at 5°C as described by Sykes at al. (1978). This method is known to destroy the manganese enzyme of the mitochondria. One unit of SOD activity was defined as the amount of the enzyme required to inhibit the oxidation of epinephrine to adrenochrome by 50% (during 1 min/mg of brain tissue protein).

CAT activity was measured in the initial supernatant after Triton X 100, a 30 min preincubation and centrifugation at 9,000 g for 30 min at 4°C. The activity was assayed as described by Aebi (1984). Rates were determined at 25°C using 10 mM hydrogen peroxide and the activity was expressed as μmole of H_2O_2 decomposed/min/mg of protein.

GSH-R activity was determined using the method of Mize and Langdon (1962) by monitoring the oxidation of NADPH at 340 nm. The reaction mixture contained 0.2 M KCl, 1 mM EDTA and 1 mM oxidized GSH (GSSG) in 0.1 M potassium phosphate buffer, pH 7.1. The reaction was initiated by the addition of NADPH to a final concentration of 0.1 mM. One unit of GSH-R oxidized 1 nmol of NADPH per min at 25°C.

SH groups were estimated according to Ellman (1959) using DTNB in whole and deproteinized brain homogenates. KCl (0.15M) was used to homogenize brain slices for the determination of SH groups and of TBA-rs.

TBA-rs content in the brain was measured using the TBA technique of Buge and Aust (1978).

Results of the experiments were expressed as means ± SD and analysed by one way analysis of variance comparing individual group. Differences with $P<0.05$ were regarded as significant.

RESULTS

Following 30 min occlusion of both carotid arteries the activity of SOD (Table I) was significantly decreased ($P<0.05$), while the activities of CAT and GSH-R were unchanged (group 1). During hypoperfusion/reperfusion lasting 60 min (group 2), the activities of SOD and GSH-R were depressed further. The activity of CAT remained at the level of the control group. After administration of spermine (group 3) the activities of SOD and GSH-R were significantly elevated and approximated those of the control group; no significant changes in CAT activity were observed.

During brain hypoperfusion the animals showed elevated values of brain TBA-rs (Table II) and decreased non-protein and total SH-groups ($P<0.05$). During subsequent reperfusion, the concentration of TBA-rs was still significantly elevated compared with the control and with the hypoperfused group. SH-groups were more decreased compared with group 1. Infusion of spermine (group 3) caused a

TABLE I

The effect of spermine on the activities of SOD, GSH-R and CAT in the whole brain tissue during hypoperfusion/reperfusion in rat

Group	SOD U/mg of protein	GSH-R IU	CAT μmol H_2O_2 /min/ mg of protein
1. Hypoperfusion	82.9±6.9	37.0±6.9	2.06±0.6
2. Hypoperfusion/reperfusion	65.1±10.3	35.5±9.1	1.79±0.5
3. Hypoperfusion/reperfusion + spermine	97.8±8.6	42.7±7.1	1.90±0.5
4. Control	98.9±6.7	42.3±9.2	1.90±0.4

Notes: Values are means ± SD. Statistically significant differences ($P<0.05$) are found between the group: SOD 1-4, 2-3, 2-4; GSH-R: 2-4, 2-3; CAT: lack of statistically significant diferences.

significant increase by 32.8% in the non-protein SH-groups in the brain in comparison with group 2. Administration of spermine led to the normalization of total and free SH-groups and of TBA-rs in the brain as well.

DISCUSSION

The activity of some enzymatic proteins and their substrates in the cells of the brain (i.e. neurones, glia, astrocytes), can be selectively modulated in some pathophysiological situations. Ischemia is a state of energy disturbances, during which the protein biosynthesis is impaired (Paschen et al. 1991). However, it seems that the reduction of some antioxidant enzyme activities such as SOD and GSH-R, observed in our studies, could rather point to their posttranslational modifications or to the impairment of their interaction with endogenous activators. It has been shown previously that cerebral ischemia leads to enhanced production of partially reduced oxygen, notably hydrogen peroxide, that can partially modify/inactivate Cu,Zn-SOD in selectively vulnerable zones (Viglino 1988).

In the model of incomplete mild cerebral ischemia induced by bilateral carotid ligature and after reperfusion, favourable action of spermine has been shown. Infusion of spermine during hypoperfu-

TABLE II

The effect of spermine on TBA-rs and non-protein and total SH-groups in the whole brain tissue during hypoperfusion/reperfusion in rat

Group	TBA-rs nmol/mg of protein	SH-compounds mmol/mg of protein	
		non-protein	total
1. Hypoperfusion	3.1±0.4	33.1±10.0	136.3±41
2. Hypoperfusion/reperfusion	3.2±0.5	26.3±5.8	125.1±24
3. Hypoperfusion/reperfusion + spermine	2.1±0.5	37.4±9.1	150.0±29
4. Control	2.0±0.4	38.2±8.3	157.9±23

Notes: Values are means ± SD. Statistically significant differences ($P<0.05$) are found between the group: BA-rs: 1-4, 2-3, 2-4; SH-total: 2-3, 2-4; SH-non-protein: 2-3, 2-4.

sion/reperfusion resulted in restoration of the endogenous antioxidant enzymatic defense system to normal values.

Our studies have demonstrated that spermine affects the antioxidative status *in vivo* similarly as *in vitro* (Lovaas 1991, Lovaas and Carlin 1991); however, its effect *in vivo* is more complex. It seems that spermine may inhibit generation and secretion of free radicals by suppressing the respiratory burst of neutrophiles (Ferrante et al. 1986). Furthermore, as demonstrated by Lenzen et al. (1986), spermine is a physiologically important activator of the mitochondrial Ca^{2+} uniporter in the presence of physiological Mg^{2+} concentration. By this mechanism, spermine can confer to the mitochondria an important role in the regulation of free cytoplasmic Ca^{2+} concentration in the brain cell and of free Ca^{2+} concentration in the mitochondrial matrix.

It has been previously noted that after prolonged recirculation after forebrain ischemia the levels of spermine and spermidine in severely damaged regions were significantly reduced (Paschen et al. 1987a,b). From the theoretical point of view high concentrations of polyamines could attenuate neurotoxic effects of excitatory amino acids by reducing activation of N-methyl-D-aspartate (NMDA) receptors in neuronal tissue . Therefore, in our experiments we used i.v. spermine after the hypoperfusion episode and after 60 min of reperfusion. In pilot studies we have found that the application of this compound at the start of reperfusion did not affect the antioxidant SOD and GSH-R activities and the lipid peroxidation processes. At physiological pH, spermine is fully protonated and has polycationic character, it is easily soluble in organic (lipophilic) as well as in aqueous media, thus making introduction into a wide variety of environments feasible. Consequently, spermine appears more efficacious than other substances because of its ability to cross the blood - brain barrier. Moreover, active transport of spermine (polyamines) into the brain (Pateman and Shaw 1975, Gilad and Gilad 1991a,b) and within the nervous system (Halliday and Shaw 1978) has been previously demonstrated. Numerous data have indicated that spermine might influence membrane properties and functions by electrostatic interaction with anionic compounds of phospholipid head groups of membranes (Igarashi et al. 1982, Powell and Reidenberg 1982, Tabor and Tabor 1984, Tadolini et al. 1985). An important consequence of spermine binding to membranes is its protective effect against lipid peroxidation (Kitada et al. 1981, Tadolini et al. 1985) by forming a ternary complex with iron and phospholipid polar head and changing in this way the susceptibility of Fe^{2+} to autooxidation (Tadolini 1988). The involvement of iron in both initiation and propagation of lipid peroxidation is well established (Minotti and Aust 1989) and it has been suggested that metabolic changes induced by ischemia may lead to intracellular iron delocalization, thus promoting lipid peroxidation (Halliwell and Gutterridge 1986). Our studies have demonstrated that exogenously used spermine during reperfusion after mild, compensated ischemia inhibits lipid peroxidation and dimishes the content of TBA-rs in rat brain. Moreover, it seems that spermine may be involved in protecting non-protein SH groups from their oxidation by free radicals and that it contributes, in this way, to the repair processes.

In conclusion, the present findings demonstrate an impressive and astounding efficacy of exogenously used spermine in the prevention of postischemic oxidative stress induced by bilateral transient carotid occlusion in rats.

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