Endothelial Protective Effect of Stobadine on Ischaemia/Reperfusion-Induced Injury

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Abstract. The aim of the present study was to evaluate the influence of the antioxidant stobadine on changes in the reactivity of the rat abdominal aorta induced by ischaemia and reperfusion (I/R). In anaesthetized male rats, *in vivo* ischaemia was elicited by occlusion of the abdominal aorta for 18 hours; reperfusion lasted 30 minutes. The aortal rings were taken from the reperfused portion. Decreased relaxant response to acetylcholine, as a consequence of endothelial injury, was seen after I/R. We also demonstrated I/R-induced reversible ultrastructural changes both in endothelial and smooth muscle cells, predominantly in the mitochondria. Lipid peroxidation was increased in homogenates of I/R aortae; the concentration of thiobarbituric acid reactive substances (TBARS) increased from a control value of 0.97 ± 0.03 to 2.57 ± 0.06 nmol/l/mg protein. Stobadine (2 mg/kg i.v., 5 minutes before starting reperfusion) protected the abdominal aorta against I/R-induced decrease of acetylcholine relaxation, and prevented changes in mitochondria and an increase of TBARS concentration. The protective effect of stobadine seems to be due to its antioxidant properties.

Key words: Stobadine — Ischaemia/reperfusion — Rat abdominal aorta

Introduction

The endothelium of blood vessels is a prominent target in ischaemia-reperfusion (I/R) injury. Loss of its normal responsiveness is an important indicator of vascular injury. Endothelium-dependent relaxation has been found to be disturbed after ischaemia followed by reperfusion. Changes in vascular reactivity depend on the

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duration of ischaemia and reperfusion as well as on the vessel type (Quillen et al 1990, Tao et al 1990, Summers et al 1993)

The role of oxygen free radicals as causal factors in reperfusion damage has been supported by beneficial effects of radical scavengers or antioxidants on different organs exposed to I/R (Wei et al 1981, Korthuis et al 1985) Stobadine, dihydrochloride of cis (-)-2,3,4,4a,5,9b-hexahydro-2,8-dimethyl-1H-pyrido 4,3 indole, is a scavenger of $OH^{\cdot}$ (Staško et al 1990), an inhibitor of the propagation of lipid peroxidation (Mišík et al 1991), and a quencher of $^1O_2$ (Steenken et al 1992) Stobadine, synthesized in the search for new antiarrhythmic drugs, was found to markedly delay the onset of epinephrine-induced heart rate disturbances and to shorten extrasystolic episodes in guinea-pigs and dogs It prolonged the functional refractory period in the rabbit and guinea-pig atrium and in the cat ventricle (Gibala et al 1985, Štolc et al 1988) Besides its antiarrhythmic properties, stobadine exhibited cardioprotective effects (Styk et al 1986) It was also observed to have potent alpha-adrenolytic (Sotníkova et al 1985), antihistaminic (Lukovič and Machová 1988), local anaesthetic and neuroprotective effects (Štolc et al 1997) Stobadine was found to depress the formation of malondialdehyde and conjugated diens in the rat brain in oxidative stress (Štolc and Horakova 1988)

The aim of this study was (a) to examine the influence of *in vivo* I/R on *in vitro* reactivity of the rat abdominal aorta, (b) to test the effect of the antioxidant stobadine on changes induced by I/R, and (c) to provide insight into the mechanisms involved in its potential protective activity

Materials and Methods

Ischaemia and reperfusion (I/R)

Experiments were performed on male Wistar rats (Dobrá Voda, Slovakia) weighing 270–300 g The animals were anaesthetized with pentobarbital (50 mg/kg i p) Ischaemia was induced by occlusion of the abdominal aorta 5 mm below the origin of the left renal artery After closing the abdomen and attendance to the surgical cut, the animals were let to survive 18 hours After this period, they were anaesthesised again, and 30-minute reperfusion was performed Stobadine was administered in the dose of 2 mg/kg i v into the tail vein 5 minutes before or after starting reperfusion This dose was chosen on the basis of previous observations of its protective effect in brain ischaemia and reperfusion injury (Horáková et el 1991)

The animals were divided into 4 groups A – control rats (without any manipulation), B – sham-operated rats (all manipulations were performed except occlusion of the abdominal aorta), C – rats with ischaemia only, D – rats with I/R Each group had 2 additional subgroups, one with and one without stobadine administration

Isolated rat aorta

The aorta taken from the reperfused portion was excised and transferred into oxygenated physiological salt solution (PSS). The arteries were cleaned of adherent tissue and cut into 2 rings, each approx. 2 mm long. The rings were mounted between two L-shaped hooks in water-jacketed (37°C ± 0.5°) chambers containing PSS bubbled with a mixture of 95% 0_2 and 5% CO_2 at pH 7.4. The composition of PSS was (in mmol/l): NaCl (118.0), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25.0) and glucose (11.0). The preparations were connected to an isometric transducer (M 1101, Czech Republic) and stretched passively to optimal length by imposing an optimal initial tension of 15 mN, as tested in preliminary experiments. After the application of the initial tension, the arterial preparations were equilibrated for 60 minutes. Isometric contractions were recorded on a Kutesz 185 line-recorder (Hungary). The experimental protocol was as follows: The rings were contracted with depolarising PSS (in mmol/l): NaCl (17.0), KCl (100.0), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25.0) and glucose (11.0). After the contraction plateau had been reached acetylcholine was added in the concentration of 1 μmol/l. Subsequently, the preparations were washed twice with PSS, and contraction was induced by 1 μmol/l noradrenaline. After further washing with PSS and reaching the initial tension value, the rings were contracted with depolarising PSS again, and at the plateau of contraction the response to the endothelium-independent relaxant sodium nitroprusside (10 nmol/l) was recorded.

Transmission electron microscopy

The abdominal aorta from 3 groups of animals – sham, I/R and I/R treated with stobadine 5 minutes before starting reperfusion – was prepared in the following manner: the aorta was carefuly excised from the abdomen and arrested in ice-cold buffer consisting of 8% sucrose and 100 mmol/l sodium cacodylate, pH 7.4. The aorta was cut into smaller rings which were immersion-fixed in a fixation solution consisting of 2.5% glutaraldehyde in 100 mmol/l cacodylate buffer, pH 7.4 at 4°C for 3 h. The rings were then rinsed in 100 mmol/l cacodylate buffer with 250 mmol/l sucrose and postfixed in 40 mmol/l OsO_4 in 100 mmol/l cacodylate buffer at 4°C for 1 h. After dehydration in graded series of alcohol and infiltration in propylene oxide, they were embedded in Epon 812. Toluidine blue-stained (1 μm thick) sections were examined and appropriate areas were selected for cutting of thin sections. Subsequently, these were stained with uranyl acetate and lead citrate, and examined in a Tesla BS 500 electron microscope.

Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined in rings of the abdominal aorta taken from sham-operated animals, animals exposed to I/R with and without administration of stobadine 5 minutes before reperfusion. The samples of the abdominal aorta were cleaned of

adherent tissue in an ice cold PSS within 3 minutes and then frozen in liquid nitrogen.

The frozen rings were quickly weighed on a Mettler microbalance UM 3 (Switzerland), and after cutting they were homogenised in 2 ml of physiological solution containing 5 μl butylated hydroxytoluene (BHT), previously bubbled with gas nitrogen. TBARS were determined in 250 μl of homogenate. Sodium dodecyl sulphate (SDS) (100 μl) was added to the homogenate and samples were shaken for 5 s. Then, 800 μl 0.1 mol/l HCl were added and shaken again for 5 s. After centrifugation at 3000 rpm for 10 min, 400 μl 0.5% TBA were added to the supernatant. After incubation of the samples at 90°C for 20 min and their cooling with tap water, the intensity of colouration was measured as absorbance at 530 nm, using a spectrophotometer (Spekord, Germany) Proteins were estimated by Lowry's method (Lowry et al. 1951).

Expression of results and statistical analysis

Contractions and relaxations were expressed either in mN or as percentages of maximum contractile response to 100 mmol/l KCl.

All results were expressed as mean ± S.E.M. of n experiments. Analysis of variance was used for statistical analysis; $p < 0.05$ was considered significant.

Drugs

Acetylcholine, noradrenaline, sodium nitroprusside and TBA were purchased from Sigma (St. Louis, USA). BHT, SDS and all chemicals used in transmission electron microscopy were from Serva (Heidelberg, Germany).

Results

Functional studies

In preliminary experiments we found that responses of the thoracic aorta to all drugs tested were not influenced by any experimental procedure.

As shown in Figures 1*A*,*B*, preparations from control, sham-operated and ischaemic animals showed no statistically significant differences in the responses of the rat abdominal aorta to KCl (100 mmol/l), acetylcholine (1 μmol/l), and noradrenaline (1 μmol/l). None of the experimental procedures used influenced the response of the preparations to sodium nitroprusside (10 nmol/l) (not shown, number of experiments in every experimental group was 8). After 18-hour ischaemia and 30-minute reperfusion, however, acetylcholine was found to produce a smaller relaxation in comparison to controls. It reached only 5.59 ± 2.6% of the maximal relaxation. In 80% of preparations, acetylcholine did not induce any relaxation at all (Fig. 1*C*).

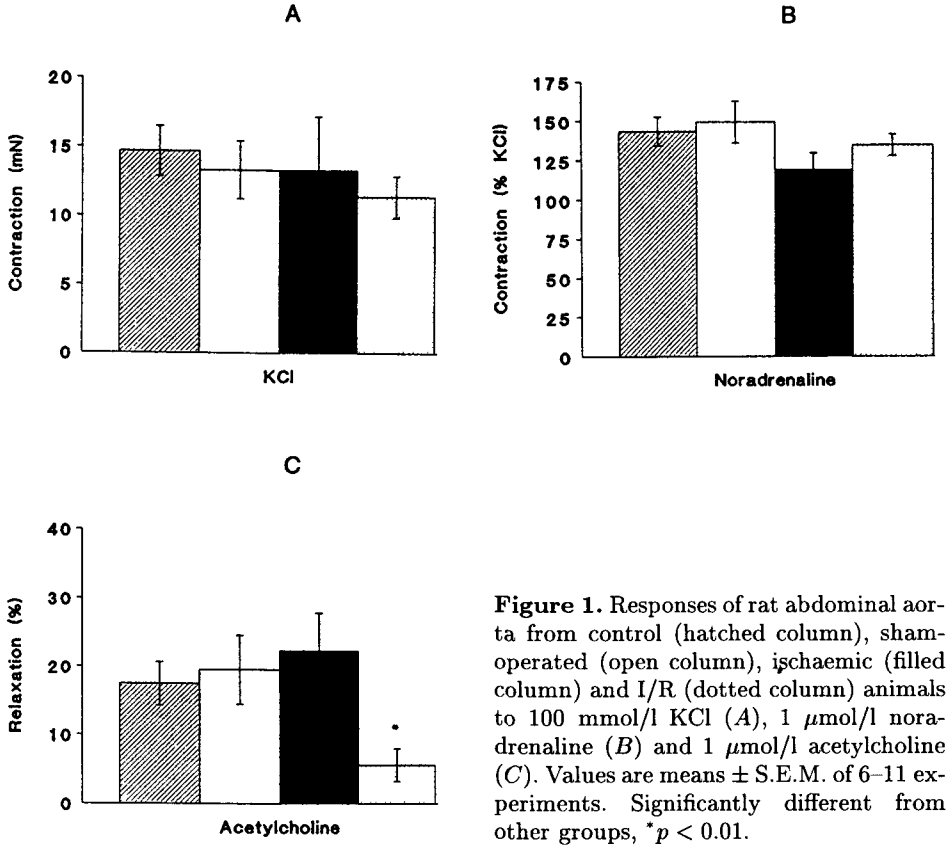

Figure 1. Responses of rat abdominal aorta from control (hatched column), sham-operated (open column), ischaemic (filled column) and I/R (dotted column) animals to 100 mmol/l KCl (*A*), 1 μmol/l noradrenaline (*B*) and 1 μmol/l acetylcholine (*C*). Values are means ± S.E.M. of 6–11 experiments. Significantly different from other groups, $^{*}p < 0.01$.

Stobadine, when administered 5 min after the onset of reperfusion, did not protect acetylcholine-induced relaxation against deleterious action of I/R. The responses to acetylcholine were not significantly different from those of the preparations taken from animals after I/R (n = 8). This experimental arrangement was therefore no longer used in further experiments.

The administration of stobadine (2 mg/kg i.v.) did not significantly change the responses of the preparations from sham-operated animals to acetylcholine (Fig. 2).

Stobadine administered 5 minutes before starting reperfusion protected the preparations against I/R-induced injury. The relaxation responses of rings to acetylcholine increased from 5.59±2.36% after ischaemia and reperfusion to 34.74±3.68% of maximal relaxation, the values exceeding those obtained for the sham samples (Fig. 2).

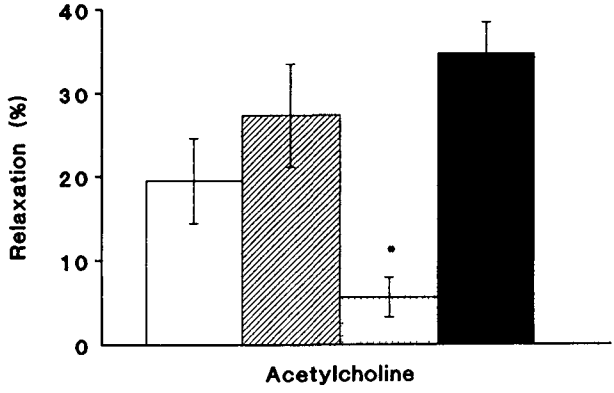

Figure 2. Relaxation induced by 1 μmol/l acetylcholine in rat abdominal aorta from sham-operated (open column), sham-operated + stobadine administered (hatched column), I/R (dotted column) and I/R + stobadine administered (filled column) animals Stobadine was administered in the dose of 2 mg/kg i v 5 min before starting reperfusion Values are means ± S E M of 6–11 experiments Significantly different from other groups, $^{*}p < 0\ 01$

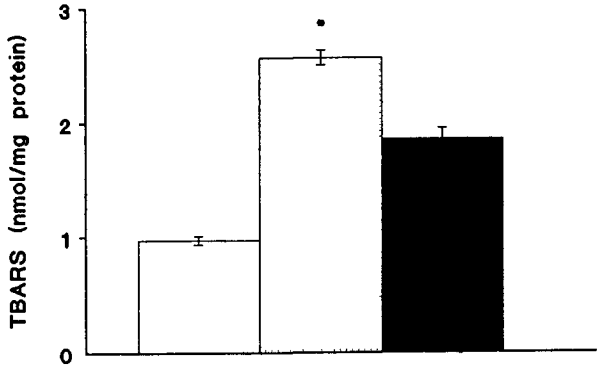

Figure 3. TBARS content in rat abdominal aorta of sham-operated (open column), I/R (dotted column) and I/R + stobadine administered (filled column) animals Stobadine was administered in the dose of 2 mg/kg i v 5 min before starting reperfusion Values are means ± S E M of 6 experiments Significantly different from other groups, $^{*}p < 0\ 01$

Determination of TBARS

The concentration of TBARS in homogenates of the rat abdominal aortae taken from sham-operated animals was 0 971 ± 0.037 nmol/mg protein. Ischaemia fol-

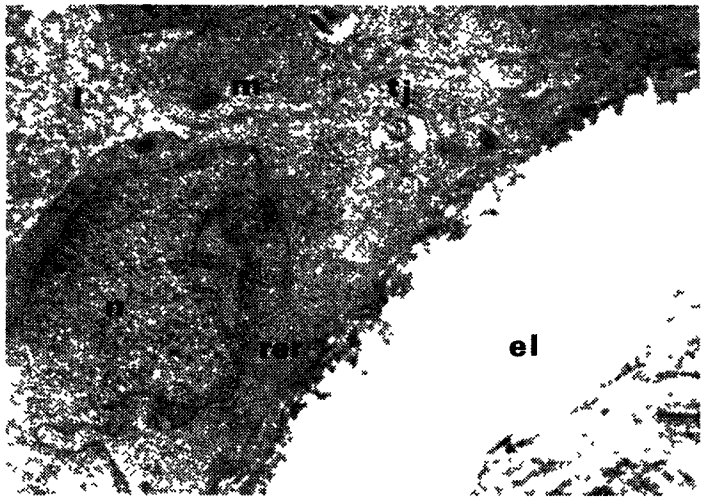

Figure 4. Intact ultrastructure of an endothelial cell of the control abdominal aorta *n* – nucleus, *m* – mitochondria, *rer* – rough endoplasmic reticulum with ribosomes, *tj* – tight junction, *l* – lumen, *el* – elastic lamina ×21,000

lowed by reperfusion induced an increase of the amount of TBARS in homogenates of the aortae to 2.573 ± 0.066 nmol/mg protein, i.e. 264.98%. After stobadine administration the concentration of TBARS decreased to 191.09% of sham values (Fig. 3).

Transmission electron microscopy

Cross sections of the rat abdominal aorta from sham controls displayed the typical architecture of the vascular wall: endothelial cells containing nuclei with dispersed chromatine, lysosomes, ribosomes, rough endoplasmic reticulum, mitochondria, and micropinocytotic vesicles. Also, electron-microscopic view of smooth muscle cells showed normal ultrastructure. The area at the pole of the nucleus, free of myofilaments contained mitochondria, Golgi apparatus, ribosomes both free, polyribosomes, and bound to the rough endoplasmic reticulum (Fig. 4).

After I/R, alterations in the mitochondria of both endothelial and smooth muscle cells were observed. The matrix of the mitochondria was more electrolucent and partially edematous, occasionally with ruptural cristae. In endothelial cells, moreover, formation of microvilli on the surface of the cells, occasionally proliferation, increased pinocytic activity and higher amount of lysosomes were found. Tight junctions between endothelial cells were also disturbed (Fig. 5).

Figure 6 shows an electron micrograph of the cells of the abdominal aorta after I/R with stobadine. The ultrastructure of the mitochondria and the tight

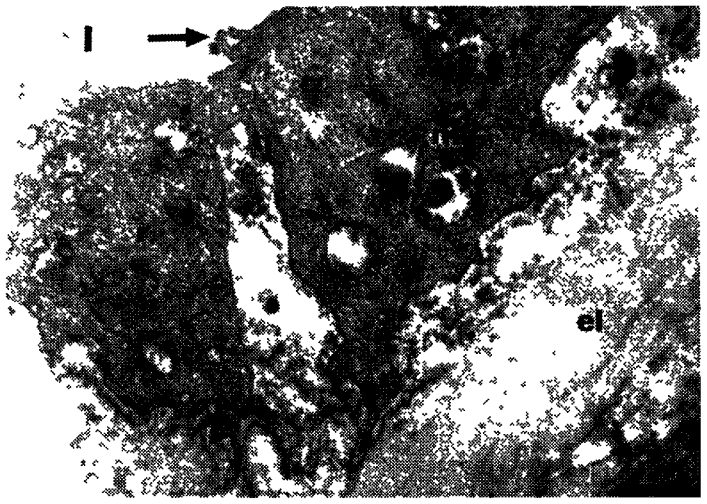

Figure 5. After ischaemia followed by reperfusion, mitochondria (m) of endothelial cells are partially edematous, with occasional ruptural cristae Microvillous projections (arrow) were observed l – lumen, tj – tight junction, el – elastic lamina, r – rough endoplasmic reticulum ×16,000

Figure 6. Ultrastructure of endothelial cells after ischaemia and reperfusion with stobadine preservation of the ultrastructure of mitochondria (m), proliferation of endothelial cells, intensive formation of microvilli (arrow), plasmalemmal protrusion (arrow head), numerous lysosomes (ly), pinocytic vesicles (pv) and abundand rough endoplasmic reticulum (r) were seen tj – tight junction, l – lumen, n – nucleus ×11,000

junctions between endothelial cells were preserved. Proliferation of the endothelial cells was observed along with an intensive formation of microvillous projections, plasmalemmal protrusion, numerous pinocytic vesicles and abundant rough endoplasmic reticulum.

Discussion

In the present study, 18-hour ischaemia did not induce any significant changes in the functional responses of the abdominal aorta to the drugs tested. A similar study of Summers et al. (1993) on the rabbit femoral artery showed no evidence of permanent endothelial dysfunction after up to 5 hours of ischaemia without reperfusion. The ability of the endothelium of large arteries to produce NO seems to be resistant to ischaemic injury. Our experiments showed that 18 hours of ischaemia followed by 30 minutes of reperfusion induced functional injury, manifested by inhibition of the relaxation response of aortal rings to acetylcholine. The long ischaemic period before reperfusion was needed to reveal changes of the endothelium. In feline coronary arteries, the ability of the endothelium to produce EDRF/NO was found to be reduced at 2.5 min of *in vivo* reperfusion after 90-min ischaemia (Tao et al. 1990). Quillen et al. (1990) found that 1-hour ischaemia and 1-hour reperfusion impaired the endothelium-dependent relaxation of canine coronary microvessels, though the authors did not find changes in EDRF/NO responses after up to 3-hour ischaemia and 1-hour reperfusion in canine conduit coronary arteries. Summers et al. (1993) reported that 4 hours of ischaemia followed by 1 hour of reperfusion of the rabbit femoral artery resulted in reduced production of NO. The size of the arteries seems to account for the differences in their sensitivity to I/R; in large arteries, such as the aorta, a longer time of ischaemia followed by reperfusion is needed for changes in EDRF/NO-induced responses to become manifest. Moreover, both the ischaemic abdominal aorta in our experiments and the rabbit femoral artery in experiments of Summers et al. (1993) were relatively at rest, with a minimal metabolic demand. In contrast, in coronary arteries exposed to ischaemia in the working heart, metabolic byproducts accumulate, and these may contribute to increased free radical formation during reperfusion.

In our experiments, after 18 hours of ischaemia followed by 30-min reperfusion, the ability of aortal rings to dilate in response to acetylcholine was abolished or strongly reduced. Responses to noradrenaline, KCl and to sodium nitroprusside, however, were not changed, showing that the relaxant as well as contractile properties of the aorta were not influenced by this procedure. Since acetylcholine-induced relaxation is elicited by the release of NO from endothelial cells (Palmer et al. 1987), its reduction indicates endothelial injury. In the present work, electron microscopy showed changes of the endothelium ultrastructure: formation of microvilli on the surface of the endothelial cells and occasional proliferation, more frequent

occurrence of pinocytic vesicles and lysosomes suggesting increased enzymatic hydrolytic activity of endothelial cells induced by I/R. Ultrastructural changes of the mitochondria of both endothelial and smooth muscle cells indicate changes not only in intracellular ATP levels but also an increased production of free oxygen radicals. As a consequence of I/R, the permeability of endothelial cells may be altered due to the injury of tight junctions.

In agreement with the changed ultrastructure of aortal preparations, in the arterial homogenates we found an increased amount of lipid peroxidation end-products, TBARS. Thus, increased peroxidation of membrane lipids appears to be involved in I/R-induced endothelial injury.

Stobadine administered before starting reperfusion prevented a decrease of the relaxation response of the aorta to acetylcholine and protected cell membranes against peroxidation, as TBARS concentrations in the homogenates declined to control values. Lukáčová et al. (1993) reported that stobadine, administered 2 min before recirculation, reduced the increase in lipid peroxidation of rabbit spinal cord homogenates induced by *in vivo* I/R. The ability of stobadine (2mg/kg) to prevent lipid peroxidation was also tested in incomplete rat cerebral ischaemia induced by 4-hour ligation of the common carotid arteries with subsequent 10-min reperfusion (Horáková et el. 1991). The significantly increased levels of TBARS in brain cortex samples from animals subjected to ischaemia followed by reperfusion decreased to control values in animals treated with stobadine administered immediately before reperfusion. The donation of hydrogen from the indole nitrogen group of stobadine to lipid radicals terminating lipid peroxidation may have accounted for the protective effects observed (Ondriaš et al. 1989). In addition to its antioxidant effect, stobadine may also prevent superoxide radical generation (Horáková et al. 1991).

The beneficial effect of stobadine in our experiments was manifested not only by the preservation of the ultrastructure of the mitochondria but also of the tight junctions. Since the mitochondria are the site of reactive oxygen species generation, the I/R-induced changes in the mitochondrial structure may reflect overproduction of free oxygen radicals. Stobadine is known to scavenge OH (Staško et al. 1990) and to quench $^1O_2$ (Steenken et al. 1992), and by these mechanisms it could exert its protective effect on the mitochondria. Inhibition of the propagation of lipid peroxidation (Mišík et al. 1991) may be another mechanism participating in the protection of the mitochondria and the tight junctions. The ultrastructure of endothelial cells showed increased pinocytic activity, as well as increased amounts of lysosomes, ribosomes, and the formation of microvilli. This may indicate that the repair process which started during reperfusion was promoted by the administration of stobadine.

Stobadine, when administered 5 min after the onset of reperfusion, had no protective effect against I/R-induced injury of the aortal endothelium. It has thus to be present in the tissue throughout reperfusion, i.e. when generated free rad-

icals induce injury It is then the scavenging activity of stobadine that provides protection to the endothelium

In conclusion, I/R of the rat abdominal aorta induced injury of the endothelium, which was manifested by decreased relaxation response to acetylcholine, damaged mitochondria and increased production of TBARS Most probably due to its scavenging properties, stobadine proved able to prevent these deleterious changes to occur

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