Changes in the Function and Ultrastructure of Vessels in the Rat Model of Multiple Low Dose Streptozotocin-Induced Diabetes

R. Sotnikova[1], S. Skalska[1], L. Okruhlicova[2], J. Navarova[1], Z. Kyselova[1], J. Zurova[1], M. Stefek[1], R. Hozova[3] and V. Nosalova[1]

[1] *Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia*
[2] *Institute for Heart Research, Slovak Academy of Sciences, Bratislava, Slovakia*
[3] *Institute for Drug Research, Modra, Slovakia*

Abstract. In this study we investigated functional changes in the femoral artery and ultrastructural alterations in mesenteric vessels and capillaries in the rat model of multiple low dose streptozotocin (STZ)-induced diabetes. Participation of oxidative stress in this model of diabetes was established by studying the effect of the pyridoindole antioxidant stobadine (STB) on diabetes-induced impairment.

Experimental diabetes was induced by i.v. bolus of STZ (20 mg/kg) given for three consecutive days to male rats. At the 12th week following STZ administration, the animals revealed typical signs of diabetes, such as polyphagia, polydypsia and polyuria. There was no weight gain in the diabetic groups throughout the experiment. No exitus occurred in any group. Diabetes was characterised with high levels of plasma glucose, no significant changes in lipid metabolism, decreased serum levels of glutathione, increased serum levels of the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAGA), injured endothelial relaxant capacity of the femoral artery and alterations in ultrastructure of mesenteric arteries and capillaries.

Antioxidant STB in the dose of 25 mg/kg body weight i.p. (5 times *per* week) did not influence glucose levels, however, it mitigated biochemical, functional and ultrastructural changes induced by diabetes, suggesting a role of reactive oxygen species in diabetes-induced tissue damage.

Key words: Rat multiple low dose STZ-diabetes — Femoral artery — Mesenteric artery — Endothelium — Antioxidant stobadine

Correspondence to: Ruzena Sotnikova, Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dúbravská cesta 9, 841 04 Bratislava 4, Slovakia
E-mail: exfarosa@savba.sk

Introduction

Diabetes mellitus is a metabolic syndrome that requires long-term medical attention both to limit the development of disease and to delay or mitigate accompanying complications. Among the most frequent diabetic complications are angiopathies, i.e micro- and macrovasculopathies. In the pathogenesis of impaired vessel function, endothelial dysfunction plays an important role. During diabetes, persistent hyperglycaemia causes increased production of oxygen free radicals that may lead to disruption of cellular functions and oxidative damage of membranes (Wolf et al. 1991; Baynes and Thorne 1999). Increased vascular reactive oxygen species (ROS) production reduces endothelial nitric oxide (NO) not only by direct inactivation but also as a consequence of increased oxidation of tetrahydrobiopterin and inhibition of dimethylarginine dimethylaminohydrolase. Increased activity of NAD(P)H oxidase, a major vascular oxidant enzyme system, is likely to represent an important source of ROS. Moreover, NAD(P)H oxidase has been shown to cause endothelial NO synthase "uncoupling" which in turn generates superoxide anion (O_2^-) in addition to NO. NAD(P)H oxidase also promotes xanthine oxidase-dependent O_2^- production (Landmesser et al. 2005). Besides enhanced ROS generation, hyperglycaemia also attenuates endogenous antioxidative mechanisms through glycation of scavenging enzymes and depletion of low molecular antioxidants, e.g. glutathione (Gugliucci 2000), and reduces vascular activity of antioxidant enzymes, such as extracellular superoxide dismutase (Landmesser et al. 2005).

All these events lead to disturbations in endothelial function and consequently to the development of vessel wall remodelling. Therefore, treatment of endothelial dysfunction in diabetes has become an attractive therapeutic target. An important group of pharmacological active substances are antioxidants which could be useful in prevention or delay of vascular complications in diabetes.

Stobadine (STB), a synthetic pyridoindole, is known to be an efficient antioxidant which is able to scavenge hydroxyl, peroxyl and alkoxyl radicals, to quench singlet oxygen, to repair oxidised amino acids and to preserve oxidation of SH groups by one-electron donation. These effects originate from its ability to form a stable nitrogen-centred radical on indole nitrogen. Consequently, it diminishes lipid peroxidation and protein impairment under oxidative stress. Oxidation of low-density lipoproteins (LDLs), which plays a major role in the development of atherosclerosis, was decreased by STB *in vitro*. Both lipid and protein (apolipoprotein B) components of LDL were protected against Cu^{2+}-induced oxidation by this agent (Horakova and Stolc 1998). These findings, along with its high oral bioavailability (Kallay et al. 1990), efficient detoxification pathways (Stefek et al. 1987; Stefek et al. 1989) and toxic safety (Ujhazy et al. 1994; Gajdosikova et al. 1995) render STB a promising agent in prevention of diabetic complications in experimental models of diabetes.

Streptozotocin (STZ) is a widely used diabetogenic agent, however, a single high dose STZ injection leads to early mortality of experimental animals. For this reason we administered STZ in multiple lower doses to avoid mortality and to

induce a mild onset of diabetes. The aim of this study was i) to characterise the low dose STZ model of diabetes in rats using biochemical investigation, ii) to study changes of endothelium-dependent relaxation in the femoral artery as an indicator of altered vessel function, iii) to study ultrastructural alterations in resistant vessels and capillaries, and iv) by studying the effect of the pyridoindole antioxidant STB on diabetes-induced impairment to establish the participation of oxidative stress in this model of diabetes.

Materials and Methods

Disease model

The investigation conforms with the Guide for the Care and Use of Laboratory Animals. Male Wistar rats, 8–9 weeks old, weighing 200–230 g, were used. The animals were of monitored conventional quality and came from the Breeding Facility of the Institute of Experimental Pharmacology (Dobrá Voda, Slovak Republic). Experimental diabetes was induced by i.v. bolus of STZ (20 mg/kg) given for three consecutive days. STZ was dissolved in 0.1 mol/l citrate buffer, pH 4.5. The animals were fasted overnight prior to STZ administration. Water and food were available immediately after dosing. Ten days after STZ administration, the postprandial plasma glucose levels were determined and animals with values >20 mmol/l were considered diabetic. Control animals (C group) received 0.1 mol/l citrate buffer. Diabetic animals were randomly assigned to two groups: a) vehiculum-treated diabetic rats (STZ group) and b) diabetic rats treated with STB hydrochloride (25 mg/kg body weight i.p. – STZ-STB group), administered 5 times *per* week.

During the experiment, the animals were housed in groups of two in cages of the type T4 Velaz (Prague, Czech Republic) with bedding composed of wood shaving (exchanged daily). Tap water and pelleted standard diet KKZ-P-M (Dobrá Voda, Slovak Republic) were available *ad libitum*. The animal room was air-conditioned and the environment was continuously monitored for the temperature of $23 \pm 1\,^{\circ}\mathrm{C}$ and relative humidity of 40–70 %.

Twelve weeks following STZ administration, the animals were killed by decapitation in thiopental anaesthesia (65 mg/kg i.p.). Samples of blood, the mesenteric and femoral arteries were taken for biochemical, functional and electron microscopic studies.

Biochemical studies

Preprandial and postprandial plasma glucose levels were measured using the commercial Glucose (Trinder) kit (Sigma, St. Louis, USA).

The activity of the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAGA) was assayed in rat serum according to standard methods (Barrett and Heath 1977), reduced glutathione (GSH) was determined by the method of Tietze (1969).

Total cholesterol, billirubin and triglycerides were determined by the commercial Bio-La-Test kits (Lachema, Brno, Czech Republic).

Functional vessel studies

In functional studies, the femoral artery was rapidly removed, immersed in physiological salt solution (PSS) composed of (in mmol/l): NaCl 122, KCl 5.9, NaHCO$_3$ 15, glucose 10, MgCl$_2$ 1.25, and CaCl$_2$ 1.25, gassed with a mixture of 95 % O$_2$ and 5 % CO$_2$, and carefully cleaned of all fat and connective tissue. Rings of femoral artery (approximately 2 mm long) were mounted in a tissue chamber containing PSS, gassed and maintained at 37℃, and attached to an isometric force transducer. Rings were passively stretched to optimal length by imposing the optimal initial tension of 15 mN found in previous studies. After the 60-min stabilisation period, the rings were precontracted with 1 μmol/l phenylephrine (PE) and relaxant responses of the preparations to acetylcholine (ACh, 0.01–100 μmol/l) were tested at the plateau of the contraction. Responses to ACh are expressed as percentages of PE-induced contraction.

Contractile responses to 100 mmol/l KCl and to PE (1 μmol/l) are expressed in mN/mg tissue wet weight.

Electron microscopy

Mesenteric arteries of the first order were removed for electron microscopic examination. Arteries were excised from the mesentery and cut into small rings. Tissue samples were immersely fixed in 2.5 % glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) for 3 h. After washing in cacodylate buffer, the tissue was postfixed in 1 % OsO$_4$ buffered with 0.1 mol/l sodium cacodylate. The tissue was dehydrated in an ethanol series, infiltrated by propylene oxide and embedded in Epon 812. Toluidine-blue-stained sections (1 μm thick) were examined by light microscopy, and appropriate areas of tissue were selected for cutting thin sections (Ultramicrotome Huxley, Cambridge). The sections were stained with uranyl acetate and lead citrate, and examined using an electron microscope Tesla BS 500 (Brno, Czech Republic).

Statistical analysis

One way analysis of variance was performed and any significant ($p < 0.05$) differences were assigned to individual between-group comparisons using Student's t-test and the Bonferroni's correction for multiple comparisons (INSTAT GraphPad, San Diego, CA, USA). All data are expressed as means $\pm$ SEM.

Results

At the 12th week following STZ administration, preprandial and postprandial blood glucose levels were significantly elevated in diabetic groups compared to control rats (14.95 $\pm$ 5.21 *vs.* 5.26 $\pm$ 1.00 and 33.53 $\pm$ 6.30 *vs.* 7.34 $\pm$ 1.20, respectively; Fig. 1). The administration of STB had no effect on blood glucose levels. Beside hyperglycaemia, the STZ-treated animals revealed further typical signs of diabetes, such as
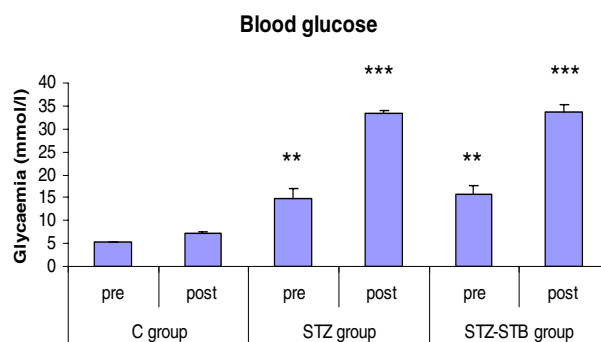
Blood glucose

Figure 1. Preprandial (pre) and postprandial (post) plasma concentrations of glucose 12 weeks after streptozotocin (STZ) administration. C group, control group; STZ group, diabetic group treated with vehiculum; STZ-STB group, diabetic group treated with stobadine. Results are means ± SEM of 8 experiments. ** $p < 0.01$, *** $p < 0.001$ against respective C group.
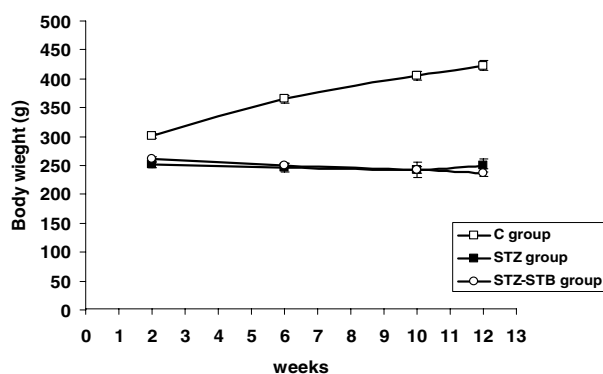

Figure 2. Body weight of rats from the control group (C group), diabetic group treated with vehiculum (STZ group) and diabetic group treated with stobadine (STZ-STB group).

polyphagia, polydypsia and polyuria. Dispite polyphagia, there was a slight weight loss in the diabetic groups at the beginning of the experiment, with no weight gain throughout the experiment. STB treatment did not affect this parameter (Fig. 2). No exitus occurred in any group.

Biochemical estimation of plasma cholesterol, triglycerides and billirubin showed that diabetes induced a slight, however, not significant increase in these biochemical parameters. STB exhibited a tendency to return diabetes-induced changes to control values (Table 1). STZ-induced diabetes was accompanied by reduction in serum levels of GSH and, on the other hand, by increase in serum levels of NAGA.

Table 1. Effect of streptozotocin (STZ)-induced diabetes on plasma levels of cholesterol, triglycerides and billirubin

	n	Cholesterol (mmol/l)	Triglycerides (mmol/l)	Total billirubin (μmol/l)
C group	8	1.3 ± 0.087	0.674 ± 0.08	7.1 ± 1.421
STZ group	7	1.686 ± 0.146	0.83 ± 0.11	10.05 ± 2.912
STZ-STB group	7	1.463 ± 0.087	0.573 ± 0.062	6.706 ± 1.486

C group, control group; STZ group, diabetic group treated with vehiculum; STZ-STB group, diabetic group treated with stobadine. Results are means $\pm$ SEM of n experiments.

Table 2. Effect of streptozotocin (STZ)-induced diabetes on N-acetyl-β-glucuronidase (NAGA) and reduced glutathione (GSH) serum levels

	n	NAGA (μg 4-nitrophenol/min·mg protein)	GSH (μg/mg protein)
C group	8	0.669 ± 0.014	0.289 ± 0.028
STZ group	7	1.1 ± 0.101*	0.133 ± 0.027**
STZ-STB group	7	0.858 ± 0.133	0.161 ± 0.022*

C group, control group; STZ group, diabetic group treated with vehiculum; STZ-STB group, diabetic group treated with stobadine. Results are means $\pm$ SEM of n experiments. * $p < 0.05$, ** $p < 0.001$.

Table 3. Responses of femoral artery rings to single concetration of KCl and phenylephrine (PE), expresed in mN/mg wet weight

	n	KCl (100 mmol/l)	PE (1 μmol/l)
C group	4	53.41 ± 9.68	23.56 ± 6.61
STZ group	9	37.00 ± 10.45	11.23 ± 3.06
STZ-STB group	12	39.41 ± 5.88	17.62 ± 3.69

C group, control group; STZ group, diabetic group treated with vehiculum; STZ-STB group, diabetic group treated with stobadine. Results are means $\pm$ SEM of n experiments.

In the group of STB-treated diabetic rats, levels of NAGA returned and those of GSH tended to approach to control values (Table 2).

In vessel functional studies, the endothelium-dependent relaxation of femoral arteries was found to be injured in diabetic rats. It was manifested by decreased responses of preparations to Ach – the maximal relaxation achieved $64.82 \pm 11.38\%$ compared to control values of $24.54 \pm 11.60\%$ of contraction. EC_{50} values (the concentration of ACh when the responses reached 50% of the maximal value) were not significantly changed. STB administration led to an improvement of endothelium-
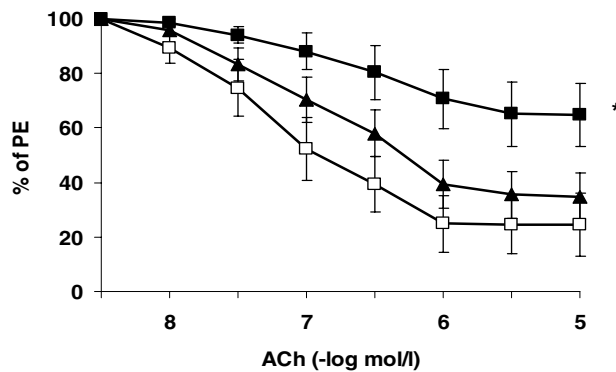
Figure 3. Responses of phenylephrine (PE)-precontracted femoral arterial rings to acetyl-choline (Ach). □ control group (C group), ■ diabetic group treated with vehiculum (STZ group), ▲ diabetic group treated with stobadine (STZ-STB group). Relaxation is expressed as percentages of 1 μmol/l PE-induced contraction. Results are means $\pm$ SEM of 12 experiments. * $p < 0.05$ against C group.

dependent relaxation; the maximal relaxation was $44.81 \pm 8.62\%$ of contraction (Fig. 3).

In diabetic animals, contractile responses of vessel rings to PE were not statistically different, however, they tended to be lower compared to controls. Alike after STB treatment, contractions were mildly but not significantly reversed towards control values (Table 3).

Electron microscopic analysis of the mesenteric artery of the first order wall from diabetic rats revealed weak to moderate local subcellular alterations of endothelial cells in comparison with age-matched controls (Fig. 4). Most endothelial cells showed a normal architecture. A part of them was oedematous with electrolucent cytoplasm and low amount of caveoles, others contained vacuoles and clumped chromatin in the nucleus. Tight junctions between endothelial cells were extended. Smooth muscle cells were not altered and they showed a conventional architecture. Apparent subcellular changes were observed in endothelial cells of mesenteric capillaries in diabetic rats. They contained many vacuoles, indicating changes in their permeability and function. In close vicinity of capillaries, an increased number of mast cells at different stage of their maturation was observed. Vacuoles in cytoplasm demonstrated mast cell degranulation. Diffusely distributed collagen fibres were also found in extracellular space (Fig. 5). STB supplementation to diabetic rats resulted in preservation of endothelial integrity of both small arteries and capillaries. In contrast to fibroblasts, where increased amount of vacuoles persisted, endothelial cells of arteries were without considerable defects (Fig. 6). Endothelial cells of capillaries contained vacuole reduction.
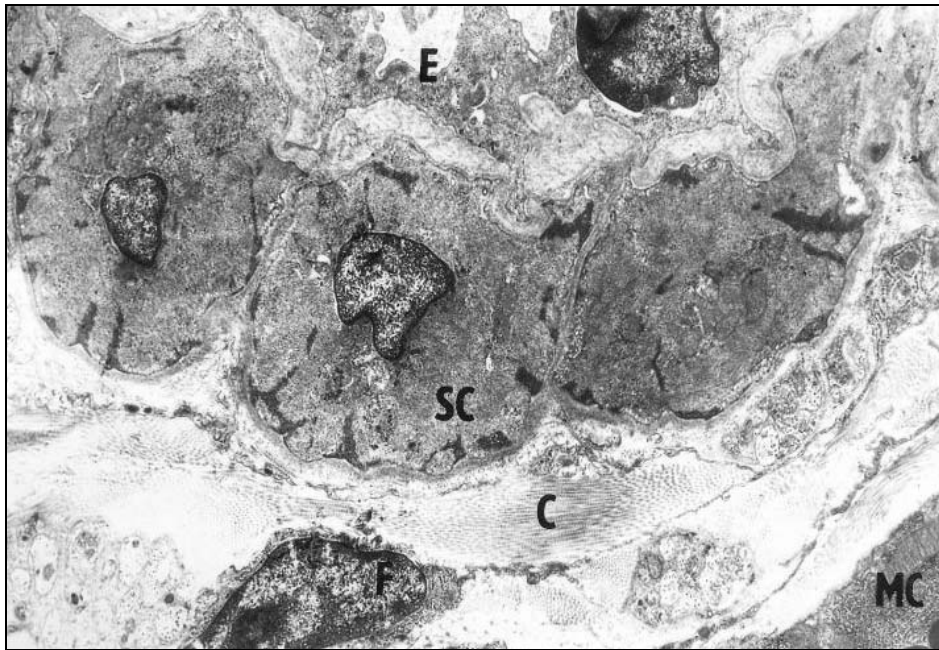
Figure 4. Electron micrograph of control small mesenteric artery with conventional architecture of endothelial and smooth muscle cells. C, collagen; E, endothelial cells; F, fibroblast; MC, mast cell; SC, smooth muscle cells. Magnification ×7,600.

Discussion

In contrast to mice, the rat model of multiple low dose STZ-induced diabetes has been rarely used (e.g. Taha and Raza 1996; Hrabak et al. 2006). Routinely diabetes is induced by a single high dose of STZ (about 60 mg/kg and more), which, however, is connected with relatively high mortality of animals. For instance, Gajdosik et al. (1999) has reported 100 % mortality of Wistar rats on 2–3 day after intravenous administration of 70 mg/kg STZ. To solve the problem of mortality, in our experiments we administered STZ in low repetitive dose of 20 mg/kg. After 3 months, this model of STZ administration led to the development of signs of diabetes: preprandial as well as postprandial values of plasma glucose were elevated with typical manifestations of diabetes, such as polyphagia, polydypsia, polyuria, and body weight loss.

In our model of diabetes, no mortality was observed after administration of STZ and hyperglycaemia developed steeply. Plasma triglycerides and cholesterol were not increased significantly, pointing at not very serious metabolic failure. Decrease in serum levels of GSH indicated the participation of oxidative stress in these conditions. In hyperglycaemia, endogenous antioxidant mechanisms are impaired due to glycation and glycooxidation of ROS-scavenging enzymes and to

depletion of low-molecular-weight antioxidants, e.g. GSH. Thus, low GSH levels are associated with oxidative stress in diabetes (Konukoglu et al. 1999). Decreased GSH levels result also from the competition between aldose reductase and glutathione reductase for NADPH during the increased flux of glucose in the polyol pathway (Kinoshita 1990).

In our study, increased NAGA serum concentrations were established. Increased activity of lysosomal enzymes in blood is one of the first signs of tissue injury as a consequence of inadequate oxygen supply. A comparison of serum levels of various lysosomal enzymes revealed a highly significant correlation of increased NAGA levels with decreased GSH levels (Waters et al. 1992). Increased serum NAGA activity was detected also in an insulin-independent diabetes model of genetically modified rats (Uehara et al. 1997).

In our experiments, 12 weeks lasting diabetes was found to induce injury of the femoral artery function. Reports concerning the magnitude of endothelium-dependent relaxation in diabetes are conflicting – from decreased (e.g. Kamata et al. 1989) to increased (e.g. Altan et al. 1989). These discrepances are probably due to differences in experimental models, the artery used, and duration of diabetes. Based on our experience with high dose STZ models of diabetes, changes in endothelium-dependent relaxation of arteries seem to begin approximately from the 5th week (e.g. Sotnikova et al. 2001). These changes are supposed to be connected with decreased bioavailability of NO during hyperglycaemia which leads to dysfunction of the endothelium manifested by decreased relaxant response of vessels to endothelium-dependent relaxants, e.g. ACh. During diabetes mellitus, O_2^- production is increased in arteries. Increased O_2^- production contributes to reduced vascular NO bioavailability and endothelial dysfunction in experimental models of diabetes. O_2^- inactivates the potent vasodilator NO by forming peroxynitrite ($ONOO^-$), which results in impaired vasorelaxation. $ONOO^-$ oxidises lipids and thiol residues, damages lipid membranes and decreases the redox potential of the cell, thus inducing multiple vascular pathologies. The uncoupled NO-synthase and NAD(P)H oxidase have been suggested as the most important sources of O_2^- in diabetes mellitus (Guzik et al. 2002). Decreased levels of GSH as well as increased NAGA levels measured in our experiments support the suggestion that injured endothelium-dependent relaxation of the femoral artery is connected with depressed NO bioavailability by increased production of ROS during diabetes.

In our experiments, contractile properties of the femoral artery were not influenced significantly by diabetes. It seems that our experimental model of multiple low dose STZ evoked changes in endothelial but not in smooth muscle function of the artery tested.

In electron microscopic studies on mesenteric arteries we found changes in the endothelium ultrastructure of small vessels and capillaries, though not in smooth muscle cells. Subcellular alterations of endothelium manifested by reduced amount of pinocytic vesicles, enhanced presence of vacuoles and changes in nucleus chromatin represent structural forms of endothelial dysfunction (Okruhlicova et al. 2005). Reduced amount of pinocytic vesicules indicates disturbances in transport
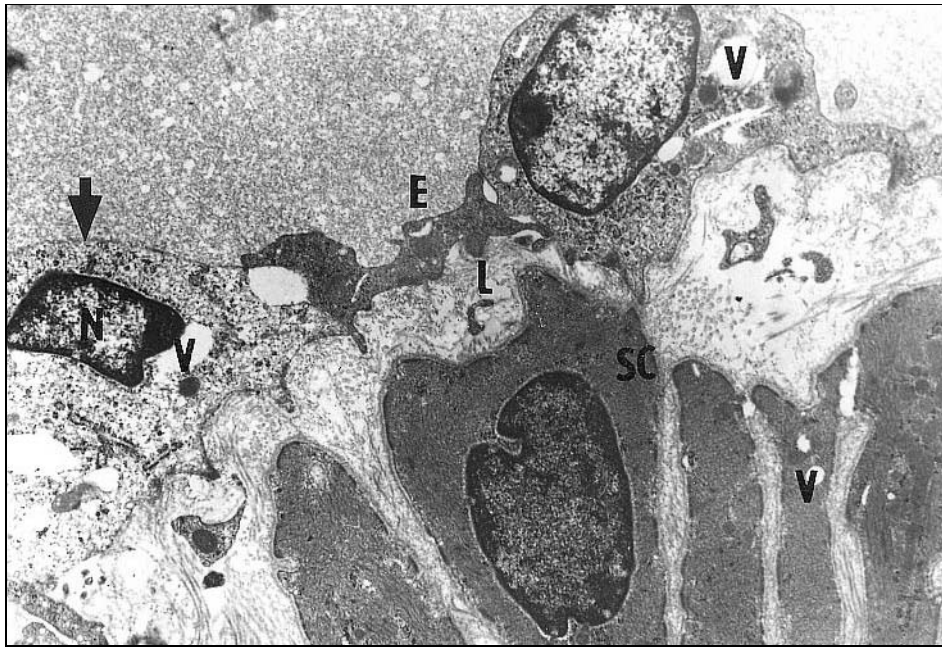
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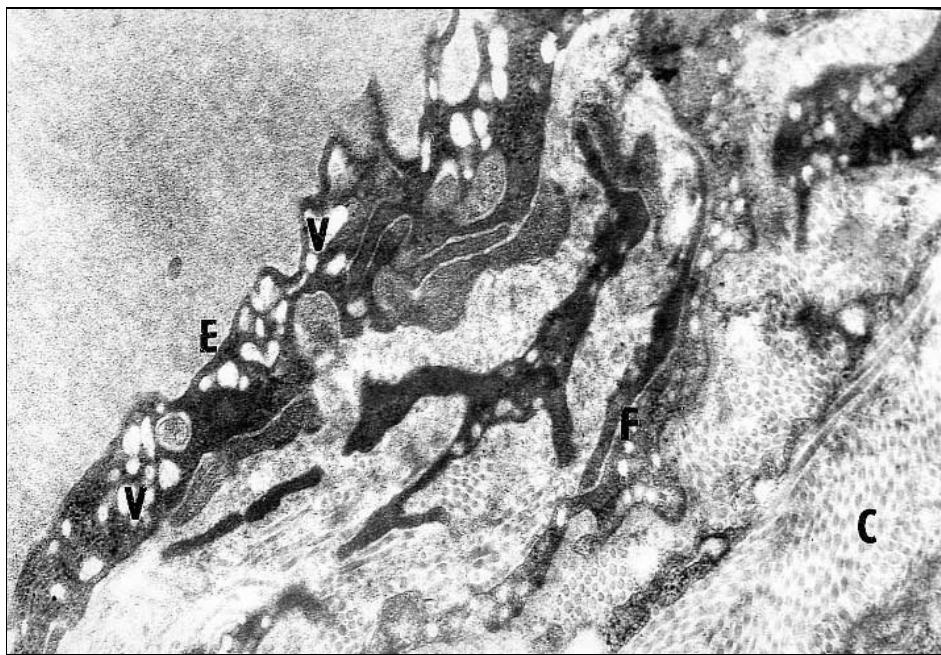
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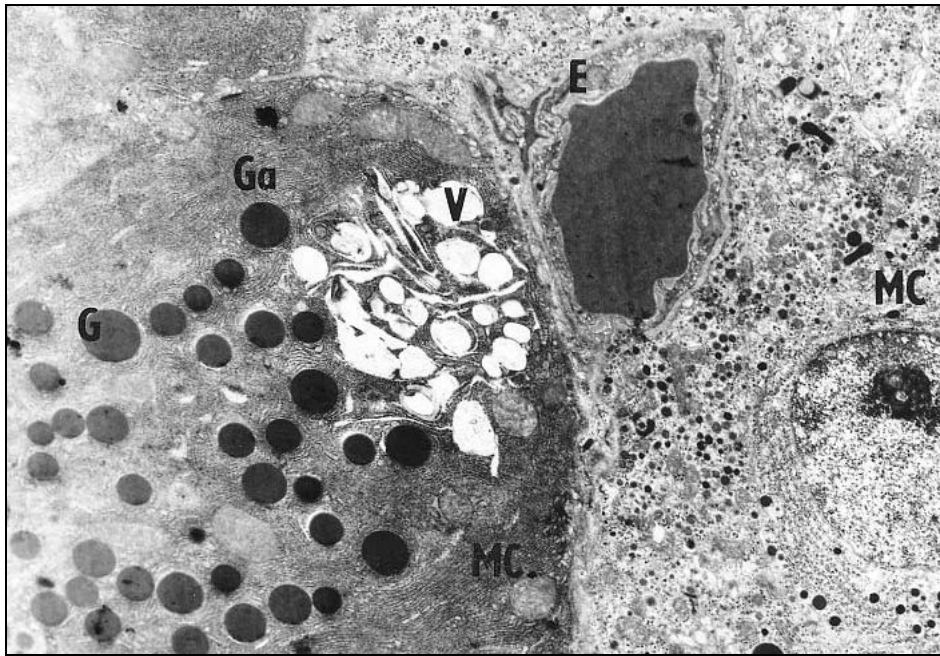
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Figure 5. A. Diabetes-induced subcellular alterations in wall cells of rat mesenteric artery: different cytoplasm density of endothelial cells, perinuclear clumping of chromatin, decreased number of pinocytic vesicules, presence of vacuoles in the endothelial and smooth muscle cells, lipid droplets in subintima. **B.** Ultrastructure of mesenteric capillary and fibroblasts in diabetic rats: cells contained many vacuoles. **C.** Mast cells at different stage of maturation surrounding mesenteric capillary. Abbreviations: C, collagen; E, endothelial cells; F, fibroblast; G, mast cell granules; Ga, Golgi complex; L, lipid droplets; MC, mast cell; N, nucleus; SC, smooth muscle cells; V, vacuoles; arrow, edematous endothelial cells. Magnification ×10,000.

of nutrients throughout the endothelium, and impaired signalling pathways (Frank et al. 2003). In adition, extended tight junctions may contribute to increased entry of neutrophils into the subintima. These alterations can contribute not only to changes in function of the endothelium but also to changes of its adhesive properties. Increased amount of degranulated mast cells observed in the vicinity of vessels in diabetic animals indicates that released mast cell mediators, e.g. cytokines, proteases tryptase and chymase, histamine, metaloproteinases, can also modulate a vessel function and production of extracellular collagen.

The pathogenetic role of ROS has been confirmed in high dose STZ-induced diabetes by experiments with antioxidants, which were able to mitigate consequences of ROS effects on the endothelium of the aorta (Sotnikova et al. 2000, 2001; Irat et al. 2003) and other organs (Stefek et al. 2000, 2002; Karasu et al. 2002; Kyselova
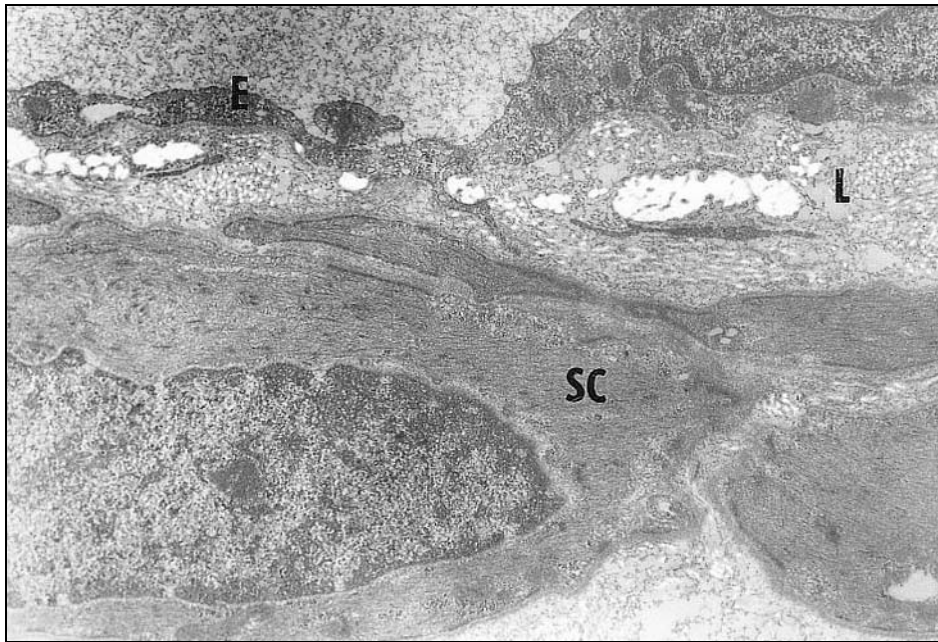
Figure 6. Electron micrograph of mesenteric artery of stobadine (STB)-treated diabetic rat showing preservation of endothelial cell structure in small artery. L, lipid droplets; SC, smooth muscle cells. Magnification ×10,000.

et al. 2005). STB partially protected vessels from the deleterious effect of reactive species, probably by diminishing lipid peroxidation and protein impairment under oxidative stress evoked by hyperglycaemia (Horakova and Stolc 1998; Pekiner et al. 2002) and thus stabilising cell and lysosomal membranes.

In our experiments we found that administration of STZ in the dose of 20 mg/kg i.v. for 3 consecutive days induced diabetes characterised with high levels of plasma glucose, no significant changes in lipid metabolism, decreased antioxidant defense status, injured endothelial relaxant capacity of the femoral artery and alterations in ultrastructure of small arteries and capillaries. The pyridoindole antioxidant STB was found to mitigate these changes, suggesting a role of ROS in diabetes-induced tissue damage.

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