Research Paper

# Rosmarinic acid administration attenuates diabetes-induced vascular dysfunction of the rat aorta

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## Abstract

**Objectives** Oxidative stress as well as inflammation processes are engaged in diabetic vascular complications. Rosmarinic acid, a natural phenol antioxidant carboxylic acid, was found to have multiple biological activity, including anti-inflammatory and antitumour effects, which are a consequence of its inhibition of the inflammatory processes and of reactive oxygen species scavenging. The aim of this work was to study effects of rosmarinic acid administration on vascular impairment induced by experimental diabetes in rats.

**Methods** Diabetes was induced by streptozocin ($3 \times 30$ mg/kg daily, i.p.) in Wistar rats. Rosmarinic acid was administered orally (50 mg/kg daily). Ten weeks after streptozocin administration, the aorta was excised for functional studies, evaluation by electron microscopy and real time PCR analysis.

**Key findings** In the aorta of diabetic rats, decreased endothelium-dependent relaxation was accompanied by overexpression of interleukin-1β, tumour necrosis factor-α, preproendothelin-1 and endothelin converting enzyme-1. Structural alterations in the endothelium, detected by electron microscopy, indicated aortic dysfunction caused by diabetes. The diabetes-induced aortic disorders were prevented by rosmarinic acid administration.

**Conclusions** Rosmarinic acid protected aortic endothelial function and ultrastructure against diabetes-induced damage. Both antioxidant and anti-inflammatory effects of rosmarinic acid seemed to participate in the mechanism of this protection.

## Introduction

In spite of significant achievements in diagnosis and treatment, cardiovascular disorders are the most common complications of diabetes and major causes of morbidity and mortality in diabetic patients.[1,2] Changes in vascular responsiveness to several vasoconstrictors and vasodilators are signals of development of some vascular complications in diabetes. It is becoming increasingly clear that oxidative stress is an important contributor to the onset and development of micro- and macrovascular complications of the disease. Indeed, recent in-vitro and in-vivo studies have shown that short-term and long-term antioxidant interventions could improve endothelial function in diabetes, suggesting a pathological role for oxygen-derived free radicals

in the impaired vascular responses.[3] Along with oxidative stress, inflammation processes are engaged in diabetic vascular complications. Hyperglycaemia was found to induce signalling pathways that give rise to the inflammatory profile of the disease. Inflammatory cytokines, like interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α), were reported to exert multiple effects leading to prothrombotic and proinflammatory changes on the vascular endothelium.[4] Chronic hyperglycaemia accelerates the formation of advanced glycation end products (AGEs) and accumulation of AGEs in various tissues. According to recent knowledge on diabetic vascular complications, AGEs, by means of their receptors together with high-

mobility-group box-1 protein and Toll-like receptors, play a key role in initiating the inflammatory state triggered by hyperglycaemia.[5]

Some phenolic compounds were found to improve reactivity of diabetic vessels by their antioxidant activity. For instance, soy isoflavones like genistein are beneficial in preventing some diabetic complications via attenuating oxidative stress in the aortic tissue.[6] Salvianoic acid A, the main effective, water-soluble constituent of *Salvia miltiorrhiza*, a well-known herb of traditional Chinese medicine, was reported to significantly improve glucose metabolism and inhibit oxidative injury, as well as to protect against impaired vascular responsiveness in streptozocin-induced diabetes in rats.[7]

Rosmarinic acid (2'R')-2-[[(2'E')-3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]]oxy]-3-(3,4-dihydroxyphenyl)propanoic acid) is a natural phenol carboxylic acid. It is a secondary metabolite found in many Lamiaceae herbs used commonly as culinary herbs, such as lemon balm, rosemary, oregano, sage, thyme and peppermint.[8] Extracts from Lamiaceae plants possess a wide scale of beneficial effects. For example, extracts of *Mentha piperita* leaves, containing rosmarinic acid as the main phenolic constituent, are used for antioxidant and anti-inflammatory purposes.[9] Rosmarinic acid has multiple biological activity, including anti-inflammatory and antitumour effects.[10,11] The latter are consequences of its inhibition of inflammatory processes and of reactive oxygen species scavenging.[12,13] Anti-inflammatory activity of rosmarinic acid was documented in experiments with apolipoprotein E-deficient mice, where it inhibited progression of atherosclerotic plaques by decreasing blood lipids and serum levels of proinflammatory cytokines TNF-$\alpha$ and IL-1$\beta$.[14] The capability to inhibit inflammatory processes and to scavenge oxygen free radicals makes rosmarinic acid a suitable candidate for protection of vessels against detrimental effects of long-lasting hyperglycaemia. Since there are no accessible publications dealing with the effect of rosmarinic acid in diabetic complications, the aim of this work was to study the impact of rosmarinic acid administration on vascular impairment induced by experimental diabetes in rats.

## Materials and Methods

### Streptozocin-induced diabetes

Male Wistar rats (13-weeks old, 300–325 g) from the Breeding Facility of the Institute of Experimental Pharmacology and Toxicology Dobrá Voda (Slovak Republic) were used. Animal experiments were conducted under the guidelines of the Ethics Committee of the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences and were approved by the State Veterinary and Food Administration of the Slovak Republic (number of the decision: Ro 1664/08-221d, July 18, 2008).

Diabetes was induced by a repetitive intraperitoneal dose of streptozocin (30 mg/kg; Sigma-Aldrich, St Louis, MO, USA) for three consecutive days. This protocol was chosen based on our pivotal studies evaluating different methods of streptozocin administration with the aim to induce a gradual onset of diabetes. Streptozocin was dissolved in 0.1 mol/l citrate buffer, pH 4.5. Animals with blood glucose concentration > 15 mmol/l were considered diabetic. Diabetes was induced in all rats administered streptozocin. Control animals received buffer only. The animals were divided into four groups containing seven animals: C, control rats; R, control rats treated with rosmarinic acid; D, diabetic rats; DR, diabetic rats treated with rosmarinic acid. Starting on the first day after the third dose of streptozocin, rosmarinic acid (Sigma-Aldrich Chemie Gmbh, Munich, Germany) was administered by an intragastric probe at a daily dose of 50 mg/kg.

Ten weeks after streptozocin administration, the rats were sacrificed by exsanguination in thiopental anaesthesia and the aorta was excised for functional studies, evaluation by electron microscopy and real time PCR analysis.

The weight of animals was registered weekly, water consumption daily. Blood glucose, blood pressure and heart rate were monitored at the end of the experiment. Plasma glucose levels were measured using the commercial Glucose (Trinder) kit (Sigma-Aldrich). Plasma thiobarbituric acid reactive substances (TBARS) level as a measure of lipid peroxidation was determined according to Esterbauer.[15] Blood pressure was measured noninvasively by tail cuff plethysmography using the AD Instruments, PowerLab MLT 125/R (Colorado Springs, CO, USA).

### Rat isolated aorta

The thoracic aorta was excised and transferred into oxygenated physiological salt solution (PSS). The arteries were cleaned of adherent tissue and cut into eight rings, each approximately 2–3-mm long. Special care was taken not to damage the endothelium. The rings were mounted between two hooks in water-jacketed (37°C) chambers containing PSS bubbled with a mixture of 95% $O_2$ 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 force transducer (Experimetria Hungary), stretched passively to 20 mN and equilibrated for 60 min.

After the equilibration period, contraction was induced by a submaximal concentration of phenylephrine ($10^{-6}$ mol/l). At the plateau of the contraction, the effect of acetylcholine in the cumulative concentrations of $10^{-8}$–$10^{-5}$ mol/l was tested as a measure of endothelial function. After washing

with PSS and reaching the initial tension value, concentration–response curves of sodium nitroprusside ($10^{-10}$–$10^{-7}$ mol/l) were performed in phenylephrine-precontracted preparations.

## Isolation of RNA and real-time PCR

Total RNA was isolated from 3-mm aortic rings using Tri-Reagent (Ambion, Grand Island, NY, USA). Isolated RNA was verified to be intact by using agarose gel electrophoresis. Reverse transcription to cDNA was performed using High Capacity cRNA RT Kit with RNAse inhibitor (Applied Biosystems, Grand Island, NY, USA), 500 ng RNA were reverse-transcribed to cDNA. Levels of preproendothelin-1 (ET-1), $ET_A$ receptor, $ET_B$ receptor, endothelin converting enzyme-1 (ECE-1), interleukin-6 (IL-6), TNF-$\alpha$ and IL-1$\beta$ mRNAs were evaluated using real-time PCR (ABI Prism 7300, Applied Biosystems, USA) with gene-specific primers with SYBR green detection (Power SYBR Green PCR Master Mix, Applied Biosystems, USA). Beta actin was used as a housekeeping gene. The delta-delta Ct method was used for quantification.[16] All primers were verified to yield a single PCR product with the correct molecular weight and the absence of signal was verified when reverse transcription was omitted. Primer sequences were as follows (5′ to 3′) ET-1: forward: AACTCCGAGCCCAAAGTACC, reverse CTTGATGCTGTTGCTGATGG; $ET_A$: forward: TGGGAA GTTTCCTCCAGCCGAGA, reverse: TCCTTCCCCTTAG AGCTCCTCGGA; $ET_B$: forward: TTGCCGCTAGCCATC ACTGCGA, reverse: GGCAAACACGAGGACCAGGCAGA; ECE-1: forward: TTCCCGACGGTCACTCACGC, reverse: GCCGTGGAATTTTCCAGCAGATGC, IL-6: TCTCTCCG CAAGAGACTTCC, reverse: GTCTCCTCTCCGGACTT GTG; TNF-$\alpha$: forward: AACTTCGGGGTGATCGGTCCCA, reverse: TACGACGTGGGCTACGGGCTT; IL-1$\beta$: forward: AATCCCTGTGGCCTTGGGCCTC, reverse: GGATCCA CACTCTCCAGCTGCAGG; actin: forward: CCGCGAGTA CAACCTTCTTG, reverse: GCAGCGATATCGTCATCCA.

## Western blotting

In the aorta, expressions of endothelial nitric oxide synthase (eNOS), caveolin-1 (cav-1), caveolin-3 (cav-3), heat shock protein 90 (hsp90), manganese superoxide dismutase (MnSOD) and housekeeping protein actin were evaluated in tissue homogenates using SDS-PAGE and immunoblot analysis with chemiluminescent detection.[17]

## Transmission electron microscopy

Cleaned aortic rings 3-mm long were immersion fixed in 2% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (Serva, Heidelberg, Germany) (pH 7.4) for 3 h at 4°C. After washing, the samples were postfixed in 1% $OsO_4$ for

30 min, dehydrated in a series of alcohol, infiltrated in propylene oxide and embedded to Epon 812 (Sigma-Aldrich). Ultrathin sections using the Ultramicrotome (LKB, Cambridge, UK) were cut with a diamond knife and mounted on nickel grids. Toluidine blue stained sections (1-$\mu$m thick) were examined under a light microscope and appropriate areas of tissue were selected for cutting thin sections. Sections were stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope Tesla BS 500 (Brno, Czech Republic).

## Data analysis

The relaxation responses to acetylcholine are expressed in mN/mg tissue. Statistical analyses were performed by using two-way analysis of variance with Bonferroni post test. Statistical significance was indicated at $P$ less than 0.05.

## Results

### Effect of rosmarinic acid on diabetic markers

Streptozocin-diabetes-induced characteristic changes were found in body weight gain, daily water consumption, and blood glucose levels. Rosmarinic acid did not influence the body weight gain or blood glucose levels of either the control or diabetic rats. Neither was blood pressure influenced by diabetes or administration of rosmarinic acid. However, the heart rate of diabetic rats was lower than that of controls ($P < 0.05$). Rosmarinic acid administration did not exert a significant effect on heart rate of control or diabetic animals. Rosmarinic acid did not affect enhanced daily urine production in diabetic rats. However, the daily water consumption was decreased by rosmarinic acid administration in both control (**$P < 0.01$) and diabetic groups (***$P < 0.001$) (Table 1).

Plasma levels of the oxidative stress indicator TBARS were increased in diabetic rats compared with controls (**$P < 0.01$). Rosmarinic acid had no effect on control values, yet it depressed those of diabetic rats (*$P < 0.05$ vs D; Table 1).

### Rat isolated aorta

As shown in Figure 1, diabetes blunted the endothelium-dependent relaxations (**$P < 0.01$ vs control and R groups). We also observed a decreased sensitivity to acetylcholine – $pD_2$ (C 6.85 ± 0.09; D 6.30 ± 0.08; *$P < 0.05$). In control rats, rosmarinic acid did not influence relaxation significantly ($pD_2$ 6.93 ± 0.07). When administered to diabetic rats, rosmarinic acid improved the relaxation to values not different from the control values, with $pD_2$ 6.62 ± 0.07. Endothelium-independent relaxation of the aorta induced

**Table 1** Effect of rosmarinic acid on characteristics of diabetes

|  | Group C | Group R | Group D | Group DR |
|---|---|---|---|---|
| Glycaemia (mmol/l[1]) | $6.0 \pm 0.2$ | $6.2 \pm 0.1$ | $29.57 \pm 0.4$*** | $29.84 \pm 0.1$*** |
| Body weight (g) | $420.0 \pm 22.5$ | $415 \pm 7.8$ | $243.6 \pm 21.3$*** | $261.4 \pm 6.7$*** |
| Blood pressure (mmHg) | $99.2 \pm 4.5$ | $110.1 \pm 4.8$ | $101.5 \pm 4.0$ | $107.2 \pm 6.1$ |
| Heart rate (beats/min) | $316.9 \pm 12.5$ | $337.1 \pm 14.3$ | $269.8 \pm 6.9$** | $283.5 \pm 8.4$* |
| Daily water consumption (ml) | $31.9 \pm 0.5$ | $27.7 \pm 0.5$*** | $175.9 \pm 4.6$*** | $155.5 \pm 4.5$ˣˣˣ *** |
| Daily urine (ml) | $0.4 \pm 0.9$ | $0.5 \pm 0.1$ | $2.0 \pm 0.2$*** | $1.9 \pm 0.2$*** |
| TBARS (mmol/mg protein) | $3.3 \pm 0.3$ | $3.2 \pm 0.2$ | $4.5 \pm 0.1$** | $3.9 \pm 0.2$ˣ |

C, control group; R, control group treated with rosmarinic acid (50 mg/kg per day); D, diabetic group; DR, diabetic group treated with rosmarinic acid (50 mg/kg per day); TBARS, thiobarbituric acid reactive substances. *$P < 0.05$ vs C; **$P < 0.01$ vs C; ***$P < 0.001$ vs C; ˣ$P < 0.05$ vs D; ˣˣˣ$P < 0.001$ vs D.
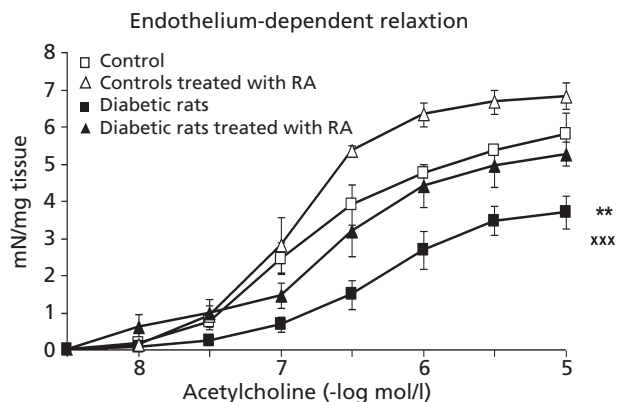


**Figure 1** Responses of the phenylephrine ($10^{-6}$ mol/l)-precontracted rat thoracic aorta to acetylcholine ($10^{-8}$–$10^{-5}$ mol/l), expressed in mN/mg tissue. Rosmarinic acid (RA) administered at 50 mg/kg per day. Data are means ± SEM of seven experiments. **$P < 0.001$ vs C; ˣˣˣ$P < 0.0001$ vs R.

by sodium nitroprusside was not influenced by diabetes or administration of rosmarinic acid (not shown).

## Inflammatory cytokines

Although experimental diabetes was not associated with increased levels of IL-6 in the aorta, treatment of diabetic rats with rosmarinic acid led to significantly decreased levels of IL-6 mRNA compared with controls (*$P < 0.05$, Figure 2). IL-1β and TNF-α were overexpressed more than twofold in the aortas from untreated diabetic rats (*$P < 0.05$). Rosmarinic acid alone had no effect on the expression of these cytokines, but it completely prevented the upregulation of IL-1β and TNF-α in diabetic rats (Figure 2).

## Components of the endothelin pathway

The expression of endothelin-1 precursor ET-1 was increased more than sixfold in the untreated diabetic rats (*$P < 0.05$; Figure 2). Rosmarinic acid treatment had no

effect on the levels of ET-1 mRNA in healthy rats nor did it prevent ET-1 upregulation in diabetic rats. Expression of the smooth muscle-specific $ET_A$ receptor was not altered by experimental diabetes, however rosmarinic acid treatment led to significant downregulation of the $ET_A$ receptor mRNA in both healthy and diabetic rats (*$P < 0.05$; Figure 2). $ET_B$ receptor mRNA levels only tended to be increased in the diabetic group ($P = NS$). Rosmarinic acid treatment decreased $ET_B$ receptor expression in healthy rats (*$P < 0.05$ vs C) and decreased expression of $ET_B$ receptor in the diabetic group (#$P < 0.05$ vs D). Finally, the expression of ECE-1 was increased in the diabetic group compared with controls (*$P < 0.05$). Rosmarinic acid prevented the upregulation of ECE-1 (#$P < 0.05$ vs D) (Figure 2).

## Western blot analysis of isolated aorta

Diabetic rats had significantly increased levels of eNOS protein in the aorta (*$P < 0.05$; Figure 3). Rosmarinic acid alone had no effect and it did not alter the upregulation associated with diabetes. Expression of hsp90, a positive allosteric modulator of eNOS, was not changed by diabetes or treatment with rosmarinic acid. We also evaluated the expression of cav-1, a negative modulator of eNOS activity. Cav-1 levels were not altered by diabetes or by rosmarinic acid. Smooth muscle-specific cav-3 expression was not changed. Expression of the superoxide-quenching enzyme MnSOD tended to be increased in diabetic groups, but the increase did not reach statistical significance and rosmarinic acid had no effect on the level of this protein (Figure 3).

## Transmission electron microscopy

Electron microscopic analysis of the endothelium of the aorta of 5-month-old healthy Wistar rats revealed generally an intact structure of the monolayer. However, weak subcellular alterations of endothelial cells were locally observed, characterized by irregular cellular shape, increased formation of luminal protrusions and endothelial denudation. These abnormalities demonstrated age-related endothelial

(a)



(b)
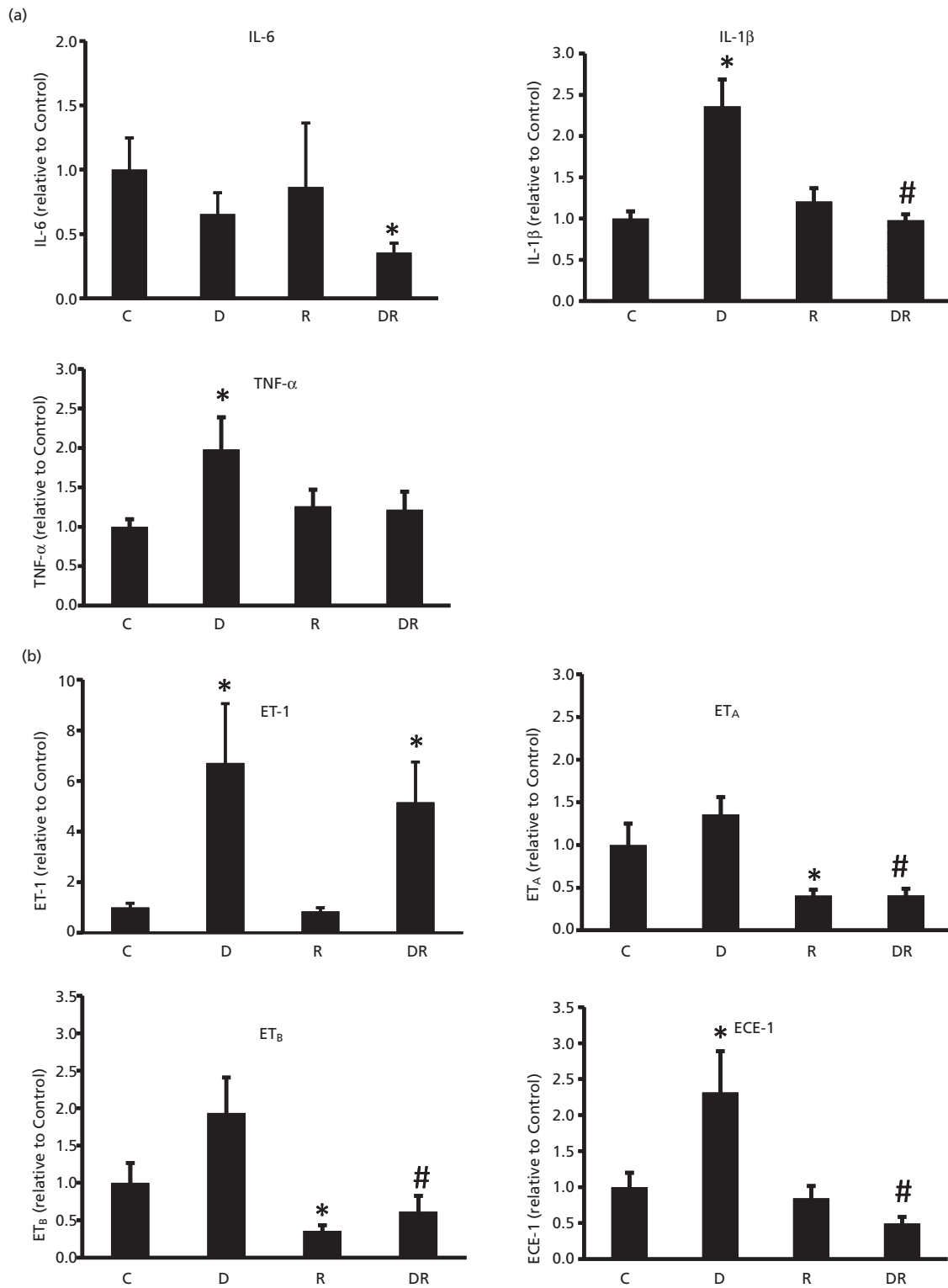


**Figure 2** Expression of (a) inflammatory cytokines and (b) endothelin-1 pathway components in the thoracic aorta evaluated by real-time PCR. IL-6, interleukin-6; IL-1β, interleukin-1β; TNF-α, tumour necrosis factor-α; ET-1, preproendothelin-1; $ET_A$, endothelin receptor type A; $ET_B$, endothelin receptor type B; ECE-1, endothelin converting enzyme-1. Beta actin was used as a housekeeping gene. C, controls; D, diabetic rats; R, controls treated with rosmarinic acid (50 mg/kg per day); DR, diabetic rats treated with rosmarinic acid (50 mg/kg per day). (a) *$P < 0.05$ vs C, #$P < 0.05$ vs R. (b) *$P < 0.05$ vs C, #$P < 0.05$ vs D.
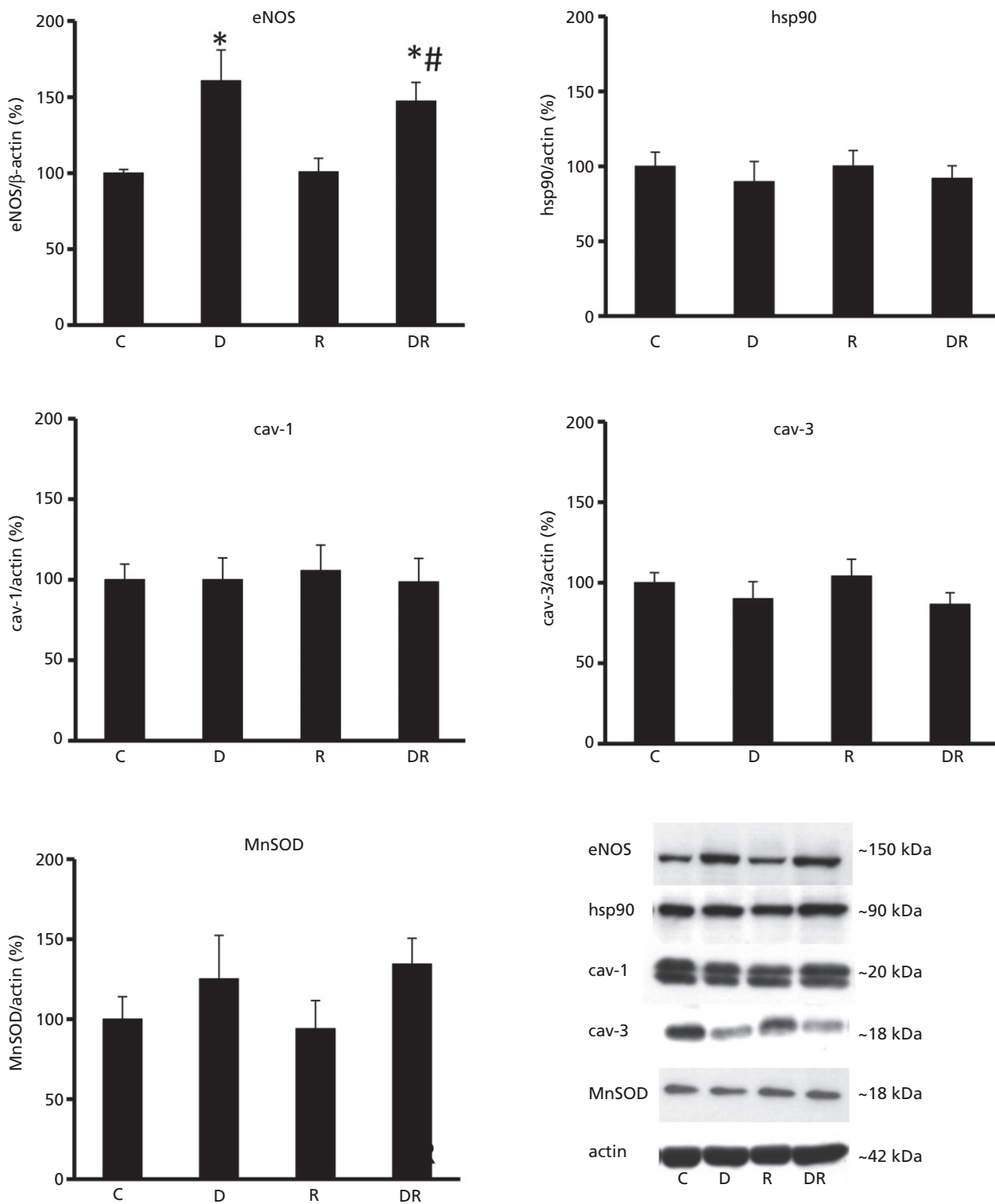
**Figure 3** Quantification of Western blot analysis of the thoracic aorta. eNOS, endothelial nitric oxide synthase; hsp90, heat-shock protein-90; cav-1, caveolin-1; cav-3, caveolin-3. Actin was used as a loading control. C, controls; D, diabetic rats; R, controls treated with rosmarinic acid (50 mg/kg per day); DR, diabetic rats treated with rosmarinic acid (50 mg/kg per day). *$P < 0.05$ vs C, #$P < 0.05$ vs R.
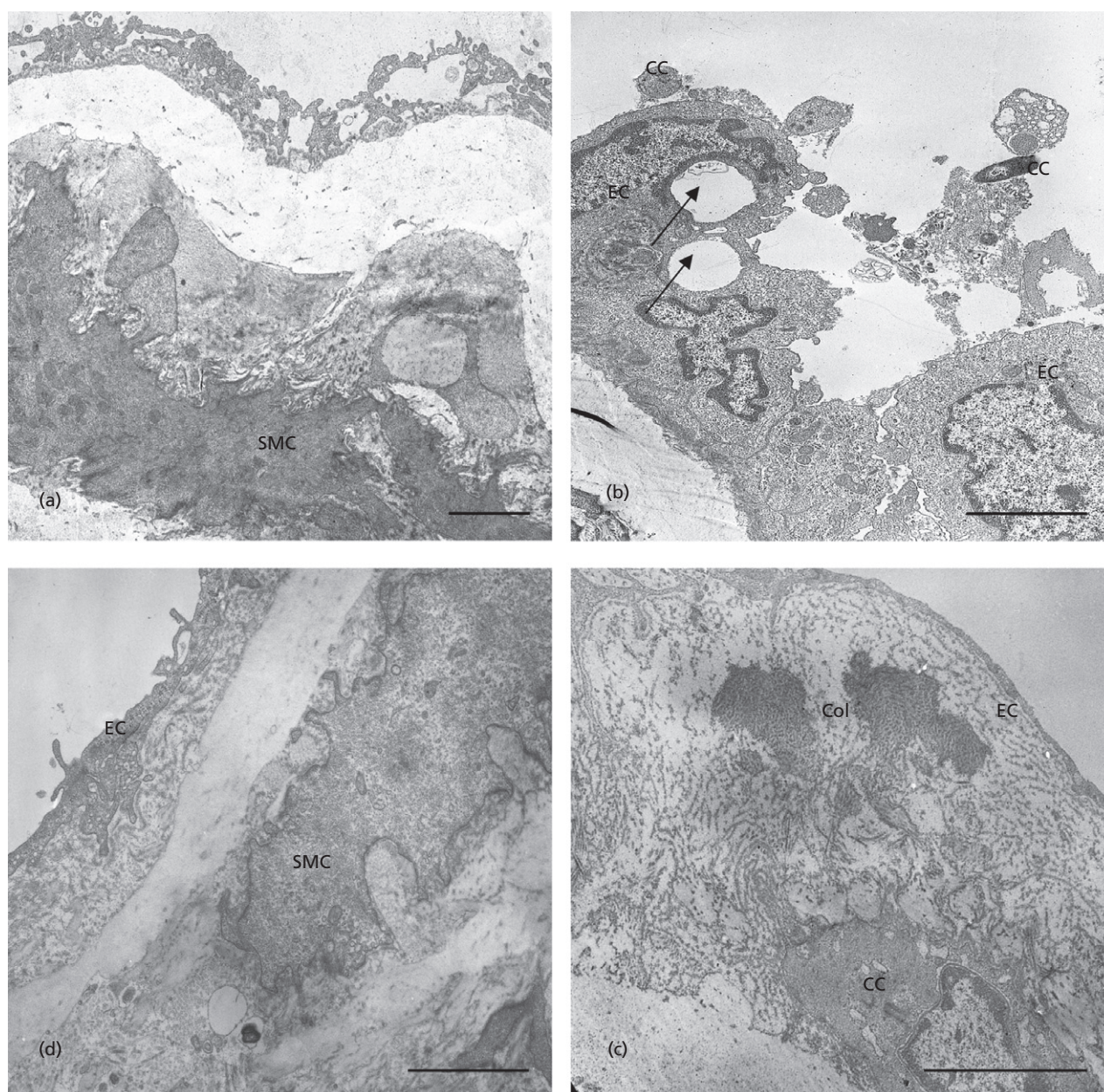
**Figure 4** Electron microgaphs of the thoracic aorta of control Wistar rats (a), diabetic rats (b, c) and diabetic rats treated with rosmarinic acid (d). EC, endothelial cells; SMC, smooth muscle cells; CC, blood circulating cells; Col, collagen fibres; arrow, widened intercellular junctions containing myelinized structures. Magnification: Bar = 1 μm.

cell alterations. We did not observe abnormalities in the ultrastructure of smooth muscle cells and the aortic wall morphology in control animals (Figure 4a). On the other hand, chronic diabetes mellitus affected adhesive properties and permeability of the endothelium, manifested by adhesion of circulating blood cells to the endothelium and extension of interendothelial connection (Figure 4b). Increased presence of collagen fibres in the subendothelium and irregular location of smooth muscle cells, locally with subcellular abnormalities in the subendothelium and tunica

media (Figure 4c), characterize diabetes mellitus-induced structural remodelling of the aortic wall. An unimpaired endothelial monolayer structure was observed in the aorta of diabetic rats treated with rosmarinic acid, suggesting protective effects of the compound. In this group of animals, in contrast to the aorta of diabetic rats, the endothelial layer was more compact, comparable with the aorta of control nondiabetic animals. Moreover, endothelial cells of irregular shape and cells filled with numerous pinocytic vesicles were occasionally found, indicating active

transport within the endothelium. In spite of the preserved endothelial integrity, we observed collagen fibres and leucocytes in the subendothelium of the aorta of diabetic rats treated with rosmarinic acid (Figure 4d).

## Discussion

Administration of streptozocin to rats evoked typical signs of diabetes – hyperglycaemia, body weight reduction, increase in water consumption and daily urine output. In the cardiovascular system, diabetes induced a decrease in heart rate but blood pressure was not affected, in accordance with Hicks *et al.*[18] who explained heart rate changes in diabetic rats by diabetes-induced changes in autonomic nervous control of cardiac function. None of the typical manifestations of diabetes were influenced by rosmarinic acid administration, except a reduction in daily water consumption, a phenomenon which would deserve further investigation.

In agreement with others, we found blunted endothelium-dependent relaxation of the aorta, yet unchanged endothelium-independent relaxation, indicating diabetic functional injury of the endothelium rather than of vascular smooth muscle.[19–21] Further, subcellular abnormalities of the endothelium indicated endothelial dysfunction, injured physical endothelial barrier and inflammatory processes. These may result in diapedesis of leucocytes and macrophages, and possibly in affecting lipid transport – mechanisms supporting stimulation of vascular wall remodelling. Inflammation induced by diabetes was manifested also by overexpression of proinflammatory cytokines IL-1β and TNF-α in the aortic tissue.

The obtained results showed protective effects of rosmarinic acid on vascular changes induced by streptozocin-diabetes in rats. Natural polyphenols are accepted to have a high antioxidant activity. These properties are desirable in prevention/treatment of chronic complications of diabetes, affecting besides others diabetic vasculopathies. Protective effects of natural antioxidants are explained mainly by their effect on oxidative stress, which is known to play a role in diabetes (see Maritim *et al.*[22]). In our previous experiments, we found rosmarinic acid to exert antioxidant activity in-vitro and also ex-vivo in the experimental model of rat mesenteric ischaemia/reperfusion (I/R), where rosmarinic acid suppressed chemiluminescence of intestinal tissue increased by I/R.[23,24] Rosmarinic acid had also a protective effect against cyclophosphamide toxicity in mice.[25] Confirming our suggestions, rosmarinic acid administered to diabetic rats protected their aortas against diabetes-induced endothelial injury. Rosmarinic acid was administered orally, as it is well absorbed from the gastrointestinal tract and was found to be effective after oral supplementation to humans.[26,27]

After rosmarinic acid administration, we observed an amelioration of relaxant responses to acetylcholine, which was a manifestation of improved vascular endothelial function. In correlation, rosmarinic acid-supplementation also suppressed hyperglycaemia-induced subcellular alterations of the aortal endothelium. In contrast with diabetic aortas, the endothelial layer of rosmarinic acid-treated aortas was more compact, comparable with the aorta of nondiabetic rats. We assumed that the observed vascular alterations found in diabetic aortas were a consequence of detrimental effect of oxidative stress and thus they could be reduced by rosmarinic acid administration. Indeed, streptozocin-diabetes was documented by typical signs of oxidative stress in plasma (increase in TBARS), which is in agreement with several experimental as well clinical reports.[28–30] Reduction of increased levels of TBARS by natural polyphenols was also reported.[31] Yet we failed to find any significant increase in the MnSOD expression in diabetic aortas. Superoxide dismutase (SOD) is an enzyme that primarily contributes to cellular defences against oxidative stress, and plays a role in the conversion of superoxide anion to $H_2O_2$. Expression of MnSOD is much lower than that of CuZn-SOD and extracellular SOD in blood vessels, nevertheless MnSOD plays a critical role in protection against mitochondrial damage induced by oxidative stress.[32] During the situations involving oxidative stress, expression of MnSOD has a biphasic time course.[33] This could be the reason why we did not observe marked changes in expression of MnSOD.

The injured endothelium-dependent relaxation of the diabetic aorta is believed to be the consequence of a decreased bioavailability of nitric oxide (NO) – an important and widespread signalling molecule. Long-lasting hyperglycaemia and dyslipidaemia during diabetes, leading to an overproduction of reactive oxygen species, have a major share in the decline in NO bioavailability. One of the reasons of decreased NO bioavailability accompanying endothelial dysfunction could also lie in an impairment of eNOS expression. However, conflicting reports exist regarding altered expression of eNOS in diabetes. Under hyperglycaemic conditions, human aortic endothelial cells have been shown to have either reduced or increased eNOS expression.[34–36] In endothelial cell cultures of mouse microvessels, high glucose levels led to increased eNOS.[37] In our experiments, diabetes induced an increase in eNOS expression and no change in its regulatory proteins cav-1, cav-3, or 28hsp90, which could affect NO production by eNOS. Cai *et al.*[38] and Leo *et al.*[39] also demonstrated that in the large vessels, NO synthesis was significantly impaired in diabetes but eNOS expression was significantly increased. In spite of increased eNOS expression, decreased NO synthesis could be caused by decreased proportion of eNOS present as a functionally coupled dimer or decreased activation of eNOS by decreasing the level of phosphorylation of Akt.[40]

Phosphorylation and dephosphorylation belong to major post-translational regulatory influences on eNOS activity. Bovine eNOS phosphorylation at serine residue 1179 (S1179), corresponding to S1177 in human eNOS, was reported to increase eNOS enzymatic activity and NO production, while proinflammatory cytokines such as interleukins and TNF-α decreased eNOS phosphorylation, thus inhibiting NO production.[41,42] Streptozocin-diabetes in our experiments induced an increase in mRNA of proinflammatory mediators IL-1β and TNF-α in the aorta, which was reduced to control values in the aortas of rosmarinic acid-treated diabetic rats. These findings support the anti-inflammatory properties of rosmarinic acid reported by several other authors. For instance, al-Sereiti *et al.*[26] found rosmarinic acid to increase the production of prostaglandin E2, reduce the production of leukotriene B4 in human polymorphonuclear leucocytes, and inhibit the complement system. Rosmarinic acid inhibited cyclooxygenase-2 expression in mouse macrophages.[43] Moreover, rosmarinic acid reduced lipopolysaccharide-induced liver injury in D-galactosamine-sensitized mice by inhibition of the expression of important inflammatory mediators, including the cell adhesion molecules intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1).[44] Rosmarinic acid administration to ovalbumin-sensitized mice inhibited protein levels and mRNA expressions of interleukins, IL-1β, IL-6 and TNF-α in the nasal mucosa tissue and spleen.[45] We speculate that rosmarinic acid could protect the endothelium and thus ameliorate endothelium-dependent relaxation by inhibiting inflammatory reactions in the aorta.

Since the endothelin-1 pathway might also be involved in endothelial dysfunction, in our experiments we investigated whether hyperglycaemia regulated expression of components of this pathway in the aorta. ET-1 is a vasoconstrictor, proinflammatory and proliferative endothelial cell-derived peptide that plays a significant role in the regulation of vascular function. The expression and functional effects of ET-1 and its receptors become markedly altered during the development of cardiovascular diseases. Oxidative stress was found to increase ET-1 generation and autocrine ET-1 activity in vascular smooth muscle cells, a mechanism that might contribute to endothelial dysfunction.[46] Further, ET-1 upregulation was reported in people with diseases connected with oxidative stress, such as type II diabetes mellitus, central obesity, and hypertension.[47] In agreement with Su *et al.*,[19] we found upregulated mRNA expression of ET-1 and ECE-1 in the thoracic aorta of diabetic rats. Although rosmarinic acid did not statistically influence upregulation of ET-1 mRNA found in diabetic aortas, it decreased $ET_A$ and $ET_B$ receptor expression and the expression of ECE-1. These results were compatible with the hypothesis that production and receptor-mediated effects of ET-1 are decreased after rosmarinic acid-administration, contributing to an improvement of endothelial function and of remodelling.

## Conclusions

Our results showed that the 10-week lasting streptozocin-diabetes in rats was accompanied by oxidative stress and inflammatory processes. We documented increased plasma levels of TBARS and injury of the endothelial function of the aorta, involving blunted acetylcholine-evoked relaxation connected with ultrastructural alterations, upregulation of inflammatory cytokines and a stimulated endothelin pathway. Rosmarinic acid protected aortic endothelium-dependent relaxation and the ultrastructure against diabetes-induced damage. The antioxidant and anti-inflammatory effects of rosmarinic acid seemed to participate in the mechanism of this protection.

## Declarations

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

### Funding

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