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RESEARCH PAPER

# Oxygen-derived free radicals mediate endothelium-dependent contractions in femoral arteries of rats with streptozotocin-induced diabetes

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**Background and purpose:** The present experiments were designed to study the contribution of oxygen-derived free radicals to endothelium-dependent contractions in femoral arteries of rats with streptozotocin-induced diabetes.
**Experimental approach:** Rings with and without endothelium were suspended in organ chambers for isometric tension recording. The production of oxygen-derived free radicals in the endothelium was measured with 2′,7′-dichlorodihydrofluorescein diacetate using confocal microscopy. The presence of protein was measured by western blotting.
**Key results:** In the presence of L-NAME, the calcium ionophore A23187 induced larger endothelium-dependent contractions in femoral arteries from diabetic rats. Tiron, catalase, deferoxamine and MnTMPyP, but not superoxide dismutase reduced the response, suggesting that oxygen-derived free radicals are involved in the endothelium-dependent contraction. In the presence of L-NAME, A23187 increased the fluorescence signal in femoral arteries from streptozotocin-treated, but not in those from control rats, confirming that the production of oxygen-derived free radicals contributes to the enhanced endothelium-dependent contractions in diabetes. Exogenous $H_2O_2$ caused contractions in femoral arterial rings without endothelium which were reduced by deferoxamine, indicating that hydroxyl radicals contract vascular smooth muscle and thus could be an endothelium-derived contracting factor in diabetes. The reduced presence of Mn-SOD and the decreased activity of catalase in femoral arteries from streptozotocin-treated rats demonstrated the presence of a redox abnormality in arteries from rats with diabetes.
**Conclusions and implications:** These findings suggest that the redox abnormality resulting from diabetes increases oxidative stress which facilitates and/or causes endothelium-dependent contractions.
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**Keywords:** endothelium-dependent contractions; endothelium-derived contracting factor; oxidative stress; oxygen-derived free radicals; streptozotocin-induced diabetes

**Abbreviations:** DCF, 2′,7′-dichlorodihydrofluorescein diacetate; DETCA, diethyldithiocarbamic acid; L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester hydrochloride; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride; S18886, terutroban, 3-[(6-amino-(4-chlorobenzenesulphonyl)-2-methyl-5,6,7,8-tetrahydronapht]-1-yl) propionic acid; SC19220, 2-acetylhydrazide 10 (11 H)-carboxylic acid 8-chloro-dibenz [b,f] [1,4] oxazepine-10 (11 H)-carboxylic acid; SOD, superoxide dismutase; STZ, streptozotocin; Tiron, 4,5-dihydroxy-1,3-benzene-disulphonic acid; TP receptor, thromboxane prostanoid receptor

## Introduction

The augmented release of endothelium-derived contracting factor, as well as decreased nitric oxide-mediated relaxation, is a hallmark of endothelial dysfunction (Cohen, 2002; Vanhoutte *et al.*, 2005). This contracting factor is derived from endothelial cyclooxygenase and activates thromboxane prostanoid receptors of vascular smooth muscle (Lüscher and Vanhoutte, 1986; Auch-Schwelk *et al.*, 1990; Yang *et al.*, 2004b; Vanhoutte *et al.*, 2005). Endothelium- and cyclo-oxygenase-dependent contractions are prominent in the aorta of the spontaneously hypertensive rat (Lüscher and Vanhoutte, 1986; Yang *et al.*, 2004a, b; Tang *et al.*, 2007), the abdominal aorta of the diabetic rabbit (Tesfamariam *et al.*, 1989) and the femoral artery of the diabetic rat (Shi *et al.*, 2007). The endothelium-derived contracting factor can be prostacyclin, thromboxane $A_2$, endoperoxides, prostaglandin $E_2$, hydroxyl radicals or superoxide anions depending on

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the animal model, the preparation and the agonist used (Katusic and Vanhoutte, 1989; Tesfamariam *et al.*, 1989; Auch-Schwelk *et al.*, 1990; Yang *et al.*, 2004a, b; Gluais *et al.*, 2005, 2006; Shi *et al.*, 2007). Oxygen-derived free radicals facilitate endothelium-dependent contractions, and antioxidants effectively decreased them in the aorta of spontaneously hypertensive rats (Tesfamariam and Cohen, 1989; Yang *et al.*, 2002, 2003b; Tang *et al.*, 2007). These findings indicate that oxygen-derived free radicals and oxidative stress are directly involved in the production of endothelium-derived contracting factor.

Oxidative stress is increased in diabetic patients and animals (De Vriese *et al.*, 2000; Alp *et al.*, 2003; Maritim *et al.*, 2003; Taniguchi *et al.*, 2005). This is due to the increased production of free radicals and/or impaired antioxidant defences. Mechanisms underlying the increased oxidative stress in diabetes include activation of transcription factors, protein kinase C, glucose oxidation and augmented production of advanced glycated end products (Bucala *et al.*, 1991; Alp *et al.*, 2003; Cosentino *et al.*, 2003; Wong *et al.*, 2003; Sheu *et al.*, 2005; Ho *et al.*, 2006). The evidence suggesting a reduced antioxidant defence in arteries from animals with streptozotocin (STZ)-induced diabetes includes the reduced activity of superoxide dismutase (SOD) and catalase, and a decreased presence or bioavailability of glutathione and nitric oxide (Mekinova *et al.*, 1995; Aragno *et al.*, 1999; Kedziora-Kornatowska *et al.*, 2000; Rauscher *et al.*, 2001; Maritim *et al.*, 2003).

The effect of STZ-induced diabetes on nitric oxide-mediated vasodilatation is still controversial; it has been reported as normal in the aorta (Head *et al.*, 1987; Harris and MacLeod, 1988; Mulhern and Docherty, 1989), mesenteric artery (Harris and MacLeod, 1988) and femoral artery (Wigg *et al.*, 2001), as blunted in aorta (Pieper *et al.*, 1997; Vallejo *et al.*, 2000), mesenteric artery (Heygate *et al.*, 1995; Vallejo *et al.*, 2000; Shi *et al.*, 2007) and femoral artery (Shi *et al.*, 2006) or augmented in aorta (Altan *et al.*, 1989). The production of vasoconstrictor prostaglandins is augmented in diabetic animals (aorta (Tesfamariam *et al.*, 1989), renal artery (Pflueger *et al.*, 1999) and femoral artery (Shi *et al.*, 2007)). Previous work from our laboratory has demonstrated an augmented endothelium- and cyclooxygenase-dependent contraction in the femoral artery of rats with STZ-induced diabetes. Furthermore, the nature of the cyclooxygenase products involved varies in the course of the disease (Shi *et al.*, 2007). Taken together, these findings prompt the hypothesis that oxygen-derived free radicals play a key role in the occurrence of endothelium-dependent contractions in this model of diabetes. Therefore, the present experiments were designed to study the contributions of reactive oxygen species to, and the effects of antioxidants (Figure 1) on, endothelium-dependent contractions of femoral arteries of rats with STZ-induced diabetes.

## Methods

*Experimental animals*

All animal procedures and protocols were approved by the institutional animal care committee.

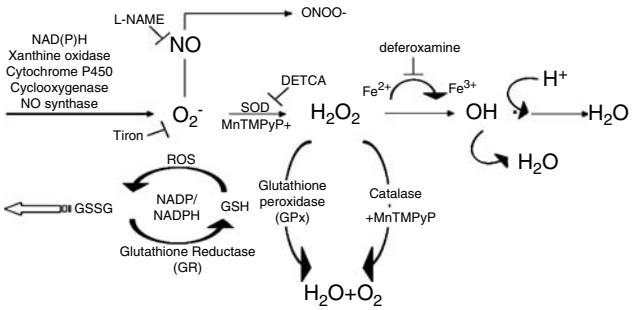


**Figure 1** Formation of oxygen-derived free radicals. Superoxide anions ($O_2^-$) can be generated from molecular oxygen, or by the action of xanthine oxidase, or of various enzymes. $O_2^-$ can be scavenged by NO. It can also be converted to highly reactive oxygen species as peroxynitrite ($ONOO^-$) when combined with NO, or $H_2O_2$ by SOD. $H_2O_2$ can be transformed to hydroxyl radicals by ferrous ions or converted to $H_2O$ by catalase and glutathione. Tiron scavenges $O_2^-$ inside cells. DETCA inhibits SOD. Deferoxamine is an iron chelator that scavenges hydroxyl radicals. L-NAME inhibits NO synthase. MnTMPyP mimics the combined effect of SOD and catalase. DETCA, diethyldithiocarbamic acid; GSH, glutathione; GSSG, glutathione disulphide; $H_2O_2$, hydrogen peroxide; L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester hydrochloride; MnTMPyP, Mn(III)-tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride; NO, nitric acid; ROS, reactive oxygen species; SOD, superoxide dismutase; tiron, 4,5-dihydroxy-1,3-benzenedisulphonic acid.

All experiments used the isolated femoral arteries of Sprague–Dawley rats. Diabetes was induced in 16-week-old male rats (450–600 g) by a single administration of STZ (55 mg $kg^{-1}$, injected in the tail vein) dissolved in citric acid–trisodium citrate (0.2 mM) buffer (pH 4.0–4.5). Seventy-two hours later, after overnight starvation, rat tail blood samples were obtained and the glucose concentration was measured using a one-touch glucometer (LifeScan Inc., Milpitas, CA, USA). Induction of diabetes was considered successful when the glucose level was higher than 16.6 mM (day 0). Non-diabetic control rats were injected with vehicle solution alone and kept under identical conditions. The animals were housed in the Laboratory Animal Unit of the University of Hong Kong, fed with regular chow and given free access to water. Twelve weeks later (day 84), they were anaesthetized with intraperitoneal pentobarbitone sodium (70 mg $kg^{-1}$), anticoagulated with heparin (0.5 U $kg^{-1}$) and exsanguinated. Non-fasting glucose level was measured on the day of death. Control rats with blood glucose higher than 11.1 mM were excluded from the study.

*Functional studies in isolated tissues*

*Preparation of the arteries.* The femoral arteries were dissected free, excised and placed into ice-cold modified Krebs–Ringer solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, $KH_2PO_4$ 1.18, calcium disodium EDTA 0.026 and glucose 11.1 (control solution). The blood vessels were cut into rings (1.5–2 mm in length). When arterial rings without endothelium were needed, the arteries were perfused with 1 ml of saponin solution (1 mg $ml^{-1}$, diluted with Krebs–Ringer solution; Samata *et al.*, 1986) before the rings were cut. The rings were suspended in organ chambers containing control

solution (37 °C) aerated with 95% $O_2$ and 5% $CO_2$. They were connected to a force transducer (Powerlab Model ML785 and ML119). Changes in isometric tension were recorded. The rings were stretched progressively to their optimal resting tension (1.0 g) determined in preliminary experiments and were allowed to equilibrate for 90 min before experimentation. Changes in tension were expressed as a percentage of the reference contraction to 60 mM KCl, obtained at the beginning of the experiment.

To study endothelium-dependent contractions, all experiments were performed under quiescent conditions in the presence of L-NAME ($N^{\omega}$-nitro-L-arginine methyl ester hydrochloride, $3 \times 10^{-4}$ M; Auch-Schwelk *et al*., 1992; Yang *et al*., 2004a; Tang *et al*., 2005). The drugs were incubated in the organ chamber for 30 min before the administration of the calcium ionophore A23187 in a cumulative manner.

Concentration–response curves to hydrogen peroxide were obtained in a cumulative manner in rings without endothelium.

*Western blotting*

Femoral arteries were dissected free and cleaned of connective tissue. To remove the remaining blood cells, arteries were rinsed three times with Krebs–Ringer solution. Blood vessels were homogenized at 4 °C in lysis buffer (Tris–HCl 10 mM, NaCl 150 mM, EDTA 1 mM, sodium pyrophosphate 25 mM, β-glycophosphate 1 mM, sodium orthovanadate 1 mM, leupeptin 2.1 μM, aprotinin 1 mg ml$^{-1}$, phenylmethylsulphonyl fluoride 1 mM and Triton X-100 1%) and incubated on ice for 10 min. Samples were then centrifuged at 3000 *g* for 10 min at 4 °C, and the supernatant was collected. Protein concentrations were determined using the Bradford assay. Lysates were used immediately or stored at −70 °C.

In all immunoblot experiments, equal amounts of protein were loaded in each lane of SDS–polyacrylamide gel electrophoresis gels. Molecular masses of immunoreactive bands were determined by loading biotinylated molecular mass standards (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, the proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories) and blocked with 5% non-fat dry milk in Tris-buffered saline (Tris–HCl 20 mM, NaCl 140 mM, pH 7.4) for an hour. Membranes were then incubated with the suggested dilution of corresponding primary antibodies overnight at room temperature, followed by three 10 min wash in 0.1% Tween-20–Tris-buffered saline. Blots were incubated with secondary antibodies at 1:1000 dilutions for 2 h at room temperature membrane, and were then washed three times for 10 min in Tween-20–Tris-buffered saline solution. Membranes were then incubated with enhanced chemiluminescence reagent (Amersham Biosciences, Buchinghamshire, UK) for 1 min and exposed to X-ray films (Fuji Super RX Medical X-ray films; Fuji Photo Film, Dusseldorf, Germany) for the recommended optimal time, depending on the signal strength. Software for electrophoresis analysis (Multi-analyst; Bio-Rad Laboratories) was used for densitometric analysis of immunoreactive bands. The presence of protein was normalized to β-actin and expressed as percentage of control.

*Fluorescence studies*

Reactive oxygen species were measured with DCF (2′,7′-dichlorodihydrofluorescein diacetate). Non-ionized DCF is membrane permeable and, therefore, diffuses readily into cells (Nasr-Esfahani *et al*., 1990). The fluorescence experiments were performed in a dark room. Rings of femoral artery were loaded with DCF solution ($10^{-5}$ M) for 15 min followed by incubation with tested agents for 30 min. After rinsing three times in control solution, the rings were opened longitudinally and placed with the endothelial layer down on a microscope stage. Reactive oxygen species were measured using a confocal laser scanning microscope. The intensity of the production was recorded after excitation at 488 nm and emission at 510 nm, using the appropriate software (Laser Sharp 2000, Bio-Rad Cell Science Division, Hemel Hempstead, UK). The production of reactive oxygen species measured from nine randomly related fields was averaged in the endothelium and the vascular smooth muscle of each preparation (Supplementary Information). To correct for nonspecific changes in fluorescence, the background signal was subtracted from the measurements and only the changes induced by A23187 are reported and discussed.

*Enzymatic activities*

Femoral arteries were dissected and cleaned free of connective tissue. The preparations were homogenized at 4 °C in lysis buffer (50 mM potassium phosphate containing 1 mM EDTA). The samples were centrifuged at 10 000 *g* for 10 min at 4 °C, and the supernatant was collected. Protein concentrations were determined using the Bradford assay. Lysates were used immediately or stored at −70 °C. The level of glutathione and catalase activity was measured following the instructions of the manufacturers.

*Data analysis*

Data are presented as means ± s.e.m.; *n* refers to the number of rats. Concentration–response curves are shown as such or as area under the curve. Statistical analysis was done by one-way analysis of variance followed with *post hoc* comparison using the Bonferroni test, or by two-way analysis of variance, as appropriate (Prism version 3a, GraphPad Software, San Diego, CA, USA). Differences were considered to be statistically significant when $P$ was less than 0.05.

*Drugs*

A23187, anti-β-actin monoclonal antibody, catalase, deferoxamine mesylate salt, DCF, DETCA (diethyldithiocarbamic acid), indomethacin, L-NAME, saponin, SC19220 (2-acetylhydrazide 10 (11 H)-carboxylic acid 8-chloro-dibenz [b,f] [1,4] oxazepine-10 (11 H)-carboxylic acid), STZ, SOD, tiron (4,5-dihydroxy-1,3-benzenedisulphonic acid) were purchased from Sigma Chemical Company (St Louis, MO, USA). Anti-catalase polyclonal antibodies were purchased from the Abcam Company (Cambridge, UK). Anti-Mn-SOD antibodies were purchased from Bethyl Laboratories Inc. (Montgomery, TX, USA). Anti-CuZn-SOD antibodies were purchased from BioVision (Mountain View, CA, USA).

Glutathione and catalase commercial kits were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Heparin was purchased from LEO Pharm (Ballerup, Denmark). MnTMPyP (Mn(III)tetrakis(1-methyl-4-pyridyl)-porphyrin pentachloride) was purchased from Calbiochem (San Diego, CA, USA). Hydrogen peroxide (30%) was purchased from Merck (Darmstadt, Germany). Secondary antibodies (ECL anti-rabbit immunoglobulin G and ECL anti-mouse immunoglobulin G) were obtained from Amersham Biosciences. S18886 (terutroban, (3-[(6-amino-(4-chlorobenzenesulphonyl)-2-methyl-5,6,7,8-tetrahydro-napht]-1-yl) propionic acid) was a kind gift of the Institut de Recherche Servier (Suresnes, France). Drug concentrations are given as final molar concentrations in the bath solution.

## Results

*General condition of animals*

At the beginning of the study, the body weights were comparable in the control and STZ-treated groups. Twelve weeks after the injection of STZ, control rats gained weight, while the STZ-treated rats lost weight significantly. The glucose level in the STZ-treated group was significantly higher than that in the controls (Table 1).

*Endothelium-dependent contractions in arterial rings*

In the presence of L-NAME ($3 \times 10^{-4}$ M), the calcium ionophore A23187 induced a concentration-dependent contraction in rings with endothelium, which was not observed in preparations without endothelium. The endothelium-dependent response was significantly larger in arteries from STZ-treated rats than those from control rats. Indomethacin ($5 \times 10^{-6}$ M) and S18886 ($10^{-7}$ M) significantly reduced the response (Figure 2).

In the presence of L-NAME, tiron ($10^{-3}$ M) significantly decreased the endothelium-dependent contraction to A23187, while SOD (120 U ml$^{-1}$) did not significantly affect it (Figure 3a). DETCA ($10^{-4}$ M), catalase (1200 U ml$^{-1}$) or deferoxamine ($10^{-4}$ M) alone significantly inhibited the endothelium-dependent contraction to A23187 (Figure 3b). MnTMPyP ($10^{-4}$ M) also significantly reduced the response to the calcium ionophore (Figure 3c).

**Table 1** Body weight, glucose level in control and streptozotocin (STZ; 55 mg kg$^{-1}$)-treated rats

| | *Control rats* | *STZ-treated rats* |
|---|---|---|
| Body weight (day 0) (g) | 545.9±7.7 | 530.9±6.2 |
| Body weight (day 84) (g) | 705.5±11.9* | 415.6±7.7*,# |
| Glucose level (day 0) (fasting; mM) | 5.50±0.01 | 21.31±0.05# |
| Glucose level (day 84) (w/o fasting; mM) | 10.31±0.30 | 29.81±0.50# |

Data shown as means±s.e.m.; $n=10$–18.
*Statistically significant differences with that on day 0 ($P<0.05$).
#Statistically significant differences with control rats on the same day ($P<0.05$).

*Exogenous hydrogen peroxide*

In rings without endothelium of femoral arteries from control and STZ-treated rats, hydrogen peroxide produced concentration-dependent contractions, which were maximal at $3 \times 10^{-5}$ M.

In arteries from STZ-treated rats, the maximal response to hydrogen peroxide was statistically significantly greater than those from control rats (Figure 4) and the concentration–response curve was shifted significantly to the left (effector concentration for half-maximum response (−log *M*): −4.87±0.20 and −5.22±0.12; for control and diabetic rats, respectively). Catalase, deferoxamine, MnTMPyP, indomethacin and S18886, but not SC19220, prevented the contractions to hydrogen peroxide (Figure 4).

*Confocal microscopy*

All experiments were performed in the presence of L-NAME $3 \times 10^{-4}$ M. In arteries from STZ-treated rats, the calcium ionophore A23187 ($3 \times 10^{-7}$ M) increased the fluorescence intensity in the endothelial layer. This increase was reduced significantly by indomethacin (Figure 5), but not by S18886 (data not shown). In control preparations, A23187 had no significant effect (Figure 5).

In the vascular smooth muscle layers from control and STZ-treated rats, the calcium ionophore A23187 did not

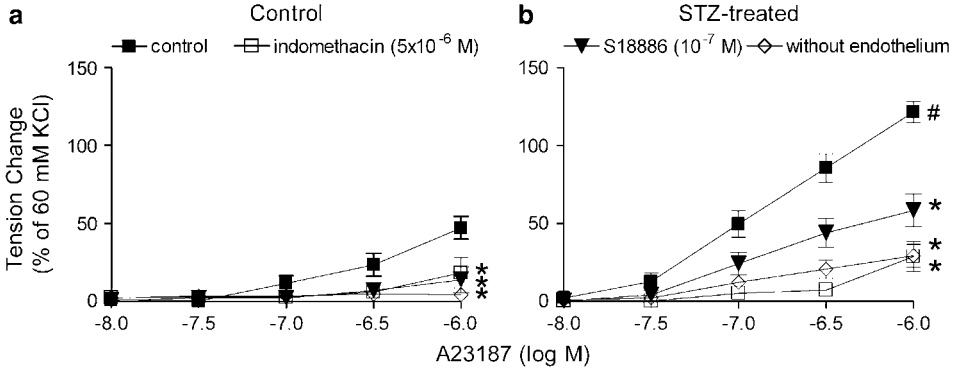


**Figure 2** Contraction to A23187 in femoral arteries from control (**a**) and STZ-treated (**b**) rats in the presence of L-NAME. Data are expressed as percentage of the control contraction to 60 mM KCl and are shown as means±s.e. mean; $n=7$. *Significant differences ($P<0.05$) from ring with endothelium under control conditions; #significant differences ($P<0.05$) from control rats. L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester hydrochloride; STZ, streptozotocin.

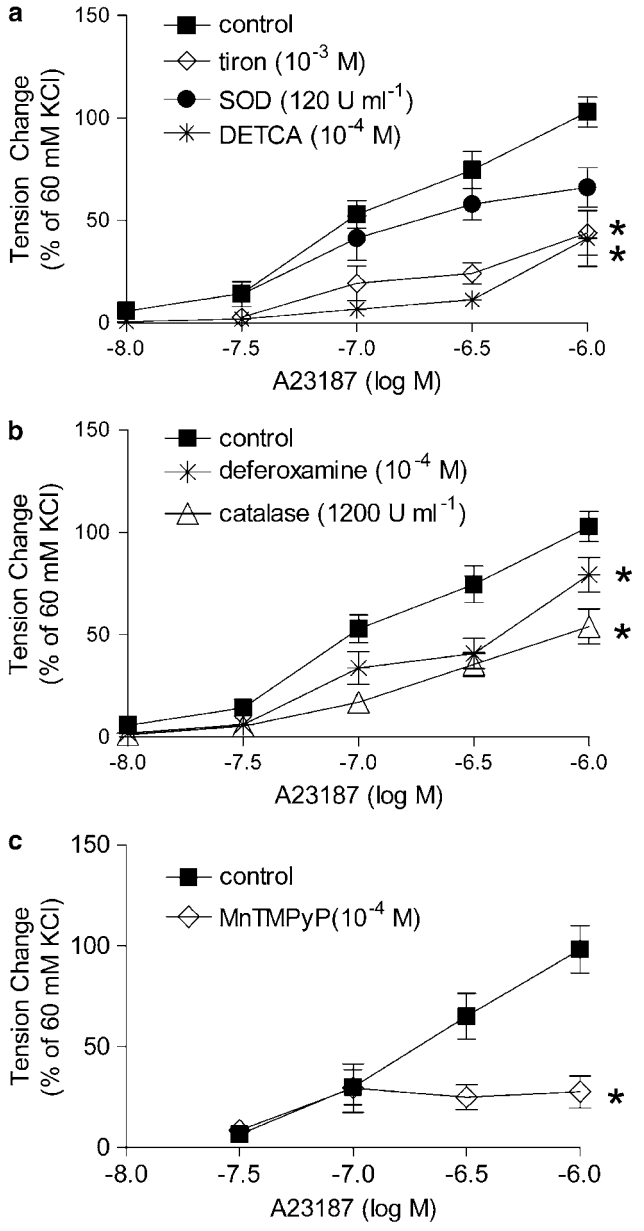


**Figure 3** Effect of antioxidants on concentration-dependent contractions to A23187 in femoral arteries with endothelium from STZ-treated rats in the presence of L-NAME. (**a**) Tiron and SOD, $n=8$–10, (**b**) deferoxamine, catalase and DETCA; $n=12$–20 and (**c**) MnTMPyP; $n=8$. Data are expressed as percentage of the reference contraction to 60 mM KCl and are shown as means ± s.e. mean; *Significant difference ($P<0.05$) from the controls (rings with endothelium). DETCA, diethyldithiocarbamic acid; L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester hydrochloride; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride; SOD, superoxide dismutase; STZ, streptozotocin.

significantly affect the fluorescence signal, in the presence of L-NAME (data not shown).

*Superoxide dismutase and catalase*

The protein level of manganese-SOD (Mn-SOD) was reduced in the femoral arteries from STZ-treated rats, whereas the protein level of copper–zinc-SOD (CuZn-SOD) was comparable (Figure 6). The expression of catalase was significantly greater in the STZ-treated group than that in the controls (Figures 7a and b).

*Antioxidant enzymes*

The activity of catalase was reduced in femoral arteries from the STZ-treated group (Figure 7c), while the total glutathione level was comparable in the two groups (data not shown).

## Discussion

Oxidative stress results from the imbalance between an increased generation of oxygen-derived free radicals and an impaired antioxidant defence. It increases under pathological conditions and along with the course of certain diseases. Oxygen-derived free radicals play an important role in the occurrence of endothelium-dependent contractions in the basilar artery of the dog and the aorta of the spontaneously hypertensive rat (Katusic and Vanhoutte, 1989; Katusic *et al*., 1993; Yang *et al*., 2003a, 2004a; Tang *et al*., 2007). Previous studies from our laboratory demonstrate that the calcium ionophore A23187 induces greater endothelium-dependent contractions in the femoral artery of diabetic rats than those of normoglycaemic animal (Shi *et al*., 2007), exemplifying the endothelial dysfunction caused by diabetes (Pieper and Gross, 1988; Heygate *et al*., 1995; Makimattila *et al*., 1996; Pieper *et al*., 1997; Vallejo *et al*., 2000). This prompted the investigation of the role of oxygen-derived free radicals in the endothelium-derived contracting factor-mediated responses of the femoral artery of rats with STZ-induced diabetes. Because acetylcholine, the most commonly used agonist to evoke such responses in the rat aorta (Katusic *et al*., 1993; Yang *et al*., 2003b; Tang *et al*., 2005), does not cause such responses in the rat femoral artery (Shi *et al*., 2007), the calcium ionophore A23187 was selected for the present experiments.

The present functional studies demonstrate that tiron, a cell-permeable non-enzymatic scavenger of superoxide anion (Malendowicz, 1972), reduced the endothelium-dependent response to A23187 while SOD, which cannot permeate into cells, had no effect. This observation resembles the findings obtained in the aorta of the spontaneously hypertensive rat (Auch-Schwelk *et al*., 1989; Yang *et al*., 2002). These observations support the concept that the superoxide anions inside endothelial cells are the primary oxidative contributor to endothelium-dependent contractions. Treatment with DETCA (Greenstock and Miller, 1975), an inhibitor of endogenous CuZn-SOD, reduced the endothelium-dependent response to A23187. Since DETCA is supposed to cause intracellular accumulation of superoxide anions (Omar *et al*., 1991; Pagano *et al*., 1993), this finding suggests that superoxide anions *per se* do not mediate the endothelium-dependent response, as they do in the canine basilar artery (Katusic *et al*., 1993). Rather, superoxide anions are the source of other oxygen-derived free radicals in the endothelial cells. Catalase, a scavenger of hydrogen peroxide (for example, Katusic and Vanhoutte, 1989) diminished the

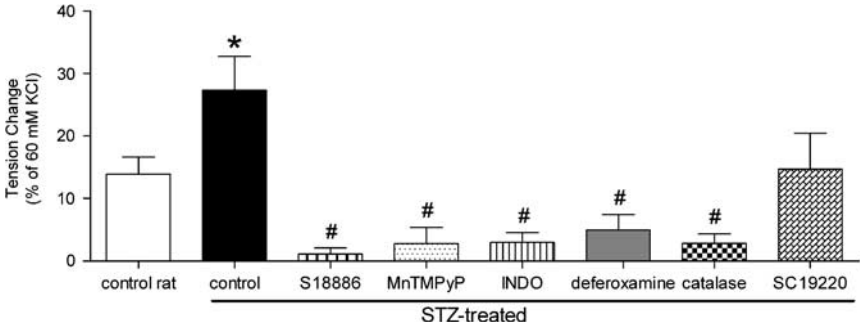


**Figure 4** Maximal contractions to hydrogen peroxide ($3 \times 10^{-5}$ M) in rings without endothelium of femoral arteries from control and STZ-treated rats. Effect of catalase, deferoxamine, MnTMPyP, INDO, S18886 and SC19220 on the response. Data are expressed as percentage of the reference contraction to 60 mM KCl and are shown as means ± s.e. mean; $n = 9$. *Significant difference from the rings from control rats; #statistically significant difference from the rings from STZ-treated rats under control conditions ($P < 0.05$). INDO, indomethacin; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride; S18886, terutroban, (3-[(6-amino-(4-chlorobenzensulphonyl)-2-methyl-5,6,7,8-tetrahydronapht]-1-yl) propionic acid; SC19220, 2-acetylhydrazide 10 (11 H)-carboxylic acid 8-chloro-dibenz [b,f] [1,4] oxazepine-10 (11 H)-carboxylic acid; STZ, streptozotocin.

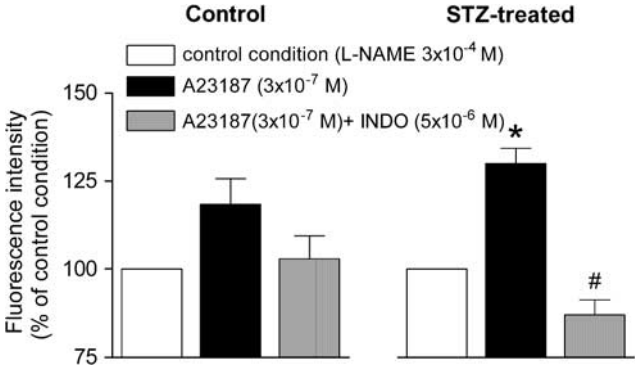


**Figure 5** Changes in fluorescence intensity in femoral arteries from control (left) and STZ (right)-treated rats in response to A23187 ($3 \times 10^{-7}$ M) and effects of indomethacin on the response. Data are expressed as percentage fluorescence value to arteries under control conditions and are shown as means ± s.e. mean; $n = 6$–7. *Significant difference ($P < 0.05$) from the arteries under control conditions; #significant difference ($P < 0.05$) from the arteries stimulated with A23187 in the absence of indomethacin. INDO, indomethacin; STZ, streptozotocin.

endothelium-dependent contractions to A23187, supporting a key role of hydrogen peroxide in the occurrence of the endothelium-dependent contractions evoked by the calcium ionophore. The role of hydrogen peroxide was confirmed by the experiments with MnTMPyP, since this SOD/catalase mimetic (Pasternack *et al*., 1986) nearly abolished the response. Ferrous ions catalyse interactions between hydrogen peroxide and superoxide anions, producing hydroxyl radicals. Deferoxamine, an iron chelator that scavenges hydroxyl radicals (for example, Auch-Schwelk *et al*., 1989), reduced the response to A23187, indicating that hydroxyl radicals are also involved in endothelium-dependent contractions in the femoral artery of rats with STZ-induced diabetes. Taken together, the findings of the present study strongly suggest that an increase in intracellular concentration of $Ca^{2+}$ leads to the generation of superoxide anions, which are converted by SOD to hydrogen peroxide that is further transformed to hydroxyl radicals, responsible in part for the endothelium-dependent contractions observed in arteries of the diabetic rats.

Thus, the present findings indicate that hydrogen peroxide, which can diffuse freely within the tissues since it is not charged (Wolin, 2000), plays an important role in the occurrence of endothelium-dependent contractions. In line with such a role, exogenous hydrogen peroxide produced moderate contractions in rings without endothelium. Since this contraction is prevented by catalase, MnTMPyP and deferoxamine, the mediators of the response are hydroxyl radicals but not hydrogen peroxide *per se*. The maximal level of contraction generated by exogenous $H_2O_2$, and thus presumably hydroxyl radicals, is comparable to the deferoxamine-sensitive part of the endothelium-dependent contraction to the calcium ionophore. A similar conclusion has been reached in the aorta of spontaneously hypertensive rats (Yang *et al*., 2002). The inhibitory effect of S18886, but not SC19220, indicates that ultimately hydroxyl radicals must cause activation of thromboxane prostanoid receptors on the vascular smooth muscle (Yang *et al*., 2002). The hyper-responsiveness of the femoral artery from the diabetic rat can be explained in part by the hyper-responsiveness of the thromboxane prostanoid receptors of its vascular smooth muscle (Shi *et al*., 2007). The fact that indomethacin, the inhibitor of cyclooxygenase, inhibited the response suggests that the hydroxyl radicals activate the production of vasoconstrictor metabolites of arachidonic acid, presumably endoperoxides or thromboxane $A_2$, in vascular smooth muscle (Auch-Schwelk *et al*., 1989; Yang *et al*., 2002, 2003b; Wolin, 2004). These data reached in functional studies with exogenous hydrogen peroxide suggest that this peroxide, at least, could be the precursor of endothelium-derived contracting factor in the femoral artery of rats with diabetes.

The measurement of oxidative stress by means of fluorescence under confocal microscopy revealed that in the presence of L-NAME, A23187, at a concentration that induced submaximal endothelium-dependent contractions in the functional studies, augmented the release of reactive oxygen species in the endothelial layer, but not in the vascular smooth muscle layer. S18886 (Simonet *et al*., 1997), a selective blocker of thromboxane prostanoid receptors, did not reduce the fluorescence in the preparation with endothelium. These findings concur with the results in the

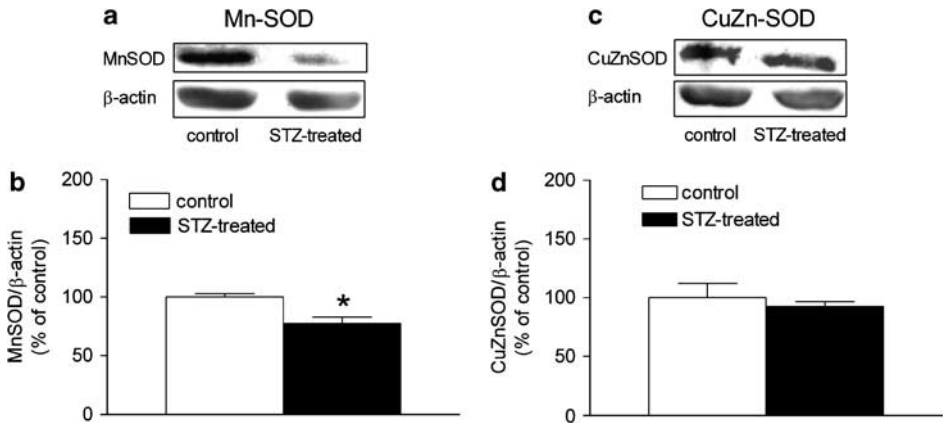


**Figure 6** Presence of Mn-SOD (**a**, **b**) and copper–zinc-SOD (**c**, **d**) in femoral arteries with endothelium from control and STZ-treated rats. (**a** and **c**) Representative western blots demonstrating the presence of proteins and (**b** and **d**) graphic representation of the data, normalized to β-actin, presented as percentage of control and shown as means ± s.e. mean; $n = 6$. *Significant difference from controls ($P < 0.05$). CuZn-SOD, copper–zinc-SOD; Mn-SOD, manganese-superoxide dismutase; STZ, streptozotocin.

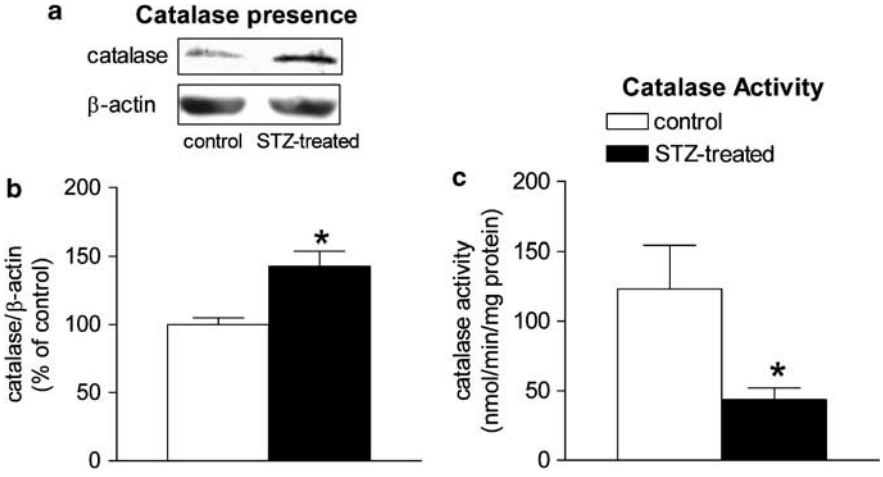


**Figure 7** Catalase protein presence and activity in femoral arteries with endothelium from control and STZ-treated rats. (**a**) Western blot demonstrating the presence of catalase. (**b**) Graphic representation of the data, normalized to β-actin, presented as percentage of control and shown as means ± s.e.m.; $n = 6$. *Significant difference from controls ($P < 0.05$). (**c**) Catalase activity in femoral arteries with endothelium from control and STZ-treated rats. Data are normalized to the protein level and are shown as means ± s.e.m.; $n = 6$. *Significant difference from controls ($P < 0.05$). STZ, streptozotocin.

vascular functional study, confirming the contribution of oxygen-derived free radicals in the endothelium-dependent contractions. Indomethacin reduced this production of reactive oxygen species, as it inhibits the endothelium-dependent contractions observed in organ chamber experiments, indicating that the release of reactive oxygen free radicals evoked by A23187 depends upon endothelial cyclooxygenase. This interpretation is consistent with that reached in the aortic endothelium of the spontaneously hypertensive rat (Tang *et al*., 2007). Cyclooxygenase, the rate-limiting enzyme in the production of endothelium-derived contracting factor, is upregulated in the aorta of spontaneously hypertensive rats and the femoral artery of diabetic rats (Ge *et al*., 1995, 1999; Shi *et al*., 2007). Therefore, the increased generation of reactive oxygen species with A23187 in arteries from STZ-treated but not control rats, in accordance with the augmented endothelium-dependent response in the organ chambers, can be attributed to the enhanced presence of endothelial cyclooxygenase. Thus, the experiments performed under confocal microscopy strongly suggest that oxygen-derived free radicals play a major role in endothelium-dependent contractions in the femoral artery of diabetic rats.

The fact that the potentiating effect of oxygen-derived free radicals on endothelium-dependent contractions is observed only in preparations from STZ-treated but not control rats resembles observations in the aorta of normotensive and spontaneously hypertensive rats (Yang *et al*., 2003b), suggesting that the redox abnormality is seen only under certain disease conditions.

In the present experiments, the protein expression of Mn-SOD, which is confined to the mitochondria, was decreased in the arteries from the STZ-treated rats, whereas the protein expression of CuZn-SOD, which mainly metabolizes superoxide anion in the cytosol, was comparable, suggesting that the anti-oxidative ability is reduced in the mitochondria in

diabetes (Sheu *et al*., 2005). Both catalase and glutathione take part in the conversion of hydrogen peroxide to water (Wolin, 2000). The net glutathione level was comparable in the two groups, suggesting that a reduction in glutathione level is not a contributing factor to the endothelial dysfunction in diabetes. However, the activity of catalase was decreased in the femoral arteries from STZ-treated rats despite an increased protein presence of the enzyme. Catalase is not efficient in eliminating low levels of hydrogen peroxide because it is difficult to saturate with hydrogen peroxide and its catalytic activity requires the interaction of two hydrogen peroxide molecules with a single active site, which is less likely to occur at low hydrogen peroxide concentrations (Halliwell and Gutteridge, 2000; Rhee *et al*., 2005). This further suggests that the accumulation of hydrogen peroxide in these arteries can be mainly attributed to a reduced activity of catalase. This interpretation is consistent with the augmented DCF fluorescence observed. Taken together, the present findings thus confirm an increased generation of reactive oxygen species, in particular hydrogen peroxide, in diabetes (Karasu, 1999; Pandolfi *et al*., 2003).

In summary, the present series of experiments demonstrated a link between oxidative stress and the occurrence of endothelium-dependent contractions in femoral arteries of diabetic rats. The oxygen-derived free radicals potentiated and/or mediated the occurrence of endothelium-dependent contractions by facilitating the production of vasoconstrictor prostanoids.

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## Conflict of interest

The authors state no conflict of interest.

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