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Cellular basis of endothelial dysfunction in small mesenteric arteries from spontaneously diabetic (*db*/*db* −/−) mice: role of decreased tetrahydrobiopterin bioavailability

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1 Endothelium-dependent and -independent regulation of vascular tone in small mesenteric arteries (SMA) from control (*db*/*db* +/?) and diabetic (*db*/*db* −/−) mice was compared.

2 Phenylephrine-induced maximum contraction, but not sensitivity, of SMA in *db*/*db* −/− compared to *db*/*db* +/? was enhanced.

3 Acetylcholine (ACh), but not sodium nitroprusside (SNP), -induced relaxation was reduced in SMA from *db*/*db* −/− compared to db/db +/?.

4 ACh-induced relaxation of SMA was inhibited by a combination of N^{ω}-nitro-L-arginine and indomethacin in db/db +/?, but not in *db*/*db* −/−.

5 Acute incubation of SMA with tetrahydrobiopterin (BH_4, 10 μM) and sepiapterin (100 μM) enhanced ACh-induced relaxation in SMA from *db*/*db* −/−, but not from *db*/*db* +/? 2,4-diamino-6-hydroxypyrimidine, an inhibitor of GTP cyclohydrolase I, (10 mM), impaired the sensitivity of SMA from db/db +/? to ACh, which was restored by co-incubation with BH_4 (10 μM).

6 BH_4 and superoxide dismutase (SOD, 150 u ml^{-1}), either alone or in combination, had no effect on either ACh or SNP-induced relaxation in SMA from eNOS −/− mice.

7 Incubation of SMA with SOD (150 iu ml^{-1}), catalase (200 iu ml^{-1}) and L-arginine (1 mM) had no effect on ACh-induced relaxation of SMA. However, the combination of polyethylene glycol-SOD (200 iu ml^{-1}) and catalase (80 u ml^{-1}) improved the sensitivity of ACh-induced relaxation in *db*/*db* −/−, but not in *db*/*db* +/?.

8 These data suggest that increased production of superoxide anions and decreased availability of BH_4 result in an 'uncoupling' of nitric oxide synthase and endothelial dysfunction in SMA from *db*/*db* −/− mice.

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Keywords: Endothelial dysfunction; type II diabetes; *db*/*db* mouse; endothelial nitric oxide synthase; tetrahydrobiopterin; superoxide anions; eNOS knock out mice

Abbreviations: ACh, acetylcholine; BH4, (6R)-5,6,7,8-tetrahydrobiopterin; DAHP, 2,4-diamino-6-hydroxypyrimidine; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-dependent relaxing factor; eNOS, endothelial nitric oxide synthase; L-NNA, N^{ω}-nitro-L-arginine; NO, nitric oxide; PE, phenylephrine; PEG-SOD, polyethylene glycol-superoxide dismutase; SMA, small mesenteric artery; SNP, sodium nitroprusside; SOD, superoxide dismutase; STZ, streptozotocin

Introduction

The discovery of endothelium-derived relaxing factor (EDRF) and its identification as nitric oxide (NO) were key contributions to our understanding of the physiology of vasodilatation (Furchgott & Zawadzki, 1980; Palmer *et al*., 1987). Endothelial dysfunction is defined as an attenuated response of the blood vessels to endothelium-dependent vasodilators such as acetylcholine (ACh) and bradykinin. Endothelial dysfunction is considered as the major risk factor for the cardiovascular complications associated with type I and type II diabetes (De Vriese *et al*., 2000).

Impaired endothelium-dependent vasodilation is primarily associated with a decreased synthesis of endothelium-derived nitric oxide (NO) and/or an increase in the production of superoxide anions (De Vriese *et al*., 2000). Reactive oxygen species such as superoxide anions can rapidly inactivate NO, resulting in the formation of vasotoxic peroxynitrite (Gryglewski *et al*., 1986; Milstien & Katusic, 1999). It has been assumed that under pathophysiological conditions, such as diabetes, an imbalance between NO and superoxide anion production may contribute to impaired endothelium-dependent relaxation to ACh (Cosentino & Luscher, 1999). It has also been reported that nitric oxide synthase (NOS), under certain conditions, produces superoxide anions, in addition to NO (Pou *et al*., 1992). Tetrahydrobiopterin (BH_4) is an essential cofactor for the synthesis of NO and, under

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physiological conditions, following binding of BH_4 to the oxidase domain of NOS, the enzyme is activated and generates NO and L-citrulline from L-arginine and oxygen (Mayer & Werner, 1995). Reduced bio-availability/activity of BH_4 has been recently reported in the fructose-fed insulin resistant rat model (Shinozaki *et al*., 1999) as well as in coronary arteries from insulin resistant patients (Shinozaki *et al*., 2001). In the presence of sub-optimal levels of BH_4, NOS generates both NO and superoxide anions resulting in the formation of hydrogen peroxide and peroxynitrite (Wever *et al*., 1997).

Thus, restoration of BH_4 to the endothelial cell should restore the activity of NOS and lead to an increased production of NO. Administration of BH_4 has been demonstrated to enhance NO production in pre-hypertensive rats (Cosentino *et al*., 1998), restore endothelium-dependent vasodilation in coronary arteries following reperfusion injury (Tiefenbacher *et al*., 1996), as well as in aortae from STZ– induced diabetic rats (Pieper, 1997), coronary resistance vessels from JCR:LA corpulent rats (Brunner *et al*., 2000) and aortae from insulin-resistant rats (Shinozaki *et al*., 2000). BH_4 supplementation improves endothelium-dependent relaxation in patients with hypercholesteromia (Stroes *et al*., 1997), endothelium-dependent relaxation in venous conduits used for coronary artery bypass graft surgery (Verma *et al*., 2000), patients with type II diabetes (Heitzer *et al*., 2000a; Verma *et al*., 2000), in normal epicardial coronary arteries (Setoguchi *et al*., 2001) and in smokers (Heitzer *et al*., 2000b). BH_4 administration has also been demonstrated to improve functional recovery following ischaemia and reperfusion; an effect ascribed to improved coronary endothelial function and reduced oxidative stress (Verma *et al*., 2002). In addition, aortic tissue from the hyperphenyalaninemic (hph-1) mouse, which has a 90% deficiency in the rate limiting enzyme, GTP cyclohydrolase I, for BH_4 synthesis, demonstrates reduced NO synthesis and heightened superoxide production (Cosentino *et al*., 2001). Furthermore, acetylcholine-mediated endothelium-dependent relaxations in aortae from hph-1, but not control, mice were inhibited by catalase and enhanced by superoxide dismutase and differences between hph-1 and control were reversed by exogenous BH_4 administration (Cosentino *et al*., 2001).

These findings from a variety of different animal models as well as from clinical studies support the hypothesis that BH_4 could be a new and an effective therapeutic approach for the treatment of endothelial dysfunction in a variety of pathophysiological conditions. The objective of the present study was to investigate endothelial function, and the role of the co-factor, BH_4, and oxidative stress in small mesenteric arteries (SMA) from spontaneously diabetic (*db*/*db* −/−) mice, an animal model of type II diabetes that lacks the leptin receptor. In our investigations we used the *db*/*db* +/? mouse, that includes the homozygous +/+ as well as the heterozygous +/−, as the control animal.

Methods

Animals

Twelve to 16-week-old male C57BL/KsJ db/db mice (*db*/*db* −/−; *db*/*db* is the gene that encodes for the leptin receptor and −/− refers deficiency of leptin receptor) and non-diabetic (*db*/*db* +/?) controls, as well as eNOS −/− mice, were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.). Plasma glucose (Sigma & Co., U.S.A.), total cholesterol (Sigma & Co., U.S.A.), triglycerides (Sigma & Co., U.S.A.) and serum non-esterified fatty acid (Wako & Co., Germany) were assayed using commercial kits.

Vascular reactivity

In accordance with a protocol approved by the University of Calgary Animal Care Committee, mice were killed by cervical dislocation and the mesenteric arcade was removed. First order branches of the mesenteric artery were dissected out into cold Krebs solution of the following composition (in mM): NaCl 120, $NaHCO_3$ 25, KCl 4.8, NaH_2PO_4 1.2, $MgSO_4$ 1.2, dextrose 11.0, $CaCl_2$ 1.8, aerated with 95% O_2 and 5% CO_2. Arteries were cut into 2 mm rings and mounted on a Mulvany-Halpern myograph as previously described (Mulvany & Halpern, 1977). The passive tension-internal circumference was determined by stretching to achieve an internal circumference equivalent to 90% of that of the vessel under a transmural pressure of 100 mmHg. All experiments were performed at 37°C.

Experimental protocols

After a 45-min equilibration period, the vascular reactivity to phenylephrine (PE) was studied in SMA from *db*/*db* +/? and *db*/*db* −/− mice. After 30 min stabilization, endothelium-dependent relaxation to ACh and endothelium-independent relaxation to SNP were recorded in preparations pre-contracted with a sub-maximal concentration of PE (EC_{75-80}).

The first series of experiments was performed in order to study the contribution of NO to the ACh-induced relaxation of SMA derived from *db*/*db* +/? and *db*/*db* −/− mice. Tissues were pre-treated with N^{ω}-nitro L-arginine (L-NNA, 100 μM) and indomethacin (10 μM) for 30 min and then a cumulative concentration-response curve to ACh was obtained.

The second series of experiments was performed in order to study the potential beneficial effect of exogenous BH_4 on ACh- and SNP-induced relaxation of SMA from *db*/*db* +/?, *db*/*db* −/− and eNOS −/− mice. BH_4 (10 μM), either alone or in combination with superoxide dismutase (SOD; 150 iu ml^{-1}), was incubated for 30 min before constructing a cumulative concentration-response curve to ACh and SNP in SMA from *db*/*db* +/?, *db*/*db* −/− and eNOS −/− mice. Further, the effects of the acute incubation with sepiapterin (100 μM) on ACh-induced relaxation of SMA from *db*/*db* +/? and *db*/*db* −/− mice was investigated. An additional set of experiments was performed to study the effect of BH_4 deficiency on endothelial function in *db*/*db* +/? mice. SMA from *db*/*db* +/? were exposed to DAHP (10 mM) for 3 h before constructing a cumulative concentration-response curve to ACh. In another group, BH_4 (10 μM) was exposed for the last 30 min in the presence of DAHP before constructing a cumulative concentration-response curve to ACh.

The third series of experiments was designed to determine the effects of oxidative stress and NOS substrate on ACh-

induced relaxation of SMA from *db/db* +/? and *db/db* −/− mice. SOD (150 iu ml^{-1}), catalase (200 u ml^{-1}), a combination of PEG-SOD (200 u ml^{-1}) and catalase (80 u ml^{-1}) or L-arginine (1 mM) were pre-incubated with the SMA for 30 min before constructing a cumulative concentration-response curve to ACh in SMA from *db/db* −/− and db/db +/?.

Drugs

All drugs were obtained from Sigma. All drugs were dissolved in distilled water except for indomethacin, which was dissolved in 95% ethanol and sepiapterin, which was dissolved in DMSO. BH_4 was prepared fresh in deoxygenated distilled water and stored in the dark until use.

Data analysis

Data are expressed as pEC_{50} values, defined as the negative logarithm to base ten of the EC_{50} value, which was used in this study as a measure of sensitivity to vasoactive drugs, and maximum relaxation/contraction, as the maximum response obtained at the highest concentration tested. In all experiments, *n* equals the number of animals used in the protocol. Relaxation is expressed as mean percentage of PE-induced tone ± standard error of mean. Statistical significance of difference between means of different groups was performed using paired or unpaired student *t*-test or one-way ANOVA. Multiple comparisons of the different groups were performed using Student Newman Keul test. A P value of <0.05 was considered statistically significant.

Results

Biochemical characteristics in spontaneously diabetic (db/db −/−) mice

Spontaneously diabetic mice showed significantly higher body weight compared to their littermates (47 ± 1 *vs* 29 ± 1 g respectively, $n=10-11$, $P<0.01$). Plasma glucose (475 ± 1 *vs* 204 ± 4 mg dl^{-1} respectively, $P<0.01$), triglycerides (83 ± 10 *vs* 43 ± 2 mg dl^{-1} respectively, $P<0.01$) and total cholesterol (143 ± 25 *vs* 43 ± 6 mg dl^{-1} respectively, $P<0.01$) were significantly elevated in *db/db* −/− compared to *db/db* +/?. Serum non-esterified fatty acid was significantly higher in *db/db* −/− compared to *db/db* +/? (2.45 ± 0.3 *vs* 1.26 ± 0.1 m Eq L^{-1} respectively, $P<0.01$).

Characterization of endothelial function in spontaneously diabetic (db/db −/−) mice

PE initiated a concentration-dependent contraction of SMA from *db/db* +/? and *db/db* −/− mice (Figure 1). Maximum contraction expressed as mN/mm, and sensitivity expressed as pEC_{50} value for *db/db* +/? and *db/db* −/− were 3.2 ± 0.2 and 6.2 ± 0.4 and 4.4 ± 0.3 and 6.4 ± 0.1 respectively. Maximum contraction to PE was significantly enhanced ($P<0.01$) without a change in the sensitivity in *db/db* −/− compared to *db/db* +/?.

ACh-induced maximum relaxation of SMA was significantly reduced ($P<0.01$) in *db/db* −/− compared to *db/db* +/?. Sensitivity and maximum relaxation to ACh for *db/db* +/? and *db/db* −/− were 6.6 ± 0.1 and 81 ± 4 and 6.5 ± 0.1 and 53 ± 6 respectively (Figure 2). Incubation of the SMA with L-NNA (100 μM) and indomethacin (10 μM) for 30 min

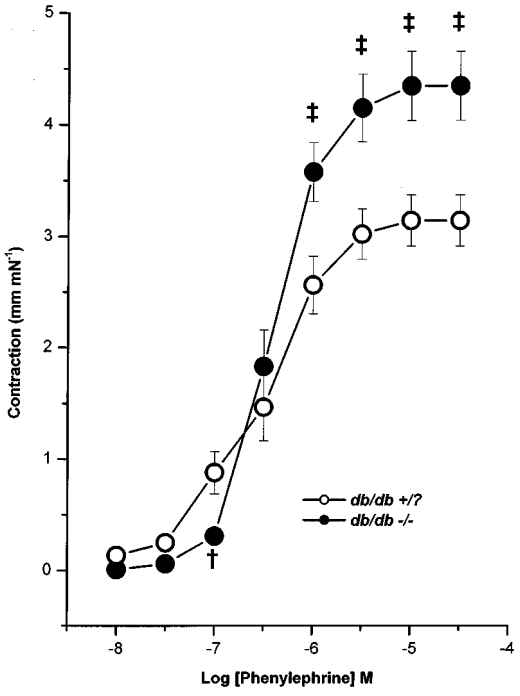

Figure 1 Mean concentration-response curves to phenylephrine in mouse mesenteric arteries from *db/db* +/? ($n=10$) and *db/db* −/− ($n=12$). Symbols are mean values with s.e.mean shown by vertical bars. †$P<0.05$ and ‡$P<0.01$ compared to *db/db* +/?.

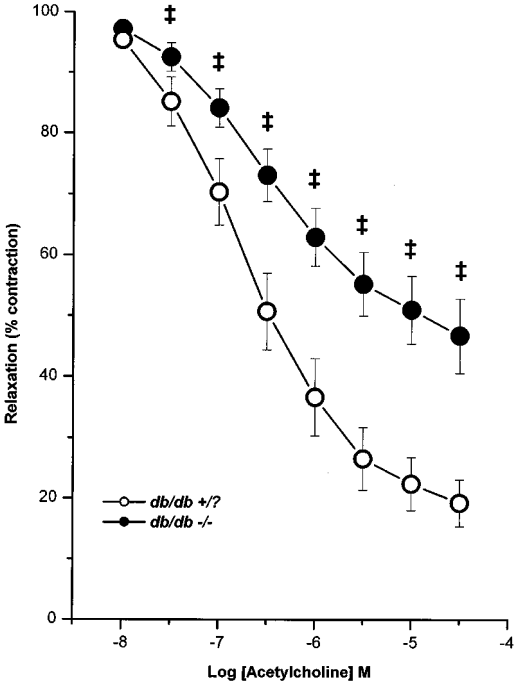

Figure 2 Mean concentration-response curves to ACh in mouse mesenteric arteries from *db/db* +/? ($n=18$) and *db/db* −/− ($n=15$). Symbols are mean values with s.e.mean shown by vertical bars. ‡$P<0.01$ compared to *db/db* +/?.

significantly reduced the maximum relaxation induced by ACh from *db/db* +/? ($P<0.01$), but not in *db/db* −/− mice ($n=10$). Sensitivity and maximum relaxation to ACh before and after incubation with L-NNA (100 μM) and indomethacin (10 μM) for 30 min were 7.0 ± 0.1 and 86 ± 3 and 6.4 ± 0.1 and 52 ± 6 respectively in *db/db* +/? and 6.8 ± 0.2 and 54 ± 7 and 7.0 ± 0.2 and 40 ± 6 respectively in *db/db* −/−. However indomethacin alone had no effect on endothelium-dependent relaxation of SMA in both *db/db* +/? and *db/db* −/− mice (data not shown).

SNP-mediated relaxation remained unchanged in both groups. Sensitivity and maximum relaxation to SNP for *db/db* +/? and *db/db* −/− were 6.6 ± 0.2 and 96 ± 1 and 6.4 ± 0.2 and 86 ± 5 respectively (Figure 3).

Effect of co-factor, tetrahydrobiopterin, on endothelium-dependent and endothelium-independent relaxation of SMA from db/db +/?, db/db −/− and eNOS −/− mice

Pre-incubation of SMA from *db/db* +/? mice with BH_4 (10 μM) alone or in combination with SOD (150 iu ml^{-1}) did not affect either the sensitivity (7.0 ± 0.1, 6.9 ± 0.2, 6.9 ± 0.1 for the ACh control, BH_4, BH_4 plus SOD group respectively) or the maximum relaxation (90 ± 2, 82 ± 5, 86 ± 3 for the ACh control, BH_4, BH_4 plus SOD group respectively) mediated by ACh (Figure 4a). However, pre-incubation with BH_4 (10 μM) and the combination with SOD (150 u ml^{-1}) significantly ($P<0.01$; Figure 4b) enhanced the sensitivity (6.5 ± 0.2, 7.5 ± 0.1, 7.5 ± 0.1 for the ACh control, BH_4, BH_4 plus SOD group respectively) to ACh in SMA from *db/db* −/− mice without affecting the maximum relaxation (61 ± 13, 73 ± 6, 83 ± 3 for the ACh control, BH_4, BH_4 plus SOD group respectively). Incubation with sepiapterin (100 μM), which is converted to BH_4 intracellularly, significantly enhanced the sensitivity to ACh without affecting the

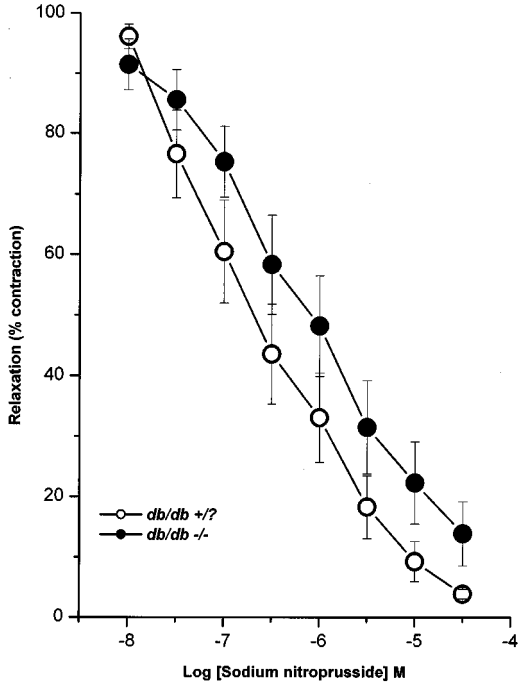

Figure 3 Mean concentration-response curves to SNP in mouse mesenteric arteries from *db/db* +/? ($n=16$) and *db/db* −/− ($n=15$). Symbols are mean values with s.e.mean shown by vertical bars.

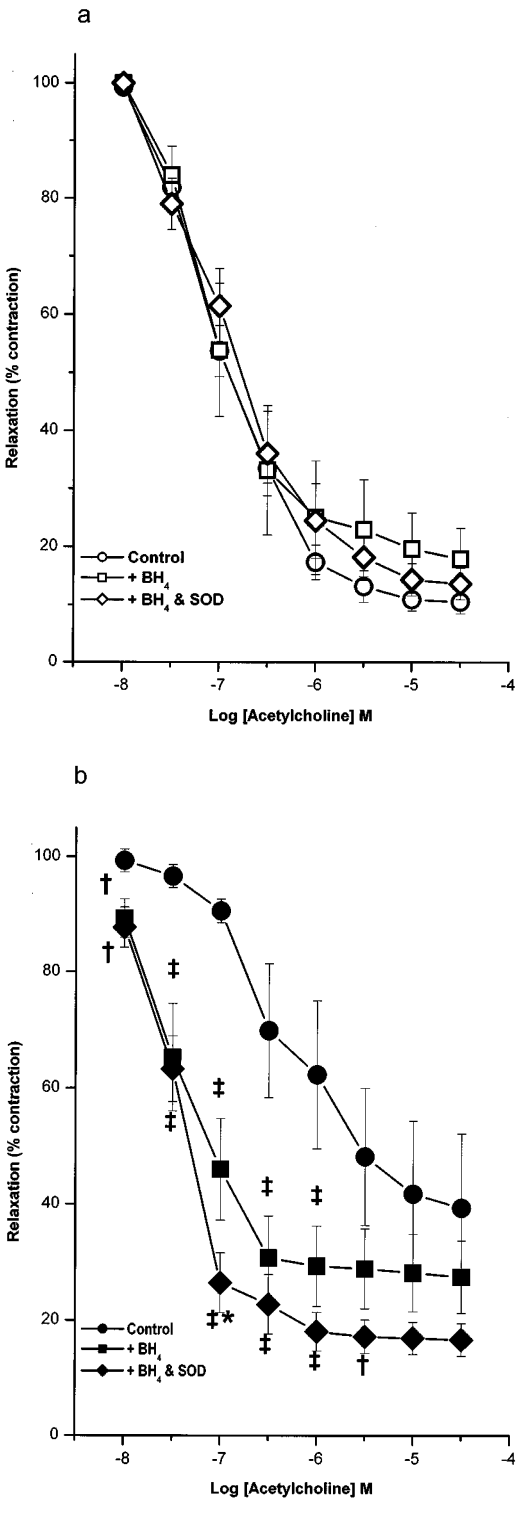

Figure 4 Mean concentration-response curves to ACh in mouse mesenteric arteries from *db/db* +/? (a) and *db/db* −/− (b) before (control) and after incubation with either BH_4 (10 μM) or combination of BH_4 (10 μM) and SOD (150 iu ml^{-1}) for 30 min ($n=7$). Symbols are mean values with s.e.mean shown by vertical bars. †$P<0.05$ and ‡$P<0.01$ compared to control and *$P<0.05$ compared to BH_4 treated group.

maximum relaxation in *db/db* −/−, but not in *db/db* +/? (Table 1).

BH_4 alone, or in combination with SOD, had no effect on SNP-mediated concentration-dependent relaxation of SMA from *db/db* +/? and *db/db* −/− (Figure 5a,b), however a small apparent potentiating effect was observed in db/db −/− mice, this did not reach a statistical significance. Sensitivity and maximum relaxation were 6.7±0.2 and 97±1, 6.7±0.2 and 96±1, 7.0±0.1 and 95±1 for SNP control, BH_4, BH_4 plus SOD group respectively for *db/db* +/? (n=5–6) and 6.1±0.2 and 82±12, 7.0±0.3 and 82±11, 6.8±0.3 and 83±10 for SNP control, BH_4, BH_4 plus SOD group respectively for *db/db* −/−.

BH_4 alone, or in combination with SOD, had no significant effect on ACh and SNP-mediated concentration-dependent relaxation of SMA from eNOS −/− mice (Figure 6a,b). Sensitivity and maximum relaxation of SMA to ACh were 6.1±0.2 and 76±9, 6.4±0.1 and 76±6, 6.4±0.2 and 73±9 for ACh control, BH_4, BH_4 plus SOD group respectively. Sensitivity and maximum relaxation of SMA to SNP were 7.2±0.2 and 99±1, 7.1±0.1 and 97±1, 7.1±0.3 and 96±1 for ACh control, BH_4, BH_4 plus SOD group respectively.

Incubation of SMA from *db/db* +/? with DAHP (10 mM), a GTP cyclohydrolase I inhibitor, for 3 h significantly ($P<0.05$) reduced the sensitivity to ACh, without affecting the maximum relaxation. Co-incubation with BH_4 (10 μM) during the last 30 min of incubation, restored the sensitivity to normal (Figure 7). pEC_{50} values and maximum relaxation to ACh were 7.3±0.2 and 85±11, 6.6±0.2 and 86±3, 7.2±0.1 and 90±4 for ACh control, DAHP, DAHP plus BH_4 group respectively.

Incubation of the SMA with BH_4 for 30 min, however, had no effect on enhanced vascular reactivity of SMA from db/db −/− to PE. Sensitivity and maximum contraction (mN/mm) to PE were 6.5±0.1 and 4.0±0.4 and 6.5±0.1 and 4.0±0.5 for before and after incubation with BH_4 respectively (n=4).

Effect of oxidative stress and substrate, L-arginine on endothelium-dependent relaxation of SMA from spontaneously diabetic mice

SOD (150 u ml^{-1}), a superoxide anion scavenger and catalase (200 u ml^{-1}), an enzyme that dismutases hydrogen peroxide, had no effect on sensitivity and maximum relaxation to ACh in db/db +/? and db/db −/− mice (Table 1). SOD also had no effect on SNP-induced relaxation in db/db +/? and db/db −/− mice (data not shown). The combination of PEG-SOD (200 u ml^{-1}), membrane permeable superoxide anion scavenger and catalase (80 u ml^{-1}) significantly ($P<0.05$) enhanced the sensitivity to ACh in db/db −/−, but not in db/db +/?, without affecting the maximum relaxation (Table 1). However, the combination of PEG-SOD and catalase had no effect on enhanced vascular reactivity to PE in db/db −/− mice. Sensitivity and maximum contraction (mN/mm) to PE were 6.5±0.1 and 4.0±0.4 and 6.7±0.1 and 3.9±0.5 for before and after incubation with the combination of PEG-SOD and catalase respectively (n= 4).

Pre-incubation with L-arginine (1 mM) for 30 min had no effect on sensitivity or maximum relaxation to ACh in db/db +/? and db/db −/− mice (Table 1). Similarly, pre-incubation with L-arginine had no effect on SNP-induced relaxation in db/db +/? and db/db −/− mice (data not shown).

Discussion

The present study demonstrates that SMA from spontaneously diabetic mice (*db/db* −/−) have enhanced vascular reactivity to PE, impaired endothelium-dependent relaxation to ACh and unaltered endothelium-independent relaxation to SNP. We have also demonstrated an improved sensitivity to ACh following acute incubation with BH_4 and with a combination of PEG-SOD and catalase, but not with catalase, SOD or L-arginine pretreatment.

The spontaneously diabetic mice used in this study demonstrated severe obesity, hyperglycaemia and dyslipidaemia, which are the characteristic features of type II diabetes. Endothelium-dependent relaxation to ACh was significantly impaired in *db/db* −/− mice compared to non-diabetic littermates, however, endothelium-independent relaxation to SNP remained unchanged in *db/db* −/− compared to *db/db* +/?. Furthermore, vessels from db/db −/− mice demonstrated enhanced vascular reactivity to PE. Thus, impaired endothelium function exists in arteries from *db/db* −/− mice and these data are in agreement with earlier reports that have studied endothelial function in the thoracic aorta (Kamata & Kojima, 1997; Piercy & Taylor, 1998) and mesenteric artery (Lagaud *et al*., 2001) from the *db/db* mouse . Furthermore, ACh-induced relaxation in *db/db* −/−, but not *db/db* +/+, was resistant to a combination of L-NNA and indomethacin suggesting that changes in NOS function was a major contributor to the attenuated ACh-mediated relaxation in pathophysiological conditions.

Table 1 Effect of the co-factor BH_4 and the substrate L-arginine for eNOS and oxidative stress on endothelial-dependent relaxation to acetylcholine in spontaneously diabetic (db/db−/−) and control (db/db+/?) mice

	db/db+/?					*db/db*−/−				
	pEC_{50}		*Max R*			pEC_{50}		*Max R*		
Drugs	*Before*	*After*	*Before*	*After*	n	*Before*	*After*	*Before*	*After*	n
Sepiapterin (100 uM)	7.2±0.1	6.7±0.5	88±2	94±4	4	6.4±0.2	7.1±0.1*	51±11	73±8	5
L-Arginine (1 mM)	6.9±0.1	6.8±0.1	84±7	86±2	4	7.0±0.2	6.9±0.1	55±17	78±8	5
SOD (150 u ml^{-1})	7.0±0.1	6.5±0.1	89±3	80±4	5	7.0±0.2	6.9±0.3	55±17	59±11	5
Catalase (200 u ml^{-1})	7.1±0.1	7.4±0.4	88±3	86±2	3	6.9±0.1	7.1±0.2	57±4	60±9	7
PEG-SOD (u ml^{-1}) and Catalase (80 u ml^{-1})	7.1±0.1	7.4±0.2	88±3	94±4	3	6.7±0.2	7.2±0.1*	62±4	57±6	4

*$P<0.05$ compared to before treatment by paired Student t-test.

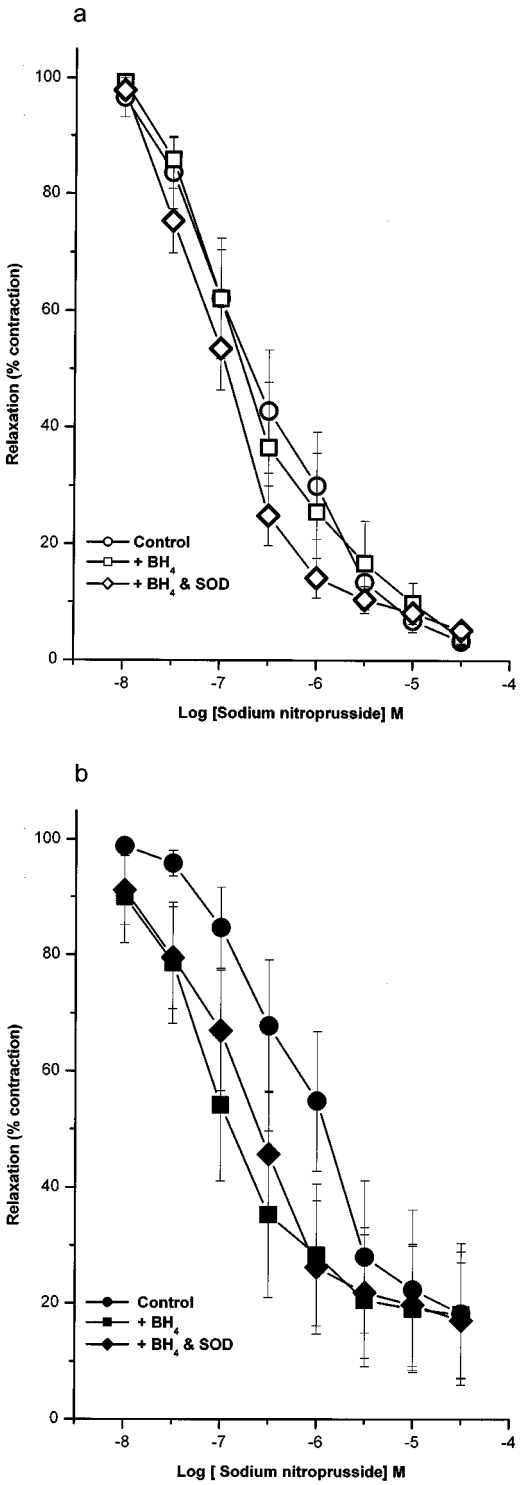

Figure 5 Mean concentration-response curves to SNP in mouse mesenteric arteries from *db*/*db* +/? (a) and *db*/*db* −/− (b) before (control) and after incubation with either BH_4 (10 μM) or combination of BH_4 (10 μM) and SOD (150 iu ml^{-1}) for 30 min ($n=6-9$). Symbols are mean values with s.e.mean shown by vertical bars.

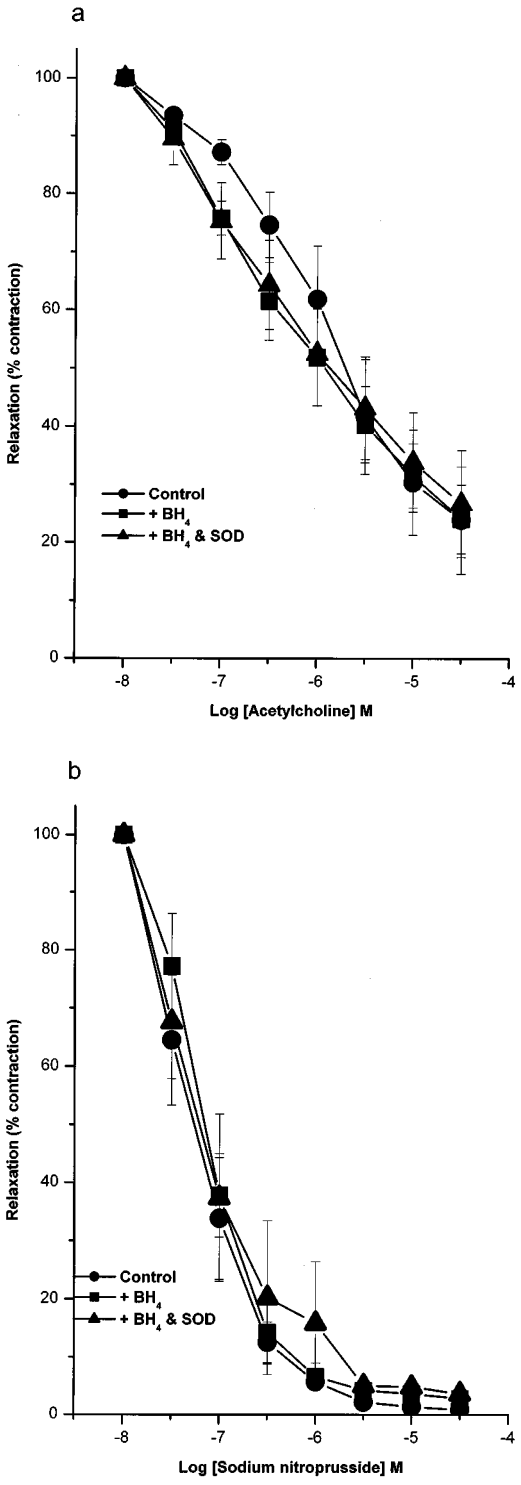

Figure 6 Mean concentration-response curves to ACh (a) and SNP (b) in mouse mesenteric arteries from eNOS −/− before (control) and after incubation with either BH_4 (10 μM) or combination of BH_4 (10 μM) and SOD (150 iu ml^{-1}) for 30 min ($n=6$). Symbols are mean values with s.e.mean shown by vertical bars.

The cellular basis for the change in NOS function may include: (a) decreased release or production of nitric oxide due to deficiency of the substrate, L-arginine or co-factor, tetrahydrobiopterin; (b) increased destruction of NO by oxygen-derived free radicals; (c) increased release of an endothelium-derived constricting factor (Harrison, 1997; Shimokawa, 1999; Hink *et al*., 2001); or (d) an increased abundance of caveolin-1, an endogenous inhibitor of eNOS.

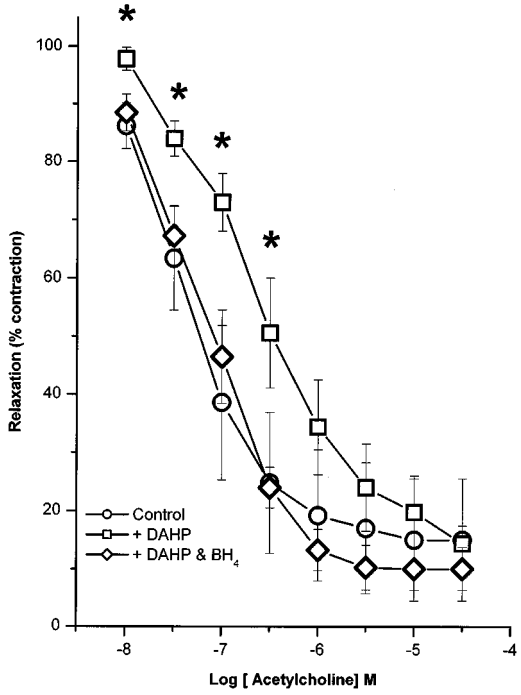

Figure 7 Mean concentration-response curves to ACh in mouse mesenteric arteries from *db/db* +/? before (control) and after incubation with either DAHP (10 mM) for 3 h or DAHP (10 mM) for 3 h plus BH_4 (10 μM) during the last 30 min ($n=4-5$). Symbols are mean values with s.e.mean shown by vertical bars. $^*P<0.05$ compared to control.

Among the cofactors for eNOS BH_4 is critical for enzyme activity and NO production. In the event of a deficiency of BH_4, constitutive NOS transfers electrons to molecular oxygen to produce superoxide instead of NO (Pou *et al*., 1992; 1999; Tiefenbacher, 2001). Supplementation with BH_4 improves endothelium-dependent relaxation to ACh in aortic tissue from STZ-induced diabetic rats (Pieper, 1997) and in human blood vessels from type II diabetic patients (Heitzer *et al*., 2000a). In this study, we demonstrated that acute incubation with BH_4 significantly enhanced the sensitivity of the SMA to ACh-mediated relaxation in *db/db* −/− mice. Auto-oxidation of BH_4 can generate superoxide anion, which in turn inactivates NO with half maximal concentration of 2 μM (Mayer *et al*., 1995), however, the generation of superoxide anions probably occurs only at high concentrations of BH_4. The generation of superoxide has been shown to have an inhibitory effect on endothelium-dependent vasorelaxation in isolated blood vessels, which can be reversed by SOD thus suggesting that these effects were mediated by superoxide (Kinoshita & Katusic, 1996). To overcome this potential problem we have also used sepiapterin, which is converted to BH_4 intracellularly through a salvage pathway (Mayer & Werner, 1995). Consistent with the results from BH_4, sepiapterin also improved the sensitivity to ACh in SMA from db/db −/− mice. Blockade of BH_4 synthesis with DAHP, a selective GTP cyclohydrolase I inhibitor, resulted in an altered or impaired endothelial function in isolated aorta, coronary (Cosentino & Katusic, 1995; Tiefenbacher *et al*., 1996) and cerebral arteries (Kinoshita *et al*., 1997). Our results indicate that incubation with DAHP reduced the sensitivity of SMA to ACh, and that sensitivity of the tissue can be restored by incubation of SMA with BH_4. Collectively these data together suggest that the dysfunction of the endothelium may be due to the lack of the co-factor BH_4. However, BH_4 had no effect on enhanced vascular reactivity to PE. This enhanced vascular reactivity, which is independent of NO and BH_4, may thus be mediated by enhanced endothelin-1 activity (Makino *et al*., 2001).

It has been reported that the BH_4 levels found in coronary artery endothelial cells from diabetic biobreeding (BB) rats were only 12% of that measured in normal rats and that this reduced level of BH_4 correlated with a reduced production of NO (Meininger *et al*., 2000). This deficiency was linked to decreased activity of GTP-cyclohydrolase I; the first and the rate-limiting enzyme in the *de novo* biosynthesis of BH_4. Moreover, the addition of sepiapterin enhanced NO production supporting the hypothesis that BH_4 deficiency is the metabolic basis for impaired endothelial NO synthesis in BB rats (Meininger *et al*., 2000). Shinozaki *et al*. (1999) reported decreased levels of BH_4 in aorta from the fructose-fed insulin-resistant rat model. Furthermore, in the same study, it was shown that acute incubation with BH_4 restored endothelium-dependent relaxation to ACh and chronic supplementation with sepiapterin in insulin-resistant rats improved endothelium-dependent relaxation of aorta and reduced oxidative stress (Shinozaki *et al*., 2000).

The mechanisms involved in BH_4-mediated improved endothelium function are not fully understood. It has been proposed that BH_4 exerts an allosteric action to stabilize the active dimeric state of NOS and to play a redox active role in stimulating NOS (Mayer & Werner, 1995). It has also been reported that BH_4 binds to NOS and facilitates the transfer of electrons and the production of NO (Hurshman *et al*., 1999; Schmidt *et al*., 2001). Other mechanisms, whereby BH_4 enhances NO activity, include increasing the binding of L-arginine to NOS (Wever *et al*., 1998) or, because BH_4 is very redox-active, scavenging reactive free radicals. In support of the later hypothesis, it has been shown that BH_4 improves endothelium function in insulin-resistant rats by reducing vascular oxidative stress (Shinozaki *et al*., 2000). ACh-mediated relaxation is impaired in SMA from eNOS −/− but is partially compensated by an up-regulation of EDHF (Waldron *et al*., 1999). In the current study we observed that BH_4 had no effect on ACh-mediated relaxation of SMA from eNOS −/− mice suggesting that the cellular basis for the action of BH_4 to improve endothelial function in db/db −/− mice is mediated through the NOS pathway.

When the endothelial cell is presented with sub-optimal concentrations of BH_4, NOS generates superoxide anions and oxygen free radicals, instead of NO (Klatt *et al*., 1992; Stroes *et al*., 1998). Reactive oxygen species readily react with NO resulting in the formation of peroxynitrite and hydroxyl ions. Peroxynitrite oxidizes BH_4 to quinonoid 5,6-dihydrobiopterin, which loses its side chain to form inactive 7,8-dihydropterin. BH_4 deficiency leads to eNOS dysfunction with the formation of reactive oxygen species (Milstien & Katusic, 1999). Thus, low levels of BH_4 by itself can mediate its own destruction. The generation of superoxide anions might be important in endothelial dysfunction in diabetes. Superoxide may impair endothelium-dependent relaxation by rapidly inactivating NO (Gryglewski *et al*., 1986) and/or by oxidation of BH4 through peroxynitrite (Milstien & Katusic, 1999; Laursen *et al*., 2001) or by

serving as a contracting factor (Katusic & Vanhoutte, 1989).

There is a body of evidence suggesting an increased oxidative stress in both experimental diabetic models as well as in clinical situations. It has been shown that free radical scavengers like SOD restore impaired endothelium-dependent relaxation in diabetic animals (Pieper & Siebeneich, 1998; Cosentino & Luscher, 1999; Kamata *et al*., 1999). In the current study, SOD and catalase had no effect on ACh-mediated relaxation. Failure of SOD and catalase to improve endothelial function may be due to the fact that their membrane permeability is poor or that their anti-oxidant action is not sufficient to scavenge overwhelming oxidative stress. Thus, in the current study, we used the membrane permeable superoxide scavenger, PEG-SOD in combination with catalase and pre-incubation of tissues with this combination improved the sensitivity of SMA from db/db $-/-$ to ACh. Thus, superoxide anions contribute to the impaired endothelium function in the *db*/*db* mouse. The cellular sources of superoxide anions need further investigation, but might be multiple viz., auto-oxidation of glucose *per se*, glucose-mediated biochemical events, such as the polyol pathway, advanced glycosylation end product and receptor interactions, diacylglycerol-protein kinase C pathway or oxidized LDL, or uncoupling of NOS (De Vriese *et al*., 2000).

A reduced availability of L-arginine seems unlikely because the concentration of L-arginine in endothelial cells is in the millimolar range and about a thousand fold higher than required for maximal activity of eNOS (Harrison, 1997). Nonetheless the beneficial effects of L-arginine supplementation have been documented *in vitro* in isolated tissue studies from diabetic rat as well as in intact models of type I diabetes (Pieper & Peltier, 1995; Pieper *et al*., 1997; Pieper & Dondlinger, 1997). However, the same groups observed that the beneficial effect is dependent on the duration of diabetes (Pieper *et al*., 1995). In the present study, pre-incubation with L-arginine (1 mM) did not enhance ACh-mediated relaxation of SMA, suggesting that lack of substrate was not the primary cause of endothelial dysfunction in SMA from spontaneously diabetic (*db*/*db* $-/-$) mice.

In conclusion, in the present study we demonstrated that SMA from spontaneously diabetic mice exhibit impaired endothelial function that can be restored by acute incubation with BH_4 and a combination of PEG-SOD and catalase. These findings indicate that the cellular basis of endothelial dysfunction in the db/db$-/-$ mouse model of type II diabetes may relate to the reduced availability of BH_4 and/or increased oxidative stress resulting in the uncoupling of eNOS leading to endothelial dysfunction.

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