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Effects of the peroxynitrite decomposition catalyst, FeTMPyP, on function of corpus cavernosum from diabetic mice

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Abstract

Peroxynitrite, the reaction product of nitric oxide and superoxide, may contribute to vascular tissue oxidant stress in diabetes mellitus. The aim was to establish whether the peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(*N*-methyl-4′ -pyridyl)porphyrinato iron III (FeTMPyP) could improve nitric oxide-dependent autonomic nerve and microvascular penile function in the diabetic mouse. Diabetes was induced by streptozotocin; duration was 6 weeks. Intervention FeTMPyP treatment (25 mg kg^{-1} day^{-1}) was given for 2 weeks following 4 weeks untreated diabetes. Corpus cavernosum were isolated in organ baths for measurement of agonist or electrical stimulation-evoked nerve-mediated tension responses. Maximum nitrergic nerve-mediated relaxation of phenylephrine-precontracted cavernosum was approximately 35% reduced by diabetes; FeTMPyP treatment reversed this deficit by 45%. The concentration response-curve for nitric oxide-mediated endothelium-dependent relaxation to acetylcholine was attenuated by diabetes; FeTMPyP restored the deficit to the nondiabetic range. Sensitivity (EC_{50}) to the nitric oxide donor, sodium nitroprusside, was reduced by approximately 0.56 $\log_{10}$ M units in diabetes; however, FeTMPyP treatment failed to significantly reverse this deficit. Therefore, the peroxynitrite mechanism contributes to nitric oxide-dependent diabetic autonomic neuropathy and vasculopathy and may be a potential target for clinical trials using peroxynitrite decomposition catalysts.

Keywords: Peroxynitrite; FeTMPyP; Diabetes

1. Introduction

The oxidation of biological macromolecules by excessive production of reactive oxygen species (oxidant stress) has been implicated in the pathogenesis of many cardiovascular diseases including the complications of diabetes mellitus (Cai and Harrison, 2000; Cameron et al., 2001a). Peroxynitrite ($ONOO^-$) is not a free radical per se but has oxidising actions that could contribute to oxidant stress. Peroxynitrite also nitrates tyrosine residues in structural proteins including neurofilaments and actin (Beckman and Koppenol, 1996). When peroxynitrite decomposes, hydroxyl radicals are liberated, which can cause lipid peroxidation, oxidative DNA damage, and depletion of important antioxidants such as glutathione (Beckman and Koppenol, 1996). Hydroxyl radicals are an important mediator of nerve and endothelium dysfunction in diabetes (Pieper et al., 1996; Mayhan and Patel, 1998; Cameron et al., 2001b). Nitric oxide generated in diabetic vasculature is rapidly scavenged by omnipresent superoxide to form peroxynitrite at a rate that is faster than the reaction between superoxide and superoxide dismutase (Bayraktutan, 2002). Thus, although potentially harmful superoxide is neutralised, beneficial nitric oxide is consumed and peroxynitrite is produced as a result.

As well as having deleterious effects, peroxynitrite relaxes vascular smooth muscle via a cGMP-dependent mechanism; however, it is much less effective than nitric oxide in penile tissue (Khan et al., 2001). Together, the endothelium and parasympathetic nitrergic nerves supply nitric oxide during the erectile process. Nitric oxide-

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mediated deficits in endothelial- and nitrergic nerve-dependent smooth muscle relaxation are evident in corpus cavernosum from diabetic animals and man (Saenz de Tejada et al., 1989; Azadzoi and Saenz de Tejada, 1992; Nangle et al., 2003a,b).

Peroxynitrite decomposition catalysts rapidly convert peroxynitrite to relatively harmless nitrate (Salvemini et al., 1998), thus reducing the formation of cytotoxic hydroxyl radicals and protein nitration. Decomposition catalyst treatment prevented nitric oxide-mediated macrovascular dysfunction in diabetic mice (Szabo et al., 2002). It is not known whether this approach can be used to improve nitric oxide-dependent nerve or microvascular function. Thus, to elucidate this point and assess the contribution of the peroxynitrite mechanism to corpus cavernosum oxidant stress, diabetic mice were treated with the peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(*N*-methyl-4′-pyridyl)porphyrinato iron III (FeTMPyP).

2. Materials and methods

2.1. Animals

Male MF1 mice were purchased from Harlan (Bicester, Oxford, UK) at 3 months and were aged between 6 and 8 months on the day of experimentation. All mice received standard laboratory chow and had access to water ad libitum. Experiments were performed in accordance with regulations specified by the United Kingdom 'Animal Procedures Act, 1986' and the National Institutes of Health 'Principles of Laboratory Animal Care, 1985 revised version'. Unless otherwise stated, all chemicals were obtained from Sigma (Poole, Dorset, UK).

2.2. Diabetes induction, treatment and anaesthesia

Diabetes was induced by 125 mg kg^{-1} i.p. streptozotocin as previously described (Nangle et al., 2003a,b); duration was 6 weeks. In addition to untreated nondiabetic and diabetic control groups, one group of diabetic mice received intervention treatment with FeTMPyP (Cayman Chemical, Ann Arbor, MI, USA) at a dose of 25 mg kg^{-1} day^{-1} i.p. for 2 weeks, following 4 weeks of untreated diabetes. A similar dose, administered acutely, markedly reduced swelling in carrageenan-induced rat hind-paw oedema; a model of acute inflammation in which peroxynitrite plays a major role (Salvemini et al., 1998). FeTMPyP exhibits good specificity as a peroxynitrite decomposition catalyst, and does not affect nitric oxide or superoxide action (Misko et al., 1998; Salvemini et al., 1998, 1999).

Mice were anaesthetised (5% halothane in air, with 0.1 ml 10% urethane in saline per 10 g body weight i.p.) and the penis was excised at its base with removal of the glans penis and connective and adventitial tissues along the shaft as previously described (Nangle et al., 2003a,b). Mice were exsanguinated following tissue removal, and plasma was stored for subsequent glucose analysis (Ascensia Esprit 2 glucose meter, Bayer Diagnostics, Dublin, Ireland).

2.3. Corpus cavernosum experiments

Experiments were conducted as previously described (Nangle et al., 2003a, b). Briefly, strips of corpus cavernosum were mounted in 10 ml organ baths containing modified Krebs–Ringer solution (144 NaCl, 5 KCl, 1.1 $MgSO_4$, 25 $NaHCO_3$, 1.1 NaH_2PO_4, 1.25 CaCl and 5.5 glucose; in mM) at 37 °C (pH 7.35) and gassed with 95% O_2–5% CO_2. Tension was monitored by isometric transducers and resting tension was set at 0.5 g. In the presence of atropine (1 μM) and guanethidine (4 μM) and against a background phenylephrine precontraction of approximately 80% of maximal tension, nitrergic nerve mediated relaxations were examined in response to electrical field stimulation (train duration 30 s; frequency 2–30 Hz; pulse duration 5 ms; 90 mA; 20 V). Futhermore, non-cumulative concentration–response curves for endothelium-dependent relaxation to acetylcholine, and cumulative endothelium-independent responses to the nitric oxide donor, sodium nitroprusside, were examined after 80% maximal phenylephrine precontraction. At the end of experiments, tissues were lightly patted dry and weighed.

2.4. Statistical analysis

Data are expressed as means±S.E.M. They were subjected to Bartlett's test for homogeneity of variances before one-way analysis of variance. Where significance was reached ($P<0.05$), between groups differences were established using the Newman–Keuls multiple comparison test. Otherwise, data were analysed by Kruskal–Wallis nonparametric one-way analysis of variance and Dunn's multiple comparison test. Concentration–response curves were fitted by sigmoid curves using the least squares method to calculate EC_{50}. Whole-curve differences were tested using two-way analysis of variance. All calculations used a standard statistical software package (Prism3, Graphpad, San Diego, CA, USA).

3. Results

3.1. Plasma glucose concentrations and body weights

Diabetes caused an approximate threefold increase ($P<0.001$) in plasma glucose concentrations (Table 1). The somewhat higher than expected values for control animals likely reflects urethane-induced hyperglycaemia (Wang et al., 2000). Additionally, there was an approximately 17% body weight loss ($P<0.001$). These diabetes-

Table 1
Body weights and plasma glucose concentrations of mice

Group	*n*	Body weight (g)		Plasma glucose ($mmol \cdot l^{-1}$)
		Start	End	
Non-diabetic	13	45.9±1.0	–	12.8±1.3
Diabetic	13	43.8±0.8	36.1±0.9[a]	34.8±3.0[b]
Diabetic+FeTMPyP	9	43.5±1.1	36.2±1.2[a]	32.8±2.9[b]

Data presented as mean±S.E.M.
[a] $P<0.001$ vs. start weight.
[b] $P<0.001$ vs. non-diabetic control.

induced changes were unaffected by intervention FeTMPyP treatment.

3.2. Corpus cavernosum study

Tissue weights did not significantly differ between groups (9.9±0.3 mg, n=13, and 9.8±0.3 mg, n=13, for nondiabetic and diabetic control, and 9.8±0.3 mg, n=9, for FeTMPyP treated diabetic cavernosum, respectively).

Electrical stimulation of corpus cavernosum elicited frequency-dependent contractions (Fig. 1A). Expressed relative to tissue weight, maximum contractions at 30 Hz did not significantly differ between groups (0.066±0.006 and 0.068±0.011 mN mg^{-1} for nondiabetic and diabetic controls, and 0.077±0.011 mN mg^{-1} for FeTMPyP treated diabetic tissue, respectively).

Electrical stimulation following phenylephrine precontraction in the presence of 1 μM atropine and 4 μM guanethidine produced frequency-dependent nitrergic nerve-mediated relaxation (Fig. 1B). Maximum relaxation at 20 Hz was reduced by approximately 35% with diabetes compared to nondiabetic control cavernosum (48.7±3.6% vs. 75.2±3.5%, $P<0.001$). This deficit was 45% corrected by FeTMPyP treatment (60.5±4.9%, $P<0.05$); however, nitrergic relaxation remained significantly depressed compared to the nondiabetic control group ($P<0.05$).

Maximum endothelium-dependent relaxation and sensitivity, assessed by (−log) EC_{50}, to acetylcholine (Fig. 2A), following phenylephrine precontraction, did not significantly differ between groups (46.3±5.3% and 6.99±0.10 mol l^{-1} for nondiabetic control; 35.5±4.6% and 6.70±0.08 mol l^{-1} for diabetic control; and 45.8±7.0% and 7.00±0.11 mol l^{-1} for FeTMPyP treated diabetic cavernosum, respectively). However, there were significant effects of diabetes and treatment ($P<0.05$) at intermediate acetylcholine concentrations (0.3 μmol l^{-1}). Furthermore, two-way analysis of variance revealed a significant diabetic deficit compared to the nondiabetic group across the whole response-curve ($P<0.001$); this was completely corrected by FeTMPyP treatment ($P<0.001$).

Maximum endothelium-independent relaxation to sodium nitroprusside (Fig. 2B), following phenylephrine precontraction, did not significantly differ between groups (67.2±4.0% and 59.5±3.5% for nondiabetic and diabetic control, and 58.5±3.8% for FeTMPyP treated diabetic cavernosum, respectively). However, sensitivity as measured by (−log) EC_{50} was reduced almost 0.6 log units by diabetes (6.12±0.15 vs. 6.68±0.10 mol l^{-1}, $P<0.01$). Treatment with FeTMPyP did not significantly alter this diabetic sensitivity deficit; (−log) EC_{50} remained depressed by approximately 0.4 log units compared to nondiabetic tissues (6.29±0.07 mol l^{-1}, $P<0.05$).

Contractile responses of corpus cavernosum to phenylephrine were not altered by diabetes or treatment. Thus, maximum tensions (mN mg^{-1}) were 0.112±0.010, n=11, and 0.114±0.011, n=12, for nondiabetic and diabetic control groups, and 0.105±0.010, n=7, for FeTMPyP treated tissue, respectively. (−log) EC_{50} values were 6.14±0.12 and 6.00±0.11 mol l^{-1} for nondiabetic and

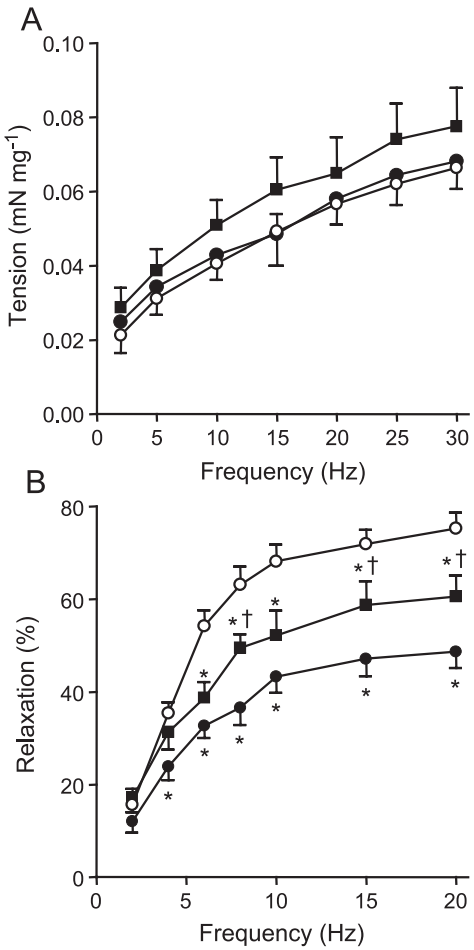

Fig. 1. (A,B) Frequency–response curves for contraction (A) and relaxation following phenylephrine precontraction in the presence of atropine and guanethidine (B) to electrical field stimulation of corpus cavernosum from nondiabetic and diabetic mice, and the effects of intervention FeTMPyP treatment. Groups: nondiabetic control (○; A and B, n=13); 6-week diabetic control (●; A, n=10; B, n=12); diabetic treated with 25 mg kg^{-1} day^{-1} FeTMPyP for 2 weeks following 4 weeks untreated diabetes (■; A and B, n=9). Data presented as mean±S.E.M. Statistics: *$P<0.05$ versus nondiabetic control group; †$P<0.05$ FeTMPyP treated group versus diabetic control group.

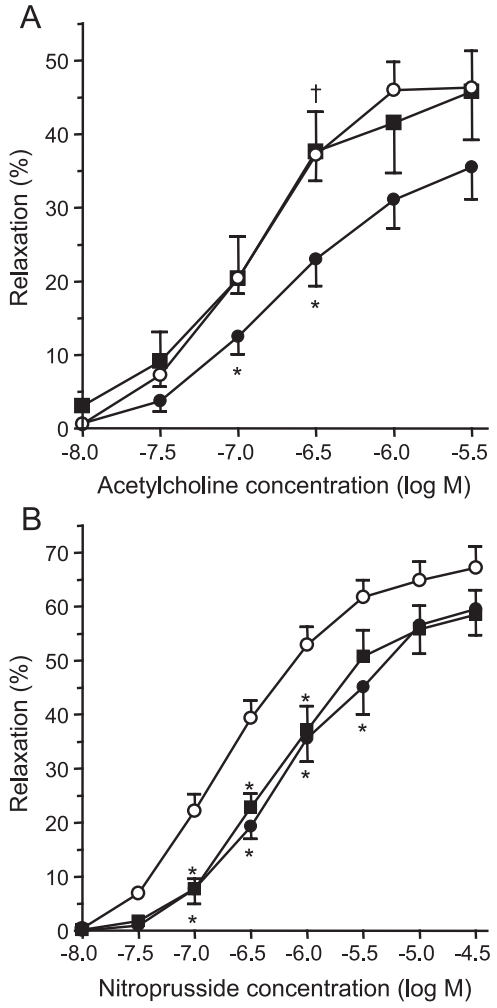

Fig. 2. (A,B) Concentration–response curves for relaxation, following phenylephrine precontraction, to acetylcholine (A; non-cumulative) and sodium nitroprusside (B; cumulative) of corpus cavernosum from non-diabetic and diabetic mice, and the effects of intervention FeTMPyP treatment. Groups: nondiabetic control (○; A and B, n=9); 6-week diabetic control (●; A, n=13; B, n=9); diabetic treated with 25 mg kg^{-1} day^{-1} FeTMPyP for 2 weeks following 4 weeks untreated diabetes (■; A, n=9; B, n=7). Data presented as mean±S.E.M. Statistics: *P<0.05 versus non-diabetic control group; †P<0.05 FeTMPyP treated group versus diabetic control group.

diabetic control groups, and 6.34±0.10 mol l^{-1} for FeTMPyP treated cavernosum.

4. Discussion

In agreement with previous studies on mouse corpus cavernosum (Gocmen et al., 2000; Nangle et al., 2003a,b), streptozotocin-diabetes attenuated nitric oxide-mediated relaxation to nitrergic nerve activation and to acetylcholine-induced endothelium-dependent mechanisms. This is also in accord with findings in man (Saenz de Tejada et al., 1989) and other experimental models including diabetic rats (Keegan et al., 1999; Cartledge et al., 2001) and rabbits (Azadzoi and Saenz de Tejada, 1992; Thompson et al., 2001).

It has been suggested that nitric oxide-linked dysfunctions arise at the level of the endothelium and nerve, as the ability of the smooth muscle to relax to nitric oxide donors was not compromised in the majority of these investigations. However, in contrast, sensitivity of cavernosum to the nitric oxide donor, sodium nitroprusside, was decreased by diabetes in the present study. Decreased responsiveness of diabetic penile tissue to nitroprusside has previously been reported in rabbits (Thompson et al., 2001) and rats (Way and Reid, 1999). In the latter case, this was normalized when the tension readings were expressed relative to tissue weight, to take account of tissue wasting. However, tissue weights did not significantly differ between groups in the present study, therefore it is unlikely that the reduced sensitivity observed is the result of any tissue degradation. Similarly diverse findings are reported in the vascular literature for diabetic patients, where responses to nitric oxide donors were either unaltered (Johnstone et al., 1993) or depressed (McVeigh et al., 1992; Arora et al., 1998). Overall, this may reflect the severity and progression of vascular damage in chronic diabetes, particularly in association with elevated low-density lipoprotein cholesterol (van Etten et al., 2002).

The discrepancy in the mouse literature may also be strain-related. Previous studies have not used the MF1 mouse, which was selected for this study based on its larger body weight, hence cavernosum tissue size. In a previous study on the more commonly used C57 counterpart, with the same duration of diabetes, there was no indication of reduced sensitivity to nitroprusside (Nangle et al., 2003b).

This is the first study to demonstrate that a peroxynitrite decomposition catalyst treatment partially corrects defective nitric oxide-dependent endothelial and nerve dysfunction in corpus cavernosum of diabetic mice, despite reduced cavernosal smooth muscle sensitivity to nitric oxide. Contractile responses to nerve stimulation and exogenous phenylephrine were unaltered either by diabetes or FeTMPyP, suggesting that diabetes does not alter adrenergic function and that the beneficial effects of treatment were not the result of any changes in the preconstriction level against which relaxations were measured.

Theoretically, peroxynitrite formation could be driven by massive production of nitric oxide, for example during inflammatory processes after stimulation of inducible nitric oxide synthase. However, as a general case in the early stages of experimental diabetes, the likely driving force is increased superoxide production in a setting of relatively normal nitric oxide synthesis (Ishii et al., 2001). One potentially important link between superoxide and diabetic neurovascular dysfunction is the production of hydroxyl radicals (Pieper et al., 1996; Cameron et al., 2001b) and the results with FeTMPyP suggest that peroxynitrite is a major mediator in this oxidant stress mechanism. The benefits of treatment with general antioxidants, such as α-lipoic acid, on corpus cavernsum function in diabetic rats (Keegan et al., 1999) are consistent with this view. In diabetic rats, nitrergic

innervation goes through a period of reversible dysfunction before eventual apoptotic loss of cell bodies (Cellek et al., 2003). It is possible that peroxynitrite contributes to this neurodegenerative state and that FeTMPyP may have protective effects on nitrergic cell bodies within the ganglia.

Experimentally, cytokines can be used to stimulate peroxynitrite formation in nervous tissue, and FeTMPyP prevented neurotoxicity in cortical microglia and dopaminergic neurons following this stress (Imam et al., 1999; Xie et al., 2002). Inducible nitric oxide synthase (iNOS) is present in penile tissue even in the absence of notable cytokine levels (Hung et al., 1995). Furthermore, corpus cavernosum function is altered in mice lacking the inducible nitric oxide synthase gene (Nangle et al., 2003c); both iNOS and peroxynitrite are increased in apoptotic penile tissue from aged rats (Ferrini et al., 2001); and iNOS is up-regulated in penile tissue from impotent diabetic men (Seftel et al., 1997). Inducible nitric oxide synthase produces greater amounts of nitric oxide than the constitutively expressed endothelial or neuronal isoforms; thus, its presence could confer upon corpus cavernosum a particular susceptibility for peroxynitrite-mediated dysfunction in diabetes.

Tyrosine nitration is used as a marker of oxidative and nitrosative stress. Although not entirely specific for peroxynitrite-mediated activity, increased nitrotyrosine has been observed in diabetic vascular tissues, including endothelium (Zou et al., 2002; Brodsky et al., 2004). However, immunohistochemical staining failed to identify increased nitrotyrosine in penile nerves of diabetic rats (Cellek et al., 1999; Pitre et al., 2001). The vascular component of diabetic neuropathy is well established (Cameron et al., 2001a); autonomic ganglion blood flow shows an early and marked reduction with diabetes in rats (Cameron and Cotter, 2001). Furthermore, treatment with a variety of antioxidants improves nerve conduction velocity and endoneurial and ganglion blood flow deficits (Cameron et al., 1994; Cameron et al., 2001a). In diabetic patients, plasma nitrotyrosine levels are doubled compared to nondiabetic subjects and those with higher levels of nitrotyrosine are more prone to develop nerve conduction velocity deficits compared to diabetic patients with lower nitrotyrosine levels (Hoeldtke et al., 2002). Therefore, in addition to, or instead of, a direct effect on nerve, it is possible that FeTMPyP improved major pelvic ganglion and nerve blood flow to an extent sufficient to protect nitrergic function.

In conclusion, the peroxynitrite decomposition catalyst, FeTMPyP, partially restored defective nitric oxide-mediated endothelial and autonomic nerve function in corpus cavernosum from diabetic mice. Thus, intervention with peroxynitrite decomposition catalysts may provide a novel therapeutic approach to erectile dysfunction caused in diabetes mellitus. In addition, the MF1 mouse model may be particularly suitable for the study of nitric oxide-mediated endothelium-independent relaxation deficits in diabetic penile tissue.

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