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Effects of the superoxide dismutase-mimetic compound tempol on endothelial dysfunction in streptozotocin-induced diabetic rats

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Abstract

Evidence exists to support the beneficial effects of superoxide dismutase on endothelial dysfunction induced by hyperglycemia in vitro. In vivo, however, studies of the effects of native superoxide dismutase preparations on the vascular complications accompanying diabetes are limited, and their therapeutic application potential has so far been disappointing. The objective of this study was to evaluate, for the first time in vivo, the effects of long-term administration of tempol, a stable superoxide dismutase-mimic compound, on diabetes-induced endothelial dysfunction in rats. Diabetes was induced by streptozotocin and rats were monitored for 8 weeks with or without treatment with tempol (100 mg/kg, s.c., b.i.d). Diabetic rats showed increased vascular levels of superoxide, which was accompanied by increased levels of the oxidative stress markers malondialdehyde and 8-epi-prostaglandin $F_{2\alpha}$. In addition, the vasorelaxant as well as the cGMP-producing effects of acetylcholine and glyceryl trinitrate were reduced in diabetic rats. Treatment with tempol abolished not only the differences in the vascular content of superoxide, malondialdehyde and 8-epi-prostaglandin $F_{2\alpha}$, but also the differences in the relaxation and cGMP responses of aortic rings to both acetylcholine and glyceryl trinitrate between control and diabetic rats. These results support the involvement of reactive oxygen species in mediation of hyperglycemia-induced endothelial dysfunction in vivo, and provide the rationale for potential utilization of stable superoxide dismutase-mimic nitroxides for the prevention of the vascular complications accompanying diabetes.

Keywords: Diabetes; Endothelial dysfunction; Nitric oxide (NO); Superoxide; Superoxide dismutase; Tempol

1. Introduction

Ample evidence exists to support the key role of reactive oxygen species in mediation of the endothelial dysfunction accompanying diabetes mellitus, atherosclerosis, hypercholesterolemia and hypertension (Giugliano et al., 1995; Cannon, 1998). In diabetes, many studies now point to decreased vascular response to endothelium-dependent (e.g., acetylcholine) and non-dependent (e.g., glyceryl trinitrate) vasodilators and to other humoral substances in both conduit (Pieper and Gross, 1988) and resistance (Diederich et al., 1994) blood vessels of experimental diabetic animals. This widespread defect in endothelial function has already been documented in humans in both type I (Johnstone et al., 1993) and type II (McVeigh et al., 1992) diabetic patients. Although the early changes in endothelial cell function in diabetes may be related either to a change in nitric oxide (NO) release or response (Hattori et al., 1991), with a longer duration of disease, both NO release and action are impaired (Kamata et al., 1992). The exact reasons for the decreased response to NO in diabetes are not yet known. However, evidence exists to support the involvement of superoxide and other reactive oxygen species in the increased destruction of NO released from endothelium or NO derived in the normal course of degradation of nitrovasodilators. This is conceivable since scavengers of reactive oxygen species such as superoxide dismutase, catalase, a scavenger of hydrogen peroxide, and deferoxamine, which prevents the formation of hydroxyl radicals, were shown to improve

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abnormal diabetic endothelial cell function (Hattori et al., 1991; Tesfamariam and Cohen, 1992). Again, this suggests that a number of free radicals are involved, possibly ultimately leading to endothelial dysfunction.

At the therapeutic level, the effects of several conventional antioxidants on endothelial dysfunction accompanying diabetes have been reported both in animals and humans (Ting et al., 1996; Palmer et al., 1998; Cameron and Cotter, 1999). Unlike these, tempol is an unconventional antioxidant with powerful superoxide dismutase-mimic activity (Mitchell et al., 1990; Samuni et al., 1991), potent activity against other reactive oxygen species (Charloux et al., 1995; Carrol et al., 2000), which acts both intra- and extracellularly, as well as at the membrane level. Based on the recent results from our laboratory showing the beneficial effects of tempol on hyperglycemia-induced endothelial dysfunction in vitro (Haj-Yehia et al., 1999), we herein report, for the first time in vivo, the effects of prolonged treatment with tempol on endothelial function in streptozotocin-induced diabetic rats.

2. Materials and methods

2.1. Animals and drugs

All animal experiments described here were reviewed and approved by the Committee on Animal Care of the Hebrew University of Jerusalem. Acetylcholine, phenylephrine, tempol and streptozotocin were purchased from Sigma (Israel). Male Sprague–Dawley rats (200 ± 20 g) were randomly divided into three groups: control, diabetic and tempol-treated diabetic rats. Rats were made diabetic by a single tail vein injection of streptozotocin (65 mg/kg) dissolved in citrate buffer (0.01 M solution, pH 4.6). Age-matched control rats were injected with the buffer solution alone. A week after streptozotocin treatment, a blood sample was obtained and diabetes verified by the existence of excessive hyperglycemia. Randomly selected streptozotocin-treated diabetic rats received tempol (100 mg/kg, s.c.) twice a day (8 AM and 8 PM) starting a week after streptozotocin treatment (tempol-treated rats) and throughout the diabetic period until the day of study (8 weeks after the injection of streptozotocin). Control rats and streptozotocin-treated diabetic rats were treated similarly with the vehicle alone.

2.2. Preparation of tissue

Eight weeks after induction of diabetes, rats were anesthetized with i.p. injection of ketamine and xylazine (50 and 10 mg/kg, respectively). A section of the descending aorta was removed and, with care taken not to damage the endothelial cell layer, dissected free of fat and connective tissue in oxygenated (95% O_2; 5% CO_2) Krebs–Henseleit solution (pH 7.4). Rings of approximately 3 mm in width were equilibrated at 37 °C in 10 ml bath chambers for 2 h under a resting tension of 2 g with bath fluid changed every 30 min. Isometric tension was measured with a force–displacement transducer and recorded on-line (Experimetria System).

2.3. Relaxation studies

After equilibration, rings were contracted with phenylephrine (5×10^{-7} M) and concentration–response curves for acetylcholine and glyceryl trinitrate were performed by cumulative addition (10^{-10}–10^{-5} M) of the drugs. Contractile response is expressed as a percentage of the maximal phenylephrine-induced contraction and the pEC_{50} values calculated from individual log concentration–response curves. Data are expressed as mean ± S.E. and statistical analysis was performed by the one-way analysis of variance with statistical significance as indicated in the figures.

2.4. cGMP content

Rings were treated as in the relaxation studies. Acetylcholine or glyceryl trinitrate (both at 10^{-6} M) was added to the chamber and, 1 min after exposure to the drug, rings were removed, freeze-clamped and stored. At the time of analysis, a known amount of tissue was homogenized and cGMP measured as previously described by us (Haj-Yehia and Benet, 1996).

2.5. Superoxide production

The production of superoxide was determined by the measurement of the superoxide dismutase-inhibitable reduction of ferricytochrome C (Heim et al., 1990) with some modifications. Ferricytochrome C (60 μM) was added to the reaction buffer at room temperature and incubated for 10 min in the presence or absence of superoxide dismutase (final concentration 40 U/ml) and reaction terminated by the addition of *N*-ethyl maleimide (1 mM). Reduction of ferricytochrome C was measured spectrophotometrically (550 nm) and the amount of superoxide produced was calculated by dividing the difference in absorbance of the samples with and without superoxide dismutase by the extinction coefficient ($E_{550\ nm} = 21.1\ mM^{-1}\ cm^{-1}$). Comparable results were also obtained when other recently reported methods for measurement of superoxide were applied (Skatchkov et al., 1998).

2.6. 8-Epi-prostaglandin $F_{2\alpha}$

Total Aortic content of 8-epi-prostaglandin $F_{2\alpha}$ was determined by established methodology using an enzyme immunoassay (Roberts and Morrow, 1997). Briefly, aortic tissue (typically pooled from six to eight animals) was homogenized, spiked with [^{3}H]8-epi-prostaglandin $F_{2\alpha}$, treated at 40 °C with 15% potassium hydroxide solution for 1 h, and acidified to pH 4 with 10% hydrochloric acid solution. 8-Epi-prostaglandin $F_{2\alpha}$ was extracted and further purified

Table 1
Weights and serum glucose concentrations of the treatment groups

Group	Serum glucose, mg/dl	Body weight, g
Control ($n=12$)	176 ± 18[a]	362 ± 18[a]
Diabetic ($n=12$)	596 ± 52	181 ± 17
Tempol-treated ($n=12$)	421 ± 43	232 ± 23
P	<0.001[b]	<0.001[b]

[a] Values are presented as means ± S.E. for number of preparations given in parenthesis.

[b] Apply for the significance of the difference between control and the other two groups, as well as between the diabetic and tempol-treated groups.

on C_{18} cartridges (Palmer et al., 1998). Total 8-epi-prostaglandin $F_{2\alpha}$ was assayed using competitive binding with mouse anti-rabbit monoclonal antibody as essentially described by the manufacturer (Oxford Biomedical). Samples were assayed in duplicates and corrected for individual recovery of [^{3}H]8-epi-prostaglandin $F_{2\alpha}$ (average of 82%).

2.7. Malondialdehyde content

Levels of malondialdehyde were determined by the thiobarbituric acid reaction (Yagi, 1975). Incubations were carried out in triplicates and were terminated by the addition of 0.5 ml of 50% trichloroacetic acid solution, followed by the addition of 1 ml of 0.67% thiobarbituric acid solution. Samples were then heated in boiling water for 15 min, cooled and centrifuged for 10 min (4000 × g at 4 °C). The concentration of thiobarbituric acid reaction products in the supernatant was determined fluorometrically (extension 515, emission 553 nm) against a standard curve prepared with malondialdehyde bis(dimethyl acetal).

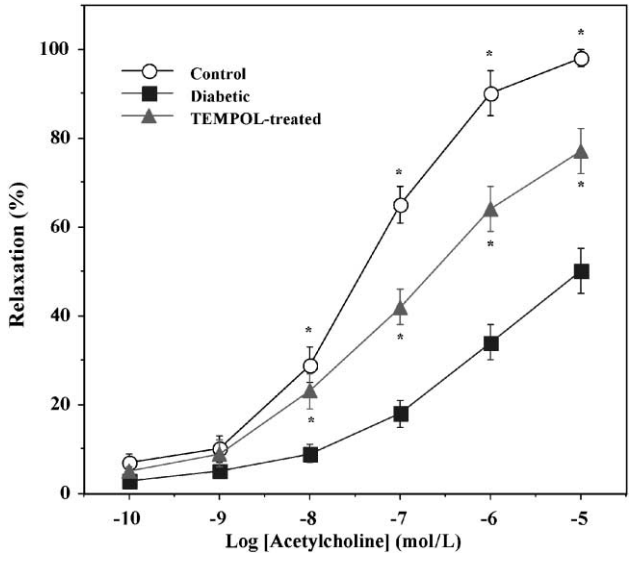

Fig. 1. Relaxation response of rat aortic rings from control, diabetic and tempol-treated diabetic rats in response to acetylcholine. *Significantly different than the corresponding values of diabetic rats ($n=8$, $P<0.05$).

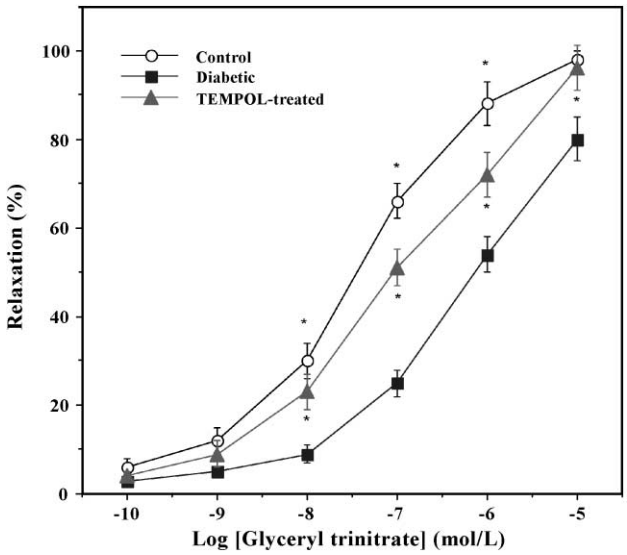

Fig. 2. Relaxations of rat aortic rings from control, diabetic and tempol-treated diabetic rats in response to glyceryl trinitrate. *Significantly different than the corresponding values of diabetic rats ($n=8$, $P<0.05$).

3. Results

3.1. Body weight and glucose concentration

Table 1 shows the weights and serum glucose concentrations in the different groups. Hyperglycemia and reduced body weights are clearly demonstrated in the diabetic and tempol-treated groups as compared to the control group. Although statistically significant, treatment with tempol did not dramatically affect the increased levels of glucose and the reduction in body weights induced by streptozotocin.

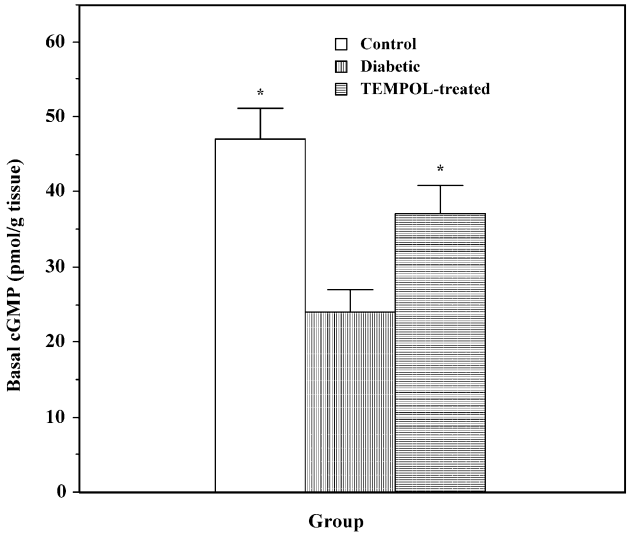

Fig. 3. Basal cGMP levels in rat aortic tissue from control, diabetic and tempol-treated diabetic rats. *Significantly greater than in diabetic rats ($n=8$, $P<0.05$).

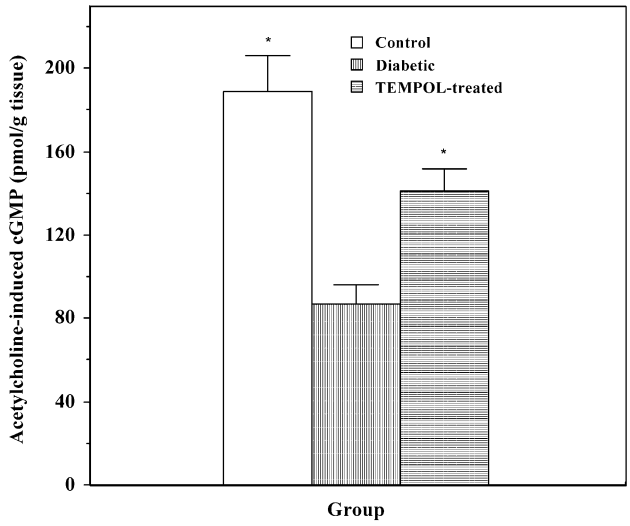

Fig. 4. cGMP levels in response to acetylcholine (10^{-6} M) of rat aortic tissue from control, diabetic and tempol-treated diabetic rats. *Significantly greater than in diabetic rats ($n=8$, $P<0.01$).

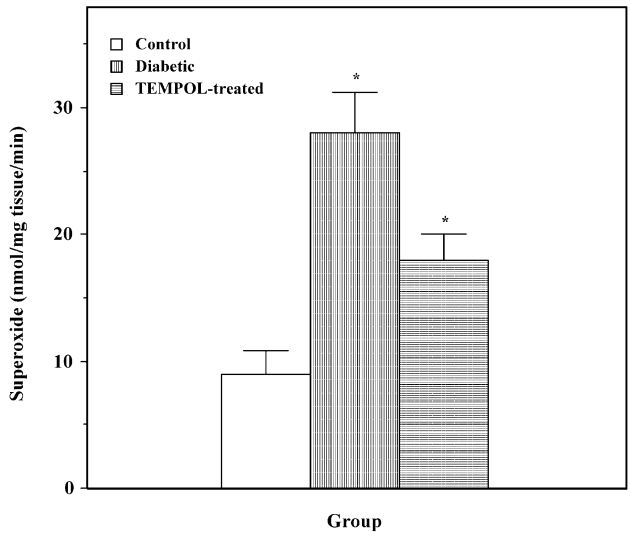

Fig. 6. Basal superoxide production in thoracic aorta from control, diabetic and tempol-treated diabetic rats. Data represents the mean ± S.E. of four experiments each representing aortas pooled from six rats. *Significantly greater than in control or tempol-treated diabetic rats ($P<0.01$).

Similar slight effects of tempol on other typical parameters of diabetes (e.g., hemoglobin A_1C and triglycerides) were also observed (data not shown).

3.2. Relaxation studies

Acetylcholine and glyceryl trinitrate relaxed aortic rings from all three groups in a concentration-dependent manner. However, the concentration–response curves for both drugs were significantly shifted to the right in the diabetic group compared to control or tempol-treated group (Fig. 1). The pEC_{50} values for acetylcholine were 7.518 ± 0.13 and 6.523 ± 0.09 in aortic rings from age-matched control and diabetic rats, respectively ($n=8$, $P<0.001$) with maximal relaxation averaging around 40% for the diabetic group as compared to 100% of the control. Similarly, the concentration-dependent relaxation produced by glyceryl trinitrate in aorta from diabetic rats was significantly different from those of control rats and tempol-treated rats; 6.527 ± 0.08 versus 7.618 ± 0.14 versus 7.287 ± 0.16, respectively (Fig. 2). However, as evident from Figs. 1 and 2, diabetes has a more profound effect on relaxation induced by acetylcholine than by glyceryl trinitrate.

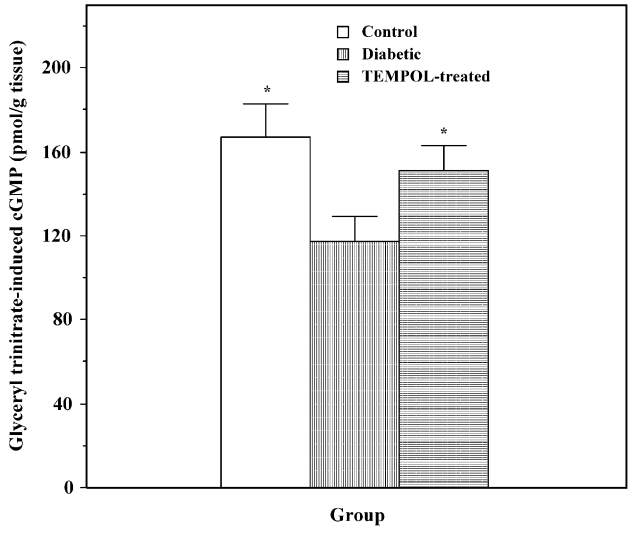

Fig. 5. cGMP levels (pmol/g wet weight) in response to glyceryl trinitrate (10^{-6} M) of rat aortic tissue from control, diabetic and tempol-treated diabetic rats. *Significantly greater than in diabetic rats ($n=8$, $P<0.05$).

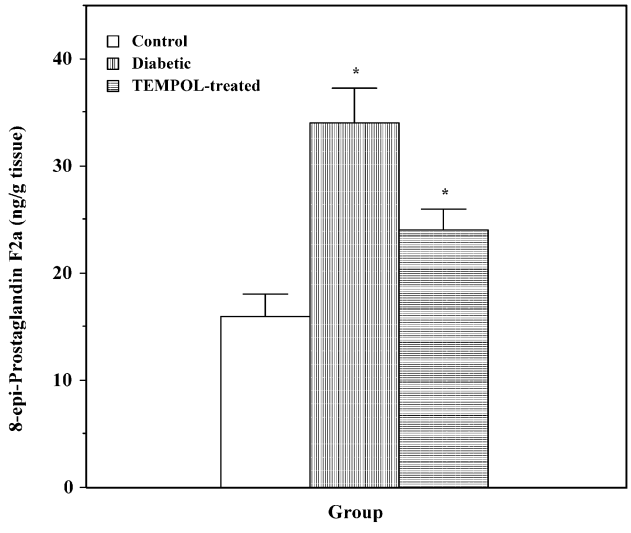

Fig. 7. Aortic 8-epi-prostaglandin $F_{2\alpha}$ content of control, diabetic and tempol-treated diabetic rats. Data represents the mean ± S.E. of four experiments each representing aortas pooled from six rats. *Significantly greater than in control or tempol-treated diabetic rats ($P<0.01$).

3.3. Cyclic GMP

Basal concentrations of cGMP in aortic rings from diabetic rats were significantly lower than in rings from tempol-treated diabetic rats or age-matched control rats (Fig. 3). In all the groups, acetylcholine and glyceryl trinitrate increased vascular cGMP levels. However, this increase was significantly lower in non-treated diabetic rats than in age-matched control and tempol-treated diabetic rats (Figs. 4 and 5). These figures also demonstrate, again, that diabetes affected more the cGMP response to acetylcholine than to glyceryl trinitrate.

3.4. Superoxide, malondialdehyde and 8-epi-prostaglandin $F_{2\alpha}$

Superoxide production, as well as the levels of malondialdehyde and 8-epi-prostaglandin $F_{2\alpha}$, both markers of oxidative stress, were significantly increased in aorta from diabetic rats as compared to control rats or to tempol-treated diabetic rats (Figs. 6–8).

3.5. Effects of tempol

Long-term treatment of diabetic rats with 100 mg/kg of tempol did not dramatically affect the elevated concentrations of glucose and reduced body weights observed in the diabetic group (Table 1). Although the effects of tempol on these parameters of the disease (body weight and serum glucose) are probably not sufficient to explain the overall effects of tempol observed in this study, they may contribute. However, treatment with tempol significantly enhanced the vasorelaxant and cGMP-producing effects of the endothelium-dependent (acetylcholine, Figs. 1 and 4) as well as those of the endothelium-independent vasodilator glyceryl trinitrate (Figs. 2 and 5). The effects of tempol were less significant at lower doses (e.g., 50 mg/kg), whereas at higher doses (e.g., 250 mg/kg), they were accompanied by a slight decrease in blood pressure with variable basal and drug-induced vascular cGMP levels. At 100 mg/kg, tempol itself did not increase basal cGMP production of aorta from control rats in a statistically significant manner, but a statistically significant increase in both basal and drug-induced cGMP production was observed in aorta from tempol-treated diabetic rats (Figs. 3–5). Tempol significantly decreased superoxide levels in vascular tissue of diabetic rats (Fig. 6). This decrease in superoxide was accompanied by a significant decrease in vascular tissue content of the oxidative stress markers malondialdehyde and 8-epi-prostaglandin $F_{2\alpha}$ (Figs. 7 and 8).

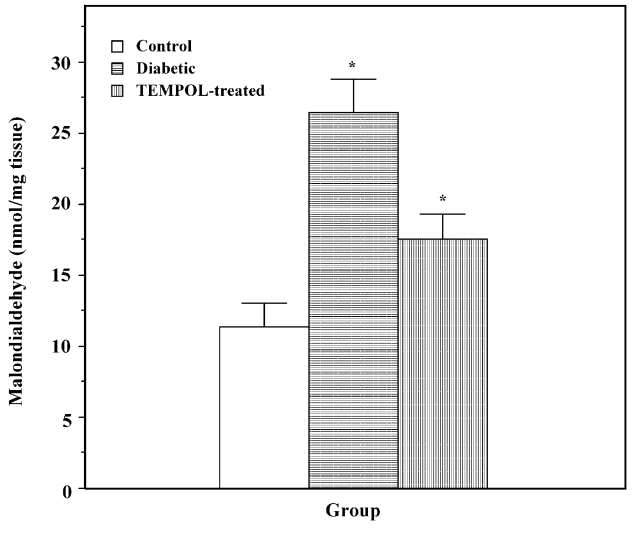

Fig. 8. Malondialdehyde content in aorta from control, diabetic and tempol-treated diabetic rats. Data represents the mean ± S.E. of four experiments each representing aortas pooled from six rats. *Significantly greater than in control or tempol-treated diabetic rats ($P < 0.01$).

4. Discussion

Diabetes is an important and independent risk factor for the development of cardiovascular complications and obstructive vascular diseases involving several body systems. Being the distinguishing feature of diabetes, hyperglycemia has been suggested to play a key role, via oxidative stress, in mediation of these complications. Upon oxidation, glucose generates reactive ketoaldehydes, free radicals and superoxide which, upon further chemical and metabolic reactions, bring about the formation of other free radicals and reactive oxygen species. These reactive oxygen species may largely participate in the formation of glycated proteins, which constitute themselves a source of superoxide, and hence, of other oxygen free radicals (Gillery et al., 1988; Sakurai and Tsuchiya, 1988).

The key role played by reactive oxygen species in the mediation of the vascular complications of diabetes is also reinforced by studies showing that antioxidants such as vitamin E, superoxide dismutase, catalase, glutathione and ascorbic acid are all decreased in blood and tissue of diabetic animals (Wohaieb and Godin, 1987; McLennan et al., 1988). This decrease in endogenously occurring antioxidants will also result in increased oxidative injury by failure of protective mechanisms. Increased flux of glucose through the polyol pathway may deplete NADPH, which is required for the generation of NO from arginine (Änggård, 1994). Furthermore, increased oxidation of sorbitol to fructose increases the ratio of cytosolic NADH/NAD^+. This redox imbalance, known as hyperglycemic pseudohypoxia, augments production of superoxide by hydroperoxidases that use NADH as a reducing co-substrate (Williamson et al., 1993).

The mechanisms by which superoxide may contribute to the abnormalities in NO actions are diverse. Superoxide may react destructively with NO and limit its biological activity (Gryglewski et al., 1986). Superoxide production may lead

to the formation of hydroxyl radicals, which may be cytotoxic to endothelial cells (Beckman et al., 1990) through direct peroxidation of lipids and proteins. In fact, more recent reports indicate that the involvement of hydroxyl radical in mediation of endothelial dysfunction in diabetes may well exceed that of superoxide or hydrogen peroxide. For example, treatment with either native preparations of superoxide dismutase or catalase failed to alter the response to acetylcholine, whereas treatment with diethylenetriaminopentaacetic acid, an inhibitor of metal-catalyzed hydroxyl radical formation, markedly enhanced relaxation to acetylcholine of aortic rings from streptozotocin-induced diabetic rats (Pieper et al., 1997). Similar results demonstrating the important role of hydroxyl radical in mediation of diabetes-induced endothelial dysfunction were also reported using the hydroxyl radical scavenger dimethylthiourea (Mayhan and Patel, 1998). Endothelial cell–platelet interactions are also altered in the presence of superoxide anion. For example, thrombin-induced platelet adherence to endothelial cell monolayers is dramatically increased after endothelial cell exposure to superoxide, suggesting that inactivation of NO may have important implications for local platelet deposition (Shatos et al., 1991). Furthermore, superoxide reacts rapidly with NO to form peroxynitrite, which serves as a potent oxidant that can transfer oxygen atoms, oxidize protein tyrosine residues or sulfhydryls, initiate lipid peroxidation or serve as a source of hydroxyl radicals (Radi et al., 1991).

Because native superoxide dismutase has limited membrane permeability and has proven to be disappointing in preventing adverse effects of superoxide in vivo, alternative agents with superoxide dismutase-mimetic activity have been investigated. However, some superoxide dismutases, such as CuZn superoxide dismutase, are metal-dependent and become ineffective intracellularly because of metal-ligand dissociation. Therefore, compounds with superoxide dismutase-like activity having low-molecular weight, biological stability, no toxicity and membrane permeability are preferred for use in vivo. Mitchell et al., 1990 and others (Samuni et al., 1991; Krishna and Samuni, 1991) have shown that tempol is a stable, metal-independent low-molecular weight superoxide dismutase-mimetic with excellent cell-permeability that possesses activity both at the membrane level and in the aqueous phase (Gelvan et al., 1991; Pou et al., 1992). In fact, it has recently been shown that tempol can normalize blood pressure in spontaneous hypertensive rats (Schnackenberg et al., 1998) and restores vasodilation in arteriols of diabetic rabbit (Schnackenberg and Wilcox, 2001).

Therefore, one may explain the beneficial effects of tempol in diabetes to be due to either direct detoxification of superoxide, inhibition of superoxide-dependent generation of other reactive oxygen species (i.e., hydrogen peroxide, peroxynitrite and hydroxyl radicals), or indirect modulation of NO levels by inhibiting the major reactive oxygen species capable of inactivating NO. These mechanisms may equally be valid also in the case of the endothelium-independent nitrovasodilator glyceryl trinitrate. Here, however, the fact that tempol augmented relaxation to glyceryl trinitrate of diabetic aorta (but not of control aorta) unmasks an additional component of glyceryl trinitrate-induced relaxation, which is specifically modified by superoxide dismutase-mimic-treated diabetic endothelium. These observations are consistent with enhanced production of superoxide and other reactive oxygen species by diabetic endothelium, which may not only alter relaxation by endogenously produced NO, but also decrease the vasorelaxant and cGMP-producing effects of exogenous NO-donors like glyceryl trinitrate. Indeed, Hink et al. (2001) have very recently demonstrated that, despite a three-fold increase in endothelial nitric oxide synthase expression in vascular tissue from diabetic animals, there was a marked decrease in the response of diabetic vascular tissue to acetylcholine. To explain this paradox, evidence was provided to show that this increase in nitric oxide synthase expression resulted in increased production of superoxide rather than of NO, which, most probably, is due to superoxide and/or other reactive oxygen species-mediated uncoupling of the enzyme (Hink et al., 2001). In addition, a significant increase in the activity of protein kinase C-mediated NADPH oxidase was found to accompany nitric oxide synthase uncoupling, thus providing an additional source for the excessive amounts of superoxide measured in diabetic animals (Hink et al., 2001). However, contrast to our present results showing a decreased response of diabetic vascular tissue to both acetylcholine and glyceryl trinitrate, Hink et al. (2001) reported a similar decrease in the response to acetylcholine with no alteration in GTN action on diabetic tissue. This discrepancy may be due to the fact that, whereas, we measured response to glyceryl trinitrate after 8 weeks of diabetes, Hink et al. (2001) measured response to the drug only 2 weeks after streptozotocin administration. This is conceivable since during an early stage of diabetes, only the response to acetylcholine is impaired and with longer duration of disease, the responses to both endothelium-dependent and independent vasodilators are impaired (Kamata et al., 1992; McVeigh et al., 1992).

It should also be noted that superoxide reacts rapidly with NO at a rate of 6.7×10^9 mol/l^{-1}/s^{-1}, which is three times faster than the reaction rate of superoxide with superoxide dismutase (including nitroxides like tempol). In other words, in a compartment in which both NO and tempol exist, there is a propensity for superoxide to preferentially react with NO rather than with tempol. Thus, under such conditions, superoxide may serve as a precursor for the formation of the more injurious reactive oxygen species, peroxynitrite, and its reaction products (hydroxyl radical), which inflicts cellular damage (Murphy et al., 1998). Thus, under diabetic conditions, it is expected that, in addition to superoxide and its dismutation product hydrogen peroxide, excessive concentrations of peroxynitrite and hydroxyl radicals are present. Under such conditions, a possibility exists that inhibition of soluble guanylyl cyclase may also occur (Mulsch et al., 1997) and thus explain the reduced

effects of both acetylcholine and glyceryl trinitrate in diabetic animals. This may, in fact, explain the only partial reversal by tempol of NO-mediated relaxation observed in this study and also indicate that nitroxides like tempol may contribute to cell protection through mechanisms independent of direct superoxide dismutation. Evidence supporting this conclusion has recently been provided by Schnackenberg and Wilcox (2001) who, using the alloxan-induced diabetes in rabbits, showed that only scavenging of superoxide by tempol may not account for the entire effects of the compound and that other mechanisms must be involved.

In support of this possibility, nitroxides like tempol were reported to suppress production and toxicity of tumor necrosis factor (TNF-α) (Pogrebiniak et al., 1991), reduce hypervalent metals such as ferryl, remove H_2O_2 by hemeproteins catalysis, convert semi-quinones to their parent quinones (Krishna and Samuni, 1991; Krishna et al., 1996), and effectively act as breakers and terminators of radical chain reactions (Nilson et al., 1989). In fact, tempol was reported to even protect cells treated with H_2O_2 in the absence of oxygen (Mitchell et al., 1990), and very recently, to also inhibit peroxynitrite formation and action (Carrol et al., 2000). Therefore, one may explain the beneficial effects of tempol observed in this study to be due to multimechanistic antioxidant actions that are not directly related to its superoxide dismutase-mimic activity.

In summary, long-term administration of the stable, nontoxic, membrane-permeable superoxide dismutase-mimetic tempol protected rats from diabetes-induced reduction of vascular response to endothelium-dependent and non-dependent NO-mediated vasodilatory action. Our data show that superoxide dismutase-mimics may prove useful for the prevention and/or reversal of endothelial dysfunction in diabetes. The effectiveness of tempol may be due to its capability to affect most reactive oxygen species at the membrane domain as well as both intracellularly and extracellularly. Considering the controversy surrounding the beneficial effects of vitamin E (Palmer et al., 1998; Paolisso et al., 2001; Visioli, 2001), the results of this work may provide the rationale for the use of alternative antioxidants like tempol for the prevention of the endothelial dysfunction accompanying not only diabetes, but also other pathologies involving oxidative stress and free radical injury.

Acknowledgements

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