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Original Research Communication

Effects of the Superoxide Dismutase-Mimic Compound TEMPOL on Oxidant Stress-Mediated Endothelial Dysfunction

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ABSTRACT

The aim of this study was to investigate the effects of oxidant stress on endothelium-dependent and endothelium-independent arterial relaxation. For this, oxidant stress was generated by preincubation of rat aortic rings (RARs) in either 25 m*M* glucose (mimicking hyperglycemic stress) or 0.5 m*M* pyrogallol (a superoxide generator) and the effects of the superoxide dismutase (SOD)-mimetic compound 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy free radical (TEMPOL) on the vasorelaxant and cGMP-producing effects of acetylcholine (ACh) and glyceryl trinitrate (GTN) in control RARs and RARs exposed to oxidant stress were examined. Pyrogallol, and to a lesser extent high glucose concentration, enhanced the contractile response of RARs to phenylephrine and markedly inhibited the vasorelaxant response to ACh. Although they existed, the inhibitory effects of high glucose and pyrogallol on the vasorelaxant response to GTN were less profound, especially with pyrogallol. Moreover, both pyrogallol and high glucose concentration inhibited the basal and the ACh-induced vascular cyclic guanosine monophosphate (cGMP) production. Treatment with TEMPOL (1–5 m*M*) slightly increased the ACh and GTN-induced cGMP levels in control RARs but had a significant effect in high glucose and pyrogallol-pretreated RARs. Additionally, concomitant treatment of RARs with TEMPOL (5 m*M*) abolished the difference in the relaxation response between control RARs and RARs exposed to either pyrogallol or high glucose concentration. These results further support the theory that reactive oxygen species (ROS), especially superoxide, play a key role in mediation of endothelial dysfunction accompanying diabetes, probably through their effects on the ability of the endothelium to synthesize, release or respond to endogenous nitric oxide (NO) or NO donated by nitrovasodilators. Antiox. Redox Signal. 1, 221–232, 1999.

INTRODUCTION

THE ENDOTHELIUM PLAYS A PIVOTAL ROLE in the mechanisms underlying both macrovascular and microvascular complications accompanying diabetes mellitus (DM). Although the exact biochemical deficits that directly follow from hyperglycemia and protein glycation need to be identified, ample evidence exists to support the key role of the reactive oxygen species (ROS)-mediated endothelial dysfunction in the pathogenesis of diabetes complications (Giugliano *et al.*, 1995; and Cannon, 1998). Many studies now point to reduction in en-

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dothelium-dependent vasodilation in response to acetylcholine (ACh) and other humoral substances in both conduit (Oyama *et al.*, 1986; Pieper and Gross, 1988) and resistance (Diederich *et al.*, 1994; Taylor *et al.*, 1995) blood vessels of experimental diabetic animals. In fact, this widespread vascular defect in endothelial function has now been documented in humans in both type I (Johnstone *et al.*, 1993; McNally *et al.*, 1994) and type II (McVeigh *et al.*, 1992) diabetic patients. The exact mechanism underlying endothelial dysfunction in DM is not yet completely understood. For example, although the early changes in DM may involve an impairment in nitric oxide (NO) release by the endothelium (Hattori *et al.*, 1991), with a longer duration of disease, both NO release and action are impaired (Abiru *et al.*, 1990; Kamata *et al.*, 1992).

The exact reasons for the decreased response to NO in DM are not yet known. However, evidence exists to support the involvement of superoxide and other ROS in the increased destruction of NO released from endothelium or NO derived in the normal course of degradation of nitrovasodilators. This is conceivable because ROS scavengers such as SOD, catalase, a scavenger of hydrogen peroxide (H_2O_2), and deferroxamine, which prevents the formation of hydroxyl radicals, were shown to improve 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. However, the insensitivity to NO in DM may also be the result of decreased guanylyl cyclase activity, increased metabolism of cGMP, or impaired cGMP-dependent protein kinase activity. In addition, Na^+/K^+ ATPase activity is known to be inhibited in diabetic smooth muscle, possibly leading to depolarization that would create resistance to the action of NO or to the action of the endothelium-dependent hyperpolarizing factor (EDHF) by either activation of potassium channels or activation of Na^+/K^+ ATPase itself (Tesfamariam and Cohen, 1992; Gupta *et al.*, 1994).

At the drug discovery level, studies on the effects of antioxidants on endothelial dysfunction are scanty, and the overall benefit of their use in DM and other diseases involving oxidative stress is controversial. For example, although generally thought of as beneficial, it has recently been reported that not only does treatment with vitamin E not prevent endothelial dysfunction in streptozotocin-induced diabetic rats, but also may by itself impair vascular function in this animal model of the disease (Palmer *et al.*, 1998).

Here we wish to report the effects of the nitroxide TEMPOL (4-hydroxy-2,2,6,6-tetramethyl-4-piperidinyloxy, free radical, I) on endothelial function after exposure of rat aortic rings (RARs) to oxidant stress *in vitro*, either by mimicking *in vivo* hyperglycemia or treatment with the superoxide generator pyrogallol. TEMPOL is a chemically stable, cell-permeable, and potentially nontoxic compound that, unlike vitamin E, has been shown to possess powerful antisuperoxide activity (Mitchell *et al.*, 1990, 1991; Krishna and Samuni, 1991; Samuni *et al.*, 1991) and also to inhibit hydroxyl radical formation (Charloux *et al.*, 1995), both in the lipid domain and the aqueous phase.

MATERIALS AND METHODS

The experiments described here were reviewed and approved by the Committee on Animal Care of the Hebrew University of Jerusalem. ACh, phenylephrine (PE), and TEMPOL were obtained from Sigma Chemical Company (Sigma, Israel). Solutions were prepared in distilled water just prior to use. On the day of experimentation, male Sprague-Dawley rats (Hebrew University, 200 ± 20 grams) were anesthetized with an intraperitoneal (i.p.) injection of ketamine and xylazine (50 and 10 mg kg^{-1}, respectively). A section of the thoracic aorta (between the aortic arch and diaphragm) 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, of the following composition: 144.0 m*M* NaCl, 5.9 m*M* KCl, 1.6 m*M* $CaCl_2$, 1.2 m*M* $MgSO_4$, 1.2 m*M* KH_2PO_4, 25.0 m*M* $NaHCO_3$, 11.1 m*M* D-glucose. In experiments involving the induction of oxidant stress by high glucose, the concentration of glucose in the buffer solution

was altered to 5.5 and 25 m*M* for control and 'diabetic' rings, as to mimic the *in vivo* hyperglycemic condition. Rings of approximately 3 mm in width were then cut, mounted between two stainless steel wires, and hooked in a 10-ml bath chambers containing continuously oxygenated Krebs-Henseleit solution. Rings were equilibrated at 37°C for 2 hr, during which tension was gradually increased to 2 grams with bath fluid changed every 30 min. Isometric tension was measured with a force-displacement transducer and recorded on line using computerized system (Experimetria®).

After equilibration, rings were contracted with PE (5×10^{-7} *M*) to induce submaximal contraction (60–80% of maximal contraction). ACh (10^{-5} *M*) was then added to assess the integrity of the endothelium, and rings that failed to attain 85% of relaxation were discarded. Rings with intact endothelium were again equilibrated and incubated for 6 hr with either 5.5 or 25 m*M* glucose with or without TEMPOL (5 m*M*). Then concentration–response curves for ACh and GTN were performed after precontracting the rings submaximally with PE (5×10^{-7} *M*) and cumulative addition (10^{-10}–10^{-5} *M*) of the drugs. Experiments involving preincubation for 1–3 min with 0.5 m*M* pyrogallol with or without TEMPOL (TEMPOL is added 5 min before pyrogallol addition) were performed on parallel rings from the same individual animals. Contractile response is expressed as a percentage of the maximal PE contraction and data are expressed as mean ± SE mean. Statistical analysis was performed by Student's unpaired *t*-test for comparison between two groups and applying the one-way analysis of variance (ANOVA) for comparison between the three groups (control, high glucose and pyrogallol) with statistical significance set at the level of 5%.

To determine the cyclic guanosine monophosphate (cGMP)-producing effect of ACh and glyceryl trinitrate (GTN), rings were treated as for the relaxation studies, except that they were not hooked to the transducer. ACh or GTN (10^{-7}) was added to the chamber and, 1 min after exposure to the drug, rings (usually 2–4) were taken, wrapped with aluminum foil, and immediately frozen at liquid nitrogen temperature and stored. At the time of analysis, rings were thawed, blotted over a dry gauze, and weighed. A known weight of tissue was homogenized in 2 ml of ice-cold modified Hank's balanced salt solution (HBSS) consisting of: 8 grams/liter NaCl, 0.4 gram/liter KCl, 1 gram/liter glucose, 0.06 gram/liter KH_2PO_4, 0.047 gram/liter Na_2HPO_4, and 0.017 gram/liter phenol red and containing 25 m*M* EDTA (disodium salt). The homogenate was centrifuged at 4,000 × *g* for 10 min at 2–4°C and the supernatant transferred into a fresh precooled test tube containing 1 ml of acetonitrile. The tubes were vortex mixed for few seconds and subsequently centrifuged at 4,000 × *g* for 5 min at 2–4°C to remove the precipitated protein.

Each supernatant was transferred into a clean test tube and evaporated to dryness under a stream of nitrogen at 55°C. The dry residue was reconstituted with 10 volumes of Tris-EDTA buffer pH 7.5 (0.05 *M* Tris containing 4 m*M* EDTA). Aliquots (100 μl) of the reconstituted solution in duplicate were used for cGMP measurements using the Amersham RIA kit. Standard curves were made with six concentrations (0, 0.5, 1, 2, 4, and 8 pmol cGMP/tube) and the assay performed as described previously by us (Haj-Yehia and Benet, 1995).

RESULTS

Contraction studies

Pretreatment of aortic rings with 25 m*M* glucose for 6 hr or with 0.5 m*M* pyrogallol for 1–3 min increased their contractile responsiveness to phenylephrine. The enhancing effect of pyrogallol was more profound than that of high glucose, as evident from the more significant shift to the left obtained with pyrogallol as compared to that following preincubation with 25 m*M* glucose (Fig. 1). In the case of pyrogallol, the maximal contractile response to phenylephrine was–increased by 15–20%, but all maximal responses were adjusted to 100% as to make comparison between groups feasible within the same figure (Fig. 1).

Relaxation studies

ACh and GTN relaxed aortic rings from all three groups (control, high glucose and pyro-

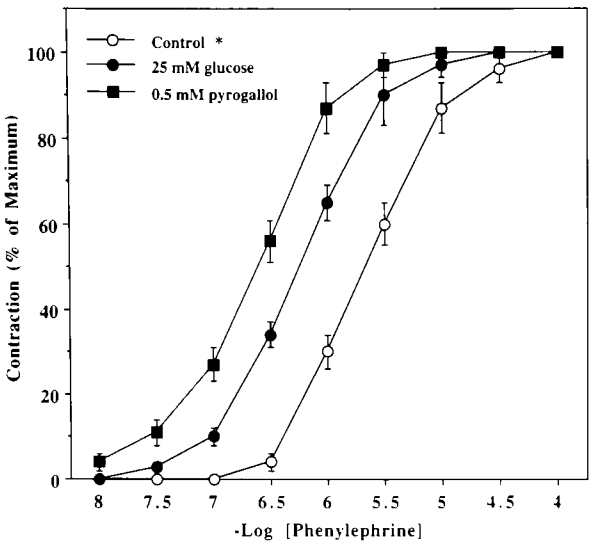


FIG. 1. Influence of glucose and pyrogallol on contractile responses to PE. RARs were incubated for 6 hr with 25 m*M* glucose or pretreated with 0.5 m*M* pyrogallol for 1 min before eliciting contractions by cumulative additions of PE. All concentrations are final bath concentrations. Contraction signifies percentage of maximal contraction observed with all curves adjusted to a common 100% maximal contraction. Values are expressed as the mean ± SE mean. (*) Significantly different from responses obtained with pyrogallol or high glucose treatment ($n = 8$, $p < 0.05$).

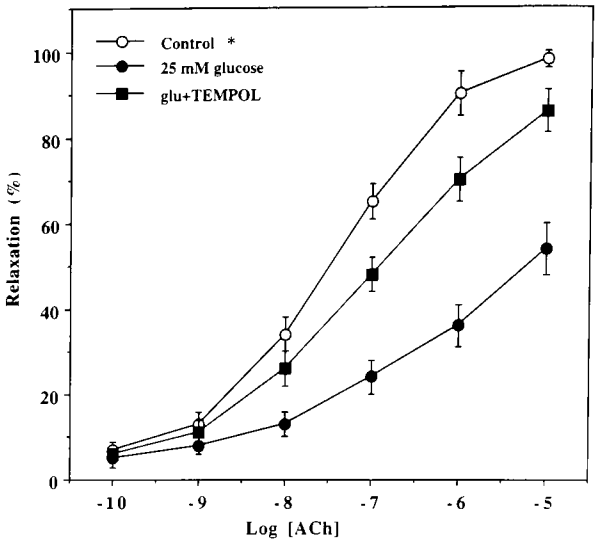


FIG. 2. Relaxation responses after precontraction with PE to cumulative additions of ACh of control RARs and RARs preincubated for 6 hr with 25 m*M* glucose with or without the concomitant presence of 5 m*M* TEMPOL. (*) Significantly different from glucose and glucose + TEMPOL groups for concentrations of 10^{-8} *M* ACh and higher ($n = 8$, $p < 0.05$).

gallol) in a concentration-dependent manner. However, the concentration–response curves for both drugs were significantly shifted to the right following pretreatment with either high glucose or pyrogallol compared to control and TEMPOL-treated groups (Figs. 2–5). The pEC_{50} values for ACh were 7.598 ± 0.13, 6.423 ± 0.11, and 6.167 ± 0.09 in control, 25 m*M* glucose and 0.5 m*M* pyrogallol-pretreated RARs (n = 8–10, $p < 0.01$). Similarly, the concentration-dependent relaxation produced by GTN in RARs preincubated with 25 m*M* glucose or pretreated with 0.5 m*M* pyrogallol was significantly different from control and TEMPOL-treated RARs (Figs. 4 and 5). The pEC_{50} values for GTN were 7.468 ± 0.14, 6.423 ± 0.11, and 7.137 ± 0.12 in control, 25 m*M* glucose, and 0.5 m*M* pyrogallol-pretreated RARs (n = 8–10, $p < 0.001$).

Cyclic GMP accumulation

The effects of high glucose concentration and pyrogallol on endothelium-dependent and endothelium-independent vascular cGMP production were examined. Basal concentrations of cGMP in RARs pretreated by either incubation with 25 m*M* glucose or 0.5 m*M* pyrogallol were significantly lower than in control or TEMPOL-treated RARs (Fig. 6). A slight, but

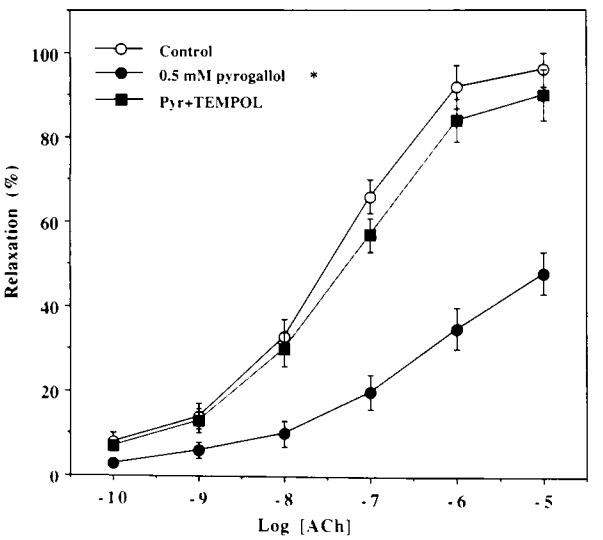


FIG. 3. Relaxation responses after precontraction with PE to cumulative additions of ACh of control RARs and RARs pretreated for 1 min with 0.5 m*M* pyrogallol with or without the concomitant presence of 5 m*M* TEMPOL (TEMPOL was added 5 min before the addition of pyrogallol). (*) Significantly different from control and pyrogallol + TEMPOL groups for all concentrations of ACh ($n = 8$, $p < 0.01$).

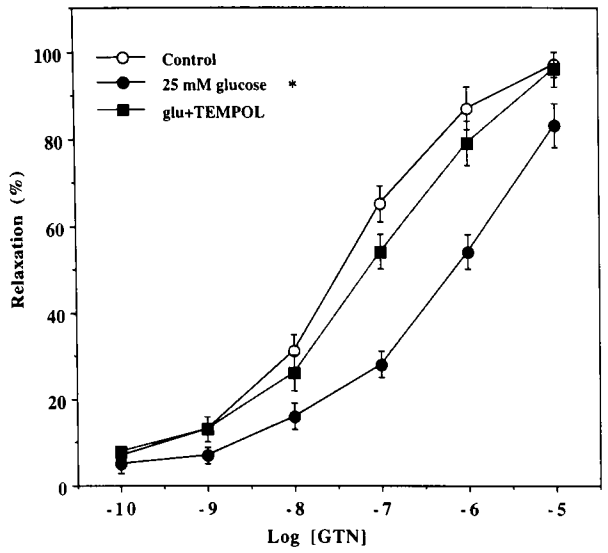


FIG. 4. **Relaxation responses after precontraction with PE to cumulative additions of GTN of control RARs and RARs preincubated for 6 hr with 25 m*M* glucose with or without the concomitant presence of 5 m*M* TEMPOL.** (*) Significantly different from control and glucose + TEMPOL groups for concentrations of 10^{-8} *M* GTN and higher ($n = 8$, $p < 0.05$).

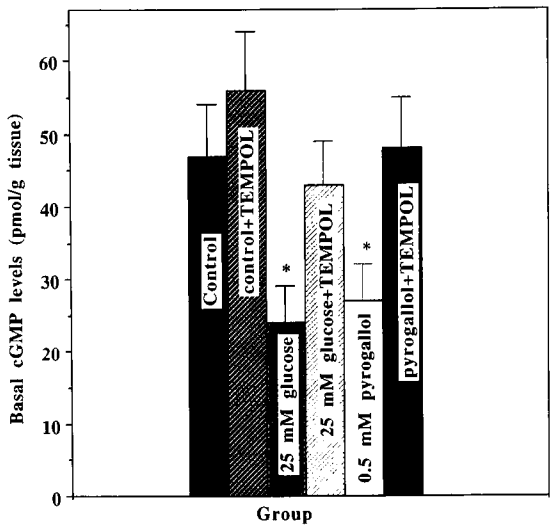


FIG. 6. **Basal concentrations of cGMP (pmol/gram wet weight of tissue) in control RARs (left black column) and RARs exposed to conditions described within the column.** (*) Significantly different from the corresponding TEMPOL-untreated group ($n = 10$, $p < 0.01$).

not statistically significant, increase in basal cGMP levels was observed in TEMPOL-treated control RARs. In all groups, ACh and GTN increased vascular cGMP levels dose dependently. This increase (measured 1 min after drug addition) was significantly greater in control and TEMPOL-treated RARs than in RARs preincubated for 6 hr with 25 m*M* glucose or treated for 1 min with 0.5 m*M* pyrogallol (Figs. 7 and 8).

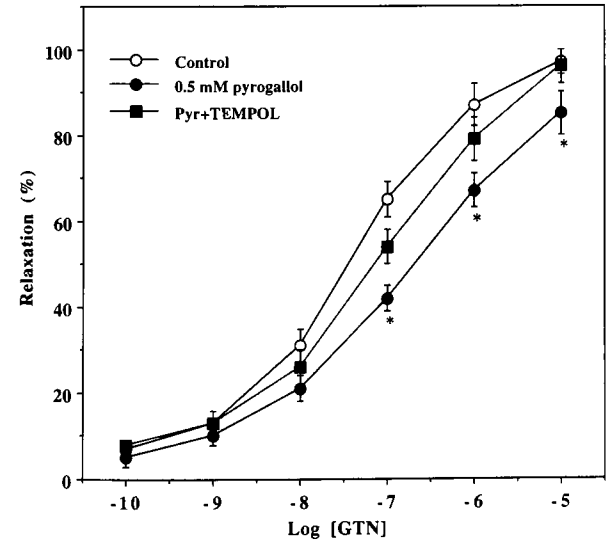


FIG. 5. **Relaxation responses after precontraction with PE to cumulative additions of GTN of control RARs and RARs pretreated for 1 min with 0.5 m*M* pyrogallol with or without the concomitant presence of 5 m*M* TEMPOL. (TEMPOL was added 5 min before the addition of pyrogallol).** (*) Significantly different from control and pyrogallol + TEMPOL groups for marked concentrations of GTN ($n = 8$, $p < 0.05$).

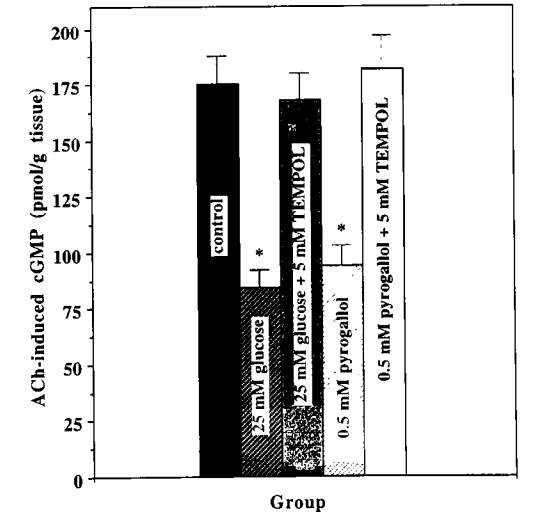


FIG. 7. **ACh-induced cGMP concentrations (pmol/gram wet weight of tissue) in control RARs (left black column) and RARs exposed to conditions described within the column.** RARs were submaximally precontracted with 5×10^{-7} *M* phenylephrine and exposed for 1 min to 10^{-7} *M* ACh. (*) Significantly different from the corresponding TEMPOL-untreated group ($n = 10$, $p < 0.01$).

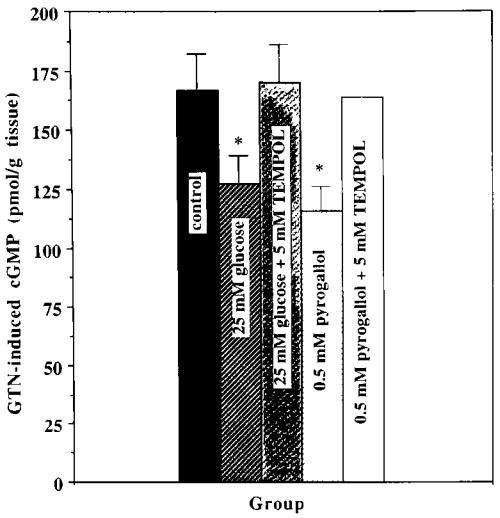


FIG. 8. GTN-induced cGMP concentrations (pmol/gram wet weight of tissue) in control RARs (left black column) and RARs exposed to conditions described within the column. RARs were submaximally precontracted with 5×10^{-7} *M* phenylephrine and exposed for 1 min to 10^{-7} *M* GTN. (*) Significantly different from the corresponding TEMPOL-untreated group ($n = 10$, $p < 0.05$).

Effects of TEMPOL

Concomitant treatment of RARs with 5 m*M* of TEMPOL significantly enhanced the vasorelaxant as well as the cGMP-producing effects of ACh (Figs. 2, 3, and 7). Although they existed, these enhancing effects were less pronounced on the vasorelaxant and cGMP-producing effects of GTN (Figs. 4, 5, and 8). TEMPOL itself did not increase basal cGMP production in control RARs in a statistically significant manner (Fig. 6), but a statistically significant increase in basal cGMP production was observed in RARs preincubated either with 25 m*M* glucose or 0.5 m*M* pyrogallol in the concomitant presence of TEMPOL as compared to preincubation with high glucose or pyrogallol alone (Fig. 6). These results demonstrate the ability of TEMPOL to abolish the difference in basal cGMP production between control RARs and RARs exposed to pyrogallol or high glucose-mediated oxidant stress. In addition to its effects on basal cGMP production, TEMPOL was found very useful in reversing the inhibition by pyrogallol and high glucose pretreatments on ACh-induced vasorelaxant and cGMP-producing effects. Similar effects of TEMPOL-treatment on relaxation and cGMP producing actions of the endothelium-independent nitrovasodilator GTN were found.

DISCUSSION

Results from this study support previous observations by others demonstrating that preincubation of vascular preparations with high glucose levels may simulate the *in vivo* hyperglycemia in DM (Tesfamariam and Cohen, 1992). In our case, we found that the higher glucose levels used in the oxygenated incubation medium, the more significant the difference is in endothelial function between control and pretreated vascular tissue. However, we chose the 25 m*M* concentration because it closely resembles the *in vivo* situation obtained in animal models of DM (*i.e.*, streptozotocin-induced DM). Similar observations were found with pyrogallol. In this case too, the higher the concentration of pyrogallol was, the more that endothelial dysfunction was observed. In fact, with higher levels of pyrogallol (especially when exposure exceeded the 5-min period), the vascular dysfunction was very profound and results in terms of both relaxation and cGMP production were less reproducible. More important, our results also demonstrate that conditions involving excessive generation of superoxide (by pyrogallol) exert a significant impact on vascular function that mimics the dysfunction obtained following incubation with high glucose levels resembling the *in vivo* condition in DM.

Thus, the principal observation of this study is that high glucose levels, like pyrogallol and SOD-mimicking compounds, cause endothelium-dependent contactile and relaxant responses, respectively. Furthermore, as in DM, pyrogallol inhibits both endothelium-dependent and endothelium-independent NO-elicited vascular smooth muscle relaxation and cGMP accumulation, whereas agents possessing SOD-like action enhance such responses. These findings are consistent with previous reports showing the inhibitory effects of pyrogallol on the relaxant and cGMP actions of NO, and the reversal of these effects with SOD (Moncada *et al.*, 1986; Ignarro *et al.*, 1988).

Being the distinguishing feature of diabetes,

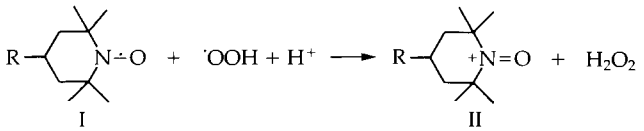
hyperglycemia has been suggested to play a key role, via oxidative stress, in mediation of diabetes complications. Upon oxidation, glucose generates reactive ketoaldehydes, free radicals, and superoxide, which, upon further chemical and metabolic reactions, bring about the formation of other free radicals and ROS. These ROS may participate largely in the formation of glycated proteins, which constitute themselves as a source of superoxide and hence of other oxygen free radicals (Gillery *et al.*, 1988; Sakurai and Tsuchiya, 1988). In fact, elevated levels of ROS products in diabetic patients have been reported (Collier *et al.*, 1990; Ceriello *et al.*, 1991; Paolisso *et al.*, 1993). In one study, the plasma concentration of superoxide was elevated in patients with insulin-dependent diabetes mellitus (IDDM), but showed a trend to normalization after strict metabolic control (Ceriello *et al.*, 1991). There was a strong correlation between plasma glucose and superoxide concentrations in both normal and diabetic subjects over a wide range of glucose concentrations. The possibility that ROS plays a role in the pathogenesis of vascular complications of diabetes is also suggested by studies showing that antioxidants such as vitamin E, SOD, catalase, glutathione (GSH), and ascorbic acid are all decreased in blood and tissue of diabetic animals (Karpen *et al.*, 1982, 1985; Wohaieb and Godin, 1987; Wolff and Dean, 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, which is hyperactive in hyperglycemia (Greene *et al.*, 1987; McLennan *et al.*, 1988), may deplete NADPH, which is required for 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 cosubstrate (Williamson *et al.*, 1993).

The mechanisms by which ROS may contribute to abnormalities in endothelium-derived relaxing factor (EDRF) action are diverse. Superoxide may react destructively with NO and limit the biological activity of EDRF (Gryglewski *et al.*, 1986). Superoxide production may lead to formation of hydroxyl radicals, which may be cytotoxic to endothelial cells (Beckman *et al.*, 1990) through direct peroxidation of lipids and proteins. In addition to these effects, intra/extracellularly generated superoxide may have harmful effects including lipid peroxidation, protein aggregation, and DNA destruction (Maxwell, 1995). Using a system of superfused endothelial cells and an arterial rings bioassay, it has been shown that SOD augments the EDRF response to ACh. In addition, the exposure of unstimulated endothelial cells to SOD produced relaxation of the bioassay ring, suggesting that basal endothelial superoxide production modulates EDRF action (Gryglewski *et al.*, 1986; Beckman *et al.*, 1990). 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 EDRF may have important implications for local platelet deposition (Shatos *et al.*, 1991). In fact, superoxide appears to enhance platelet adhesion and aggregation directly, perhaps through inactivation of NO produced during platelet aggregation (Salvemini *et al.*, 1989; Malinski *et al.*, 1993). 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; Huie and Padmaja, 1993; Goldstein and Czapski, 1995). In fact, being highly active, peroxynitrite has been proposed as the major ROS responsible for damaging vascular tissue, thereby impairing vascular function (Buttery *et al.*, 1996; Myatt *et al.*, 1996).

Previous investigators have used superoxide scavengers to reduce inflammation (Henson and Johnston, 1987), and ischemia/reperfusion injury (McCord, 1985). Because native SOD has limited membrane permeability and has proven to be disappointing in preventing adverse effects of superoxide accompanying DM *in vivo*, alternative agents with SOD-mimetic activity have been investigated. However,

some SODs such as CuZn SOD are metal dependent and become ineffective intracellularly because of metal–ligand dissociation. Therefore, compounds with SOD-like activity having low molecular weight, biological stability, no toxicity, and membrane permeability are preferred for use *in vivo*. Mitchell *et al.*, (1990, 1991) and others (Samuni *et al.*, 1991; Krishna and Samuni, 1991) have shown that TEMPOL is a stable, metal-independent, low-molecular weight SOD-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 very recently been shown that TEMPOL can normalize blood pressure and renal vascular resistance in spontaneous hypertensive rats (Schnackenberg *et al.*, 1998) and, like other SOD-mimetic compounds, can indeed prove useful for the restoration of the response to ACh of endothelium made dysfunctional by oxidant stress (Mok *et al.*, 1998; MacKenzie and Martin, 1998).

Likewise, our results also show that TEMPOL protects against oxidant stress-induced endothelial dysfunction, and thus offers a convenient alternative agent that can compensate for the loss of native SOD activity known to accompany DM and other pathologies involving excessive superoxide production. The mechanisms by which nitroxide antioxidants like TEMPOL exert their antisuperoxide activity is still controversial. The controversy mainly concerns the definition of nitroxides being either stoichiometric scavengers or true SOD mimics (Krishna *et al.*, 1996). Yet, it is universally accepted that they do possess effective antioxidant activity in various biological systems ranging from molecular, cellular, and laboratory animal level. Nitroxides are reported to catalyze superoxide dismutation through two different catalytic pathways including reductive and oxidative reaction mechanisms. In the case of TEMPOL I, it is readily oxidized by protonated superoxide, ·OOH, to yield oxoammonium cation II:



I II

which in turn oxidizes another superoxide to molecular oxygen:

The net reaction, as in the case of native superoxide dismutase, involves the removal of superoxide without affecting nitroxide concentration (Krishna *et al.*, 1996):

Therefore, one may explain the beneficial effects of TEMPOL observed in this study to be due to either direct detoxification of superoxide, inhibiting 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 ROS species capable of inactivating NO. Of these ROS, inhibition of peroxynitrite formation may have the largest impact because its effects on endothelial function are dual. First, TEMPOL may prolong the half-life of NO and thus allow it to exert a more powerful vasodilatory action. Second, by blocking its formation, TEMPOL may inhibit the peroxynitrite-mediated production, release, and/or activation of vasoconstrictor endoperoxides (Landino *et al.*, 1996). These mechanisms may equally be valid also in the case of the endothelium-independent nitrovasodilator GTN. Here, however, the fact that TEMPOL augmented relaxation to GTN of RARs preincubated with high glucose and pyrogallol (but less of control RARs) unmasks an additional component of GTN-induced relaxation, which is specifically modified by SOD-mimic-treated dysfunctional endothelium. These observations are consistent with enhanced production of superoxide and other ROS by dysfunctional 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 GTN. This inhibitory effect of ROS on vascular GTN action may be the result of either a reduction in NO formation from metabolism of GTN or due to enhanced breakdown of such NO. How-

ever, in light of reports showing a general increase in NO production under hyperglycemic states (Bank and Aynedjian, 1993), it is unlikely that constitutive NO synthase is down-regulated in these conditions. Rather, the reduced effects of either endogenous or exogenous NO on RARs exposed to high glucose concentration or pyrogallol appear to result from accelerated destruction of NO by a mechanism that can be reversed by exogenous SOD mimics. At the vascular level, similarly, both aortic rings and bioassay studies have indicated that aorta tissue of diabetic rats releases more spontaneous NO than does control tissue and that the influence of NO on vascular smooth muscle tone is masked by increased NO destruction mediated by superoxide and other ROS (Freedman and Hatchell, 1992; Lin *et al.*, 1993). Thus, a decrease in SOD activity, in the face of excess superoxide production provided by high glucose or pyrogallol, can be expected to result in an accumulation of superoxide. This accumulation may accelerate NO breakdown regardless to level and source of this NO, and may therefore underlie the reduced effect of endogenous (*i.e.*, in response to ACh) as well as to exogenous NO (*i.e.*, GTN) on RARs exposed to oxidant stress as observed in this study. Another possibility may exist which concerns the effects of ROS on guanylyl cyclase activity. This seems reasonable because the activity of this enzyme is thiol dependent, and inhibition of ROS-mediated oxidation of vital SH groups on the enzyme may play a role in TEMPOL activity. This is especially true because the intimate relationship between ROS, NO, and reduced thiols has also been documented (Ignarro *et al.*, 1981; Kontos and Wei, 1983; Burke and Wolin, 1987). Thus, our results showing a decrease in basal and in agonist (GTN and ACh)-induced cGMP production in RARs exposed to high glucose levels or pyrogallol, which is prevented by concomitant treatment with TEMPOL, are in agreement with this possibility.

In summary, concomitant treatment with the stable, membrane-permeable, nontoxic SOD-mimetic TEMPOL protected RARs from high glucose and pyrogallol-induced reduction of vascular responses to endothelium-dependent (ACh) and endothelium-independent (GTN) NO-mediated vasodilatory and cGMP-producing actions. In fact, our data show that, as in the case of hypertension (Schnackenberg *et al.*, 1998) and DM-induced neophropathy (Kazuhisa and Carmines, 1995), SOD mimics may prove superior to other antioxidants like vitamin E for prevention and/or reversal of endothelial dysfunction induced by hyperglycemia or excessive production of superoxide. The effectiveness of TEMPOL, as compared to the recently reported lack of effect of vitamin E (Palmer *et al.*, 1998), may be due to the fact that, unlike vitamin E which scavenges hydroxyl radicals only in the membrane, TEMPOL is capable of scavenging superoxide and hydroxyl radicals at the membrane domain as well as both intracellularly and extracellularly. By doing so, TEMPOL not only increases NO availability by inhibiting its destruction by excess superoxide and other superoxide-derived ROS, but also prevents the formation of hazardous peroxyl radicals. However, some investigators have observed discrepancies, even when using similar experimental conditions relating to diabetes. The effects on the vascular tissue brought about directly by superoxide, or indirectly by other ROS generated from superoxide, are complex. Also, there may be a possible involvement of ROS in either guanylyl cyclase or NO-synthase activity. Thus, the exact contribution of these pathways requires further investigation.

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ABBREVIATIONS

ACh, acetylcholine; ANOVA, one-way analysis of variance; cGMP, cyclic guanosine monophosphate; DM, diabetes mellitus; EDHF, endothelium-dependent hyperpolarizing factor; EDRF, endothelium-dependent relaxing factor; GSH, glutathione; GTN, glyceryl trinitrate; H_2O_2, hydrogen peroxide; HBSS, Hanks' balanced salt solution; IDDM, insulin-dependent diabetes mellitus; i.p., intraperitoneally; NO, nitric oxide; PE, pheneylephrines; RARs, rat aor-

tic rings; ROS, reactive oxygen species; SOD, superoxide dismutase; TEMPOL, 4-hydroxy-2,2,6,6-tetramethyl-4-piperidinyloxy free radical I).

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