Hyperglycemia increases endothelial superoxide that impairs smooth muscle cell Na\(^+\)-K\(^+\)-ATPase activity


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Diabetes is associated with a variety of metabolic abnormalities, principal among these being hyperglycemia. A number of prospective and cross-sectional studies have clearly established a link between increasing blood glucose and cardiovascular morbidity and mortality in both diabetic and nondiabetic subjects (5, 9, 17). The precise mechanism (or mechanisms) by which hyperglycemia could contribute to cardiovascular dis-

ease in diabetes, however, remains the subject of considerable controversy. Established sequelae of hyperglycemia, such as cytotoxicity, increased extracellular matrix production, and vascular dysfunction, have all been implicated in the pathogenesis of diabetes-induced vascular disease (3, 30).

One important consequence of hyperglycemia is an impairment in the bioactivity of nitric oxide (NO), an important mediator of vascular homeostasis. Normally, NO controls vascular tone, prevents inappropriate vasospasm, inhibits platelet aggregation, and limits smooth muscle cell proliferation (reviewed in Ref. 37). In diabetic patients, however, normal NO bioactivity is impaired (26), and this abnormality is reproduced both in vitro (34) and in vivo (38) by acute hyperglycemia. The mechanism responsible for altered NO bioactivity by hyperglycemia is not yet clear. On the basis of measurements for which endothelium-dependent arterial relaxation was used as an index of NO bioactivity, a number of mechanisms have been proposed including increased protein kinase C activity (34), increased aldose reductase activity (36), advanced glycation end-product formation (4), and increased oxidative stress (13).

In addition to the control of vascular tone, there is evidence that NO also plays a role in the regulation of basal Na\(^-\)-K\(^-\)-ATPase activity in arterial tissue (10, 11). Hyperglycemia inhibits Na\(^-\)-K\(^-\)-ATPase activity in rabbit aorta, and this defect is related to diminished synthesis or bioactivity of NO (10, 12). Excess vascular superoxide is known to impair NO bioactivity in diabetes (13); however, the role of superoxide in modulating NO-mediated control of Na\(^-\)-K\(^-\)-ATPase activity under hyperglycemic conditions results from the increased production of superoxide.

METHODS

Materials. Pentobarbital sodium was from Anthony Products (Kanakee, IL). Zopolrestat was a gift from Pfizer (Gro-

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KCl, 25 NaHCO₃, 1.2 NaH₂PO₄, 0.6 MgCl₂, 2.5 CaCl₂, and sodium (50 mg/kg) via lateral ear vein and exsanguinated. Male New Zealand White rabbits were killed by injection of pentobarbital 5 min before addition of 86RbCl (200 U/ml). Ouabain (0.1 mM) was added to the incubation medium 10 min before addition of 86RbCl (2 μCi/ml). In some incubations, superoxide dismutase (SOD; 150 U/ml; Cu,Zn form; Sigma) or zopolrestat (10 μM) was present throughout the 3-h incubation and during incubation with ouabain. Data were expressed as nanomoles per minute per milligram of dry tissue weight based on the specific activity of 86Rb⁻ in the incubation medium.

**Measurement of superoxide anion.** Because the detection of superoxide is dependent on both its production and its metabolism by SOD, we measured superoxide under two conditions. To estimate the net superoxide flux (i.e., production minus metabolism), we determined the superoxide flux in vessel segments with intact SOD activity. To estimate total superoxide production (i.e., that available for reaction with NO (19)), we measured the superoxide flux after inactivating Cu,Zn SOD (85% total SOD activity (19)) with the copper chelator diethyldithiocarbamate (DDC) (14). The flux of superoxide anion was estimated essentially as described previously (16, 19) except that 5 μM lucigenin was used to avoid artifactual superoxide production (21). After a 3-h incubation in Krebs buffer containing 5.5 or 44 mM glucose, rings were incubated with or without 10 mM DDC for an additional 30 min, followed by washing with Krebs buffer containing 1 mM diethyldiaminetriamine pentaacetic acid to remove any liberated transition metals. Vessel rings were then transferred into a tube containing HEPEFS-buffed Krebs, pH 7.4, of the following composition (in mM): 118.3 NaCl, 20 HEPES, 4.5 KCl, 5 NaHCO₃, 1.2 NaH₂PO₄, 0.6 MgCl₂, 2.5 CaCl₂, and 5.5 or 44 glucose with or without 5 μM lucigenin (37°C) for another 10 min in the dark. The tube was then placed in a Turner Designs model 20e luminometer (Mountain View, CA), and all measurements were done in the dark at 37°C. The luminometer was set to report arbitrary units (AU) of emitted light (analog voltage of the photomultiplier) integrated over 30-s intervals. The repeated measurements were made over 30-min periods. After initial measurements were completed, the free radical scavenger Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid; 10 mM) or EUK-8 (300 μM), a cell-permeable SOD mimic (1), was added to the tubes containing lucigenin and allowed to equilibrate for 5 min. Measurements were then repeated for another 10 min. The background signal by lucigenin alone was determined and subtracted from the experimental values. The superoxide signal was stable over the duration of the assay.

**Measurement of tissue sorbitol and fructose levels in aortic rings.** Endothelium-intact aortic rings were incubated in Krebs-bicarbonate buffer containing normoglycemic or hyperglycemic glucose concentrations with or without zopolrestat (10 μM) as described in Measurement of superoxide anion for 3 h and frozen in 0.5 ml of 6% perchloric acid. For analysis, aortic rings were thawed, homogenized by Polytron, sonicated in a bath sonicator at room temperature for 10 min, and centrifuged at 10,000 g for 10 min. The supernatant was neutralized with 3 M potassium carbonate (70 μl/0.5 ml supernatant), vortexed, and centrifuged again as described above. An aliquot of the supernatant was analyzed for sorbitol by a modification of the enzymatic method of Malone et al. (22). Final reagent concentrations were 0.2 M triethanolamine, pH 8.5, 1.2 μM resazurin, 0.3 U/ml diaphorase, and 1.0 mM NAD, with or without 1 U/ml sorbitol dehydrogenase (Boehringer Mannheim). Samples were incubated in the dark with shaking at 23°C for 60 min. Sample fluorescence was determined at a wavelength of 580 nm with excitation at 560 nm. Sorbitol content was calculated by comparison with appropriate standards and blanks. An aliquot of the supernatant was analyzed for fructose by a modification of the method of Nakashima et al. (24) with resazurin substituted for ferricyanide (33). Final reaction concentrations were 0.2 M citric acid, pH 4.5, 13.2 μM resazurin, 3.3 U/ml fructose dehydrogenase (Sigma), and 0.068% (vol/vol) Triton X-100. Samples were incubated for 60 min at 23°C in the dark on a rotary shaker. Fluorescence was read at an emission wavelength of 580 nm with excitation at 560 nm. Fructose content was calculated by comparison with appropriate standards and blanks.

**Measurement of enzyme activity in partially purified Na⁺-K⁺-ATPase preparation.** Na⁺-K⁺-ATPase enzyme activity of commercially available porcine cerebral cortex enzyme preparations (Sigma A7510) was measured at 37°C by coupling ADP production to NADH oxidation and recording the absorbance change vs. time at 340 nm for 5 min. The standard assay mixture contained 0.14 mM NADH, 1 mM phosphoenolpyruvate, 5.5 mM MgCl₂, 100 mM NaCl, 10 mM KCl, 0.1 mM EDTA, 2.8 mM Na-ATP, 12.5 μg/ml (6.25 U/ml) pyruvate kinase, 20 μg/ml (22 U/ml) lactic dehydrogenase, and 141 mM triethanolamine, pH 7.4. The reaction was started by the addition of 5 μl/ml ATPase (Sigma units), and assays were performed in the absence and presence of ouabain (0.1 mM), pyrogallol (0.1 mM, as described in Ref. 16), and/or SOD (150 U/ml). Absorbance data were converted to micromoles of NADH oxidized per minute by using the extinction coefficient of NADH (6,300 mol⁻¹ cm⁻¹) at 340 nm (2). Ouabain-sensitive activity was calculated by subtracting values obtained from parallel ouabain-treated samples. Ouabain inhibited the control rate by 97%. The flux of superoxide from pyrogallol autoxidation was quantified as the reduction of cytochrome c (horse heart, Sigma) inhibited by SOD by using an extinction coefficient of 2.1 × 10⁴ M⁻¹ cm⁻¹ at 560 nm (23).

**Data analysis.** Results are expressed as means ± SE. Data were analyzed by one-way ANOVA with a Newman-Keuls post hoc comparison. Differences were considered statistically significant when P < 0.05.

**RESULTS**

**SOD prevents hyperglycemia-induced inhibition of ouabain-sensitive 86Rb⁺ uptake.** As shown in Fig. 1, incubation of aortic rings for 3 h under hyperglycemic (44 mM glucose) conditions inhibited ouabain-sensitive 86Rb⁺ uptake by ~50% compared with rings incubated under normoglycemic conditions (5.5 mM glucose), in
agreement with earlier studies (12). SOD (150 U/ml) prevented the decrease in ouabain-sensitive $^{86}$Rb$^+$ uptake caused by hyperglycemia (Fig. 1; $P < 0.05$), whereas SOD had no effect on ouabain-sensitive $^{86}$Rb$^+$-uptake under normoglycemic conditions. These results suggest a role for superoxide in hyperglycemia-induced inhibition of Na$^+$/K$^+$-ATPase activity.

Hyperglycemia increases superoxide production by aortic rings. We next estimated the vascular steady-state superoxide flux under normoglycemic and hyperglycemic conditions. Normoglycemic incubation of endothelium-intact vessel segments produced a net superoxide signal of 0.09 ± 0.04 AU/5 min (Fig. 2; $n = 21$ experiments). The superoxide signal increased approximately threefold (0.30 ± 0.025 AU/5 min; $n = 24$) after inhibition of Cu,Zn SOD with DDC ($P < 0.05$) to reflect superoxide production. Hyperglycemia increased the net superoxide flux insignificantly to 0.12 ± 0.07 AU/5 min ($P = $ not significant; $n = 15$). However, we observed an ~50% increase in superoxide production to 0.51 ± 0.05 AU/5 min ($P < 0.01$; $n = 15$) with hyperglycemia with inhibition of Cu,Zn SOD by DDC (Fig. 2). The free radical scavenger Tiron (10 mM) and the SOD mimic EUK-8 (300 μM) both reduced the superoxide flux >95% regardless of DDC or glucose treatment (data not shown). Thus hyperglycemia produces an increase in vascular superoxide production.

The effect of hyperglycemia on vascular superoxide is endothelium dependent. Because hyperglycemia inhibits Na$^+$/K$^+$-ATPase activity in an endothelium-dependent manner (12), we sought to determine whether the increase in vascular superoxide production due to hyperglycemia also was endothelium dependent. As shown in Fig. 3, hyperglycemia produced an ~50% increase in superoxide production (DDC-treated vessels) with an intact endothelium (from 0.35 ± 0.02 to 0.51 ± 0.05 AU/5 min; $P < 0.05$). In contrast, vessels without endothelium did not demonstrate an increase in superoxide production as a function of glucose concentration (0.38 ± 0.02 vs. 0.37 ± 0.03 AU/5 min). The presence or absence of endothelium had no bearing on superoxide production in vessels that had been incubated under normoglycemic conditions (Fig. 3), consistent with a prior report (27).
L-Arginine suppresses the increased superoxide production induced by hyperglycemia. We previously reported (12) that hyperglycemia inhibits Na\(^+\)-K\(^+\)-ATPase activity most likely by decreasing the availability of NO, because this effect was reversed by L-arginine and NO donors. To determine whether the hyperglycemia-induced increased superoxide production was sensitive to NO, we examined the effect of L-arginine on the vascular superoxide production. The presence of L-arginine (300 \(\mu\)M) normalized superoxide production in vessel segments exposed to hyperglycemia (Fig. 4; \(P < 0.05\) by ANOVA). This effect of L-arginine was reversed by 100 \(\mu\)M \(N^\bullet\)-nitro-L-arginine (L-NNA), an NO synthase inhibitor (Fig. 4). However, in vessels exposed only to normoglycemia, superoxide production was not materially altered by L-arginine or L-NNA (Fig. 4).

**Aldose reductase, superoxide, and Na\(^+\)-K\(^+\)-ATPase activity.** Because reduced Na\(^+\)-K\(^+\)-ATPase activity has been linked to the polyol pathway (35), we examined the role of aldose reductase in both the superoxide flux and reduced Na\(^+\)-K\(^+\)-ATPase activity produced by hyperglycemia. Incubation of aortic rings in Krebs buffer with hyperglycemic glucose levels produced a doubling of tissue sorbitol levels (\(P < 0.05\)) that was abrogated in the presence of zopolrestat (10 \(\mu\)M), an aldose reductase inhibitor (Fig. 5A). Interestingly, zopolrestat had no effect on the 50% increase in tissue fructose induced by hyperglycemic glucose levels (Fig. 5B). Inhibition of aldose reductase also prevented the increased superoxide production associated with hyperglycemic glucose concentrations (Fig. 5C; \(P < 0.05\)) and partially reversed the effect of hyperglycemia to depress ouabain-sensitive \(^{86}\text{Rb}^+\) uptake (Fig. 5D). Zopolrestat alone did not affect either ouabain-sensitive \(^{86}\text{Rb}^+\) uptake or the superoxide production under normoglycemic glucose concentrations (data not shown).

**Superoxide anion directly reduces Na\(^+\)-K\(^+\)-ATPase activity.** To further understand the interaction between superoxide and Na\(^+\)-K\(^+\)-ATPase, we used pyrogallol to create a superoxide flux and then examined its effect on the enzyme activity using a partially purified, commercially available preparation. In the presence of a 2.23 \(\mu\)M/min superoxide flux from pyrogallol (16), Na\(^+\)-K\(^+\)-ATPase activity was inhibited by \(\sim 40\%\) (Fig. 6). Addition of SOD, which alone did not affect the enzyme activity significantly, prevented pyrogallol-induced decrease in activity. Thus superoxide inhibits Na\(^+\)-K\(^+\)-ATPase activity directly.

**DISCUSSION**

The results of the present study suggest that inhibition of vascular Na\(^+\)-K\(^+\)-ATPase activity by hyperglyc-
Super oxide and Na\(^{+}\)-K\(^{-}\)-ATPase Activity

![Graph](image)

**Fig. 6.** Effect of super oxide on partially purified Na\(^{+}\)-K\(^{-}\)-ATPase enzyme activity. Partially purified Na\(^{+}\)-K\(^{-}\)-ATPase was incubated in assay buffer alone (control) or buffer containing pyrogallol (100 μM), SOD (150 U/ml), or SOD + pyrogallol. Na\(^{+}\)-K\(^{-}\)-ATPase activity was measured as coupled NADH oxidation as described in METHODS. Results are means ± SE from 4 experiments. *P < 0.05 vs. control by 1-way ANOVA with post hoc Newman-Keuls comparison.

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Hyperglycemia is related, in part, to an increased endothelial cell flux of superoxide anion. This notion is supported by our observations that inhibition of Na\(^{+}\)-K\(^{-}\)-ATPase activity in the rabbit aorta by hyperglycemia was prevented by SOD and associated with increased production of superoxide. Furthermore, manipulation of the vascular superoxide flux was associated with concurrent changes in Na\(^{+}\)-K\(^{-}\)-ATPase activity. Specifically, l-arginine both decreased the vascular superoxide signal and limited the reduction in Na\(^{+}\)-K\(^{-}\)-ATPase activity caused by hyperglycemia. Similarly, aldose reductase inhibition with zopolrestat blunted the effect of hyperglycemia to both increase superoxide and reduce Na\(^{+}\)-K\(^{-}\)-ATPase activity in rabbit aorta. Finally, we also demonstrated direct inhibition of Na\(^{+}\)-K\(^{-}\)-ATPase activity by superoxide in a cell-free partially purified enzyme preparation. Thus our data indicate that the local availability of superoxide in the vascular wall is an important determinant of Na\(^{+}\)-K\(^{-}\)-ATPase activity.

The concept that superoxide specifically, or reactive oxygen species in general, can modify the activity of Na\(^{+}\)-K\(^{-}\)-ATPase is not new. Hexum and Fried (15) found that purified Na\(^{+}\)-K\(^{-}\)-ATPase from rat brain was inhibited by a xanthine/xanthine oxidase system. Subsequent studies in rat (6, 31), canine (18), and porcine (8, 32) tissues have yielded qualitatively similar results. The precise mechanism(s) for such observations remain a matter of debate. Superoxide has been implicated as the causative agent in a number of studies (6, 8, 15, 31) because SOD prevents inhibition of Na\(^{+}\)-K\(^{-}\)-ATPase. Other data suggest that the picture is more complex, however. For instance, activated neutrophils, which produce a number of reactive species, also inhibit Na\(^{+}\)-K\(^{-}\)-ATPase activity, and this inhibitory effect is attenuated by a combination of catalase and SOD but not by SOD alone, suggesting some role for H\(_2\)O\(_2\) (18). This contention is supported by observations that prolonged exposure to H\(_2\)O\(_2\) directly inhibits Na\(^{+}\)-K\(^{-}\)-ATPase activity (7, 8). Lipid peroxidation, an important consequence of reactive oxygen species production, also may contribute to inhibition of Na\(^{+}\)-K\(^{-}\)-ATPase (29). Thus the data presented here are in general agreement with previous reports that reactive oxygen species impair the activity of Na\(^{+}\)-K\(^{-}\)-ATPase.

Although our data implicate superoxide as an important component of reduced Na\(^{+}\)-K\(^{-}\)-ATPase activity caused by hyperglycemia, the source of increased superoxide remains a matter of controversy. One possibility relates to the fact that monosaccharides such as glucose with an α-hydroxylaldehyde structure are subject to autoxidation and superoxide formation (40). One suggested cellular mechanism of reactive oxygen species production by hyperglycemia involves a state of “pseudohypoxia” (39). According to this scenario, hyperglycemia increases glucose flux through the aldose reductase pathway, leading to an increase in sorbitol. Sorbitol oxidation, in turn, increases the NADH/NAD ratio, leading to a number of metabolic changes that mimic hypoxia. Because both hypoxia and an increase in the NADH/NAD ratio are associated with superoxide production (39), the pseudohypoxia hypothesis suggests that hyperglycemia should be associated with increased cellular superoxide levels. Our data provide some support for this hypothesis. In particular, we observed that hyperglycemia caused increases in both tissue sorbitol levels and superoxide production, two phenomena that were inhibited, in part, by the aldose reductase inhibitor zopolrestat (Fig. 5). These findings implicate sorbitol generation as a contributing factor for the increased superoxide production with hyperglycemia. Factors beyond sorbitol are likely to be involved in our observations, because aldose reductase...
inhibition provided only partial restoration of Na\(^+-\)K\(^+-\)ATPase activity (Fig. 4). In this context, one must also consider whether the mitochondria might be a contributing source of superoxide, because a recent report has linked mitochondrial oxidant production as crucial in hyperglycemia-induced oxidative stress (25). Finally, we also cannot rule out the possibility that a reduction in NO production plays a contributing role.

With respect to the site of vascular superoxide production, several points merit consideration. Because the effect of glucose on superoxide was not observed in vessels without endothelium (Fig. 3), one might infer that the endothelium is the source of increased superoxide production. This represents an attractive hypothesis, because previous data indicate that the endothelium exerts considerable influence over vascular Na\(^+-\)K\(^+-\)ATPase activity. In particular, we have previously demonstrated that endothelium-derived NO stimulates Na\(^+-\)K\(^+-\)ATPase activity in rabbit aorta (10, 12). Because superoxide and NO combine readily in a diffusion-limited reaction (20) to form peroxynitrite, a compound with limited NO bioactivity, it is reasonable to speculate that hyperglycemia-induced superoxide limits the action of NO that is necessary for Na\(^+-\)K\(^+-\)ATPase activity. On the basis of this hypothesis, one would expect that increasing endothelial NO should blunt the effect of hyperglycemia. This is precisely what we found with exogenous L-arginine, a known stimulus for increased NO bioactivity in diabetes (28). Thus our data provide evidence that the balance between NO and superoxide in the endothelium is an important determinant of vascular Na\(^+-\)K\(^+-\)ATPase activity.

Although our data provide strong evidence for endothelial superoxide as the cause of the reduced Na\(^+-\)K\(^+-\)ATPase activity in rabbit aorta induced by hyperglycemia, we cannot be certain of the precise site of superoxide action. On one hand, superoxide directly inactivates Na\(^+-\)K\(^+-\)ATPase activity in isolated enzyme preparations (Fig. 6), consistent with prior observations (6, 8, 15, 31). On the other hand, superoxide also can react with and remove NO, which we have previously shown to directly activate Na\(^+-\)K\(^+-\)ATPase in vascular smooth muscle (10). These two modes of action are depicted schematically in Fig. 7. It is attractive to speculate that the balance between NO and superoxide regulates Na\(^+-\)K\(^+-\)ATPase activity; however, this remains to be proven.

In summary, the data presented here indicate that hyperglycemia produces a decrease in vascular Na\(^+-\)K\(^+-\)ATPase activity that is caused, in part, by an increased production of superoxide in vascular endothelium. The precise source of this superoxide flux is not yet clear but appears to depend on the intracellular accumulation of sorbitol, a product of the polyol pathway. Our data are consistent with a model in which endothelial superoxide inactivates NO, a known stimulus for smooth muscle cell Na\(^+-\)K\(^+-\)ATPase activity. These findings provide further evidence that the balance between superoxide and NO in the vascular wall has important implications for normal vascular function.

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