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Early neural and vascular dysfunctions in diabetic rats are largely sequelae of increased sorbitol oxidation

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Early Neural and Vascular Dysfunctions in Diabetic Rats Are Largely Sequelae of Increased Sorbitol Oxidation

Yasuo Ido,1 Jens R. Nyengaard,2 Kathy Chang,3 Ronald G. Tilton,4 Charles Kilo,3 Banavara L. Mylari,5 Peter J. Oates,6 and Joseph R. Williamson7

Abstract

These experiments were undertaken to assess the importance of cytoplasmic (c) sorbitol oxidation versus mitochondrial (m) pyruvate oxidation in mediating neural and vascular dysfunction attributable to hyperglycemia in diabetic rats. Increased oxidation of sorbitol is coupled to enzymatic reduction of free oxidized NAD\(^+\)c to reduced NADHc, manifested by an increased ratio of NADH to NAD\(^+\)c. Likewise, increased oxidation of pyruvate is coupled to reduction of NAD\(^+\)m to NADHm, which increases the NADH/NAD\(^+\)m ratio. Specific inhibitors of sorbitol production or sorbitol oxidation normalized: increased diabetic nerve NADH\(^+\)/NAD\(^+\)c, impaired nerve-conduction velocity, and vascular dysfunction in sciatic nerve, retina, and aorta; however, they had little or no impact on increased NADH\(^+\)/NAD\(^+\)m. These observations provide, for the first time, strong in vivo evidence for the primacy of sorbitol oxidation versus pyruvate oxidation in mediating the metabolic imbalances, impaired nerve conduction, and vascular dysfunction evoked by diabetes. These findings are consistent with (a) the fact that oxidation of sorbitol produces "prooxidant" NADHc uncoupled from subsequent production of "antioxidant" pyruvate required for reoxidation of NADHc to NAD\(^+\)c by lactate dehydrogenase, and (b) the hypothesis that neural and vascular dysfunction in early diabetes are caused primarily by increased NADHc, which fuels superoxide production by NADH-driven oxidases. Antioxid. Redox Signal. 12, 39–51.

Introduction

Increasing evidence supports the importance of superoxide (O\(_2^−\)) and related reactive oxygen species (ROS) in mediating diabetic complications attributed to hyperglycemia (4, 5, 8, 20, 29, 52); however, the primary source(s) of electrons that fuel superoxide production is controversial. Two distinctly different hypotheses have been proposed: (a) increased oxidation of pyruvate (produced by increased glycolysis) in mitochondria coupled to reduction of free NAD\(^+\)m to NADHm, which promotes superoxide production by the mitochondrial electron transport chain (5, 29); and (b) increased oxidation of sorbitol (produced by increased flux of glucose via the sorbitol pathway, which does not produce pyruvate) by sorbitol dehydrogenase (SDH) to fructose coupled to reduction of cytosolic NAD\(^+\)c to NADHc (equimolar to fructose) that drives superoxide production primarily by NADH-driven oxidases (20, 30, 55):

\[
\text{Sorbitol + NAD}^+ + \text{SDH} \rightarrow \text{fructose + NADHc + H}^+;
\]

\[
\text{NADHc + O}_2 \rightarrow \text{NAD}^+ + \text{O}_2^{-}\]

The first hypothesis suggests that pyruvate supplementation might mimic or exacerbate metabolic imbalances and vascular and neural dysfunction evoked by hyperglycemia. However, pyruvate supplementation (a) normalizes/attenuates vascular dysfunction and mitochondrial imbalances evoked by hyperglycemia in several different paradigms of diabetes (16, 24, 44, 48, 50, 57), and (b) attenuates cataract formation in diabetic rats (58).

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This research was performed in the Department of Pathology, Washington University School of Medicine, St. Louis, MO.
The second hypothesis suggests that sorbitol supplementation (at normal glucose levels) might cause oxidative stress and associated metabolic imbalances and vascular dysfunction comparable to hyperglycemia and associated metabolic imbalances and vascular dysfunction (at normal glucose levels) might cause oxidative stress and vascular dysfunction evoked by diabetes, and (b) efficacy of SDI versus ARI in preventing and reversing sorbitol pathway-linked increases in NADH/NAD⁺c and associated neural and vascular dysfunction.

Materials and Methods
Animal protocols
Male Sprague–Dawley rats were purchased from Sasco (O’Fallon, MO) and cared for in accordance with guidelines of the University Committee for the Humane Care of Laboratory Animals. Rats were housed one per cage and had free access to standard rat chow (Ralston Purina, Richmond, IN) and tap water. Diabetes was induced after an overnight fast by injection of 50 mg/kg body weight (bwt) of streptozotocin (Zanosar obtained from Upjohn) in citrate saline buffer, pH 4.5. Rats were anesthetized with ketamine (Vetalar, 35 mg/kg bwt), and streptozotocin was injected in a surgically exposed femoral vein. Nonfasting blood samples were obtained 3–4 days later from a tail vein for measurement of plasma glucose levels with the hexokinase method, as in previous experiments (39). Rats with plasma glucose levels of 16.7 mM (300 mg/dl) or more were considered to be diabetic and were distributed to groups of untreated, and ARI- and SDI-treated diabetics balanced to achieve mean ± SD values of glucose levels that did not differ (p > 0.05) among the groups. SDI and ARI treatments were then initiated. Plasma glucose levels and HbA1c levels were assessed again after 2 and 4 weeks of treatment in all studies, and then at 4-week intervals, depending on the duration of diabetes, and again at the termination of each study. Plasma glucose levels shown in the Results sections and in tables are expressed as mean ± SD of glucose levels measured at intervals of 2–4 weeks and at the termination of the study.

Because neural and vascular dysfunction varies with duration and severity of diabetes, prevention studies were first performed in rats with diabetes of 5 and 18 weeks duration. Subsequently, the efficacy of SDI and ARI in reversing neural changes was assessed after 2 and 4 weeks of treatment initiated after 6 weeks of untreated diabetes. Because our observation in this study (Reversal Study 1) that MNCV deficits were normalized by the SDI were discordant with the failure of an SDI to reverse MNCV deficits evoked by diabetes in the report by Cameron et al. (6), this experiment was repeated (Reversal Study 2) to validate the reproducibility of this observation and, in addition, to assess the relation of normalization of MNCV deficits to changes in neural blood flow and vascular leakage and changes in sorbitol, fructose, and myo-inositol levels that were not assessed in Reversal Study 1.

SDI (CP-166,572 (i.e., SDI-158) (13) and two structurally distinct ARIs, zopolrestat (28) and sorbinil (38), were synthesized at Pfizer Global Research and Development, Groton, CT. SDI was added to drinking water to provide a dose of 200 mg/kg bwt/day; zopolrestat was added to chow to provide 50 mg/kg bwt/day in the 5-week study and 100 mg/kg bwt/day in both reversal studies; sorbinil was added to chow to provide 50 mg/kg bwt/day in the 18-week study. Two structurally different ARIs were used to minimize the possibility that efficacy was an off-target effect. Dosages were based on dose–response effects of the SDI and ARIs (33). Body weights were monitored weekly, and consumption of drinking water and chow was checked every 2–3 days; quantities of inhibitors were adjusted to provide the appropriate dosage.

Motor-nerve conduction
Motor-nerve conduction velocity (MNCV) was measured in rats anesthetized with intramuscular injections of ketamine (100 mg/kg body wt). Core body temperature was maintained at 37 ± 5°C with the use of heating pads and lamps controlled by a rectal temperature probe. Nerves were stimulated with a Grass stimulator (model SDP; Quincy, MA) by generating a rectangular pulse 0.2 ms in duration at a voltage sufficient to give a maximal response. The compound muscle action potential was amplified and recorded on a digital-storage oscilloscope 1425 (Gould, Oxnard, CA).

MNCV in the distal tibial branch of the sciatic nerve was quantified by insertion of stimulating electrodes through the skin at the popliteal fossa and the ankle to stimulate the nerve and insertion of a concentric bipolar recording electrode into the plantar muscle. MNCV was calculated by dividing the distance between the two stimulating electrodes by the l-
tency difference in the onset of the electromyographic (EMG) potentials in the recording electrodes evoked by the two stimulating electrodes. MNCV in the distal peroneal branch of the sciatic nerve was quantified by inserting stimulating electrodes at the sciatic notch and at the popliteal fossa to stimulate the nerve and by insertion of the concentric bipolar recoding electrode into the anterior tibial muscle. MNCV was calculated as described for the tibial nerve. MNCVs are reported in meters per second (m/s).

**Blood flow and vascular permeability measurements**

One week after the last assessment of MNCV, sciatic nerve and retinal blood flows and vascular permeability (VAP) were assessed, as previously described (39, 40), and reported as microliters per gram (μl/g) wet wt/min and μg plasma/g wet wt/min, respectively. Rats were anesthetized with thiobutabarbital (Inactin), rather than ketamine, because it has a longer duration of action needed for preparation and assessment of blood flow and VAP. Blood flow was assessed by injection of 11.3 μm 4°Sc-labeled microspheres. VAP was quantified by the injection of [125I]-bovine serum albumin (BSA) into the right subclavian artery (10). The ratio of sciatic nerve molar concentrations of free cytosolic NADHc and free NAD⁺c was estimated by the redox metabolite indicator method (54). This method is based on the near-equilibria established between (a) NADPH/NAD⁺c and mitochondrial NADH/NAD⁺m and (b) NADH/NAD⁺c and β-hydroxybutyrate/pyruvate ratios by the malic enzyme (19), and (b) NADH/NAD⁺m and β-hydroxybutyrate/acetoacetate ratios by β-hydroxybutyrate-DH (54).

**Statistical analysis**

All results are reported as mean ± SD. Multiple analysis of variance was performed on Blom-transformed data with the SAS general linear models procedure; overall differences among experimental groups for each parameter were first assessed by the Van der Waerden test, and individual pairwise group comparisons were evaluated with least-square means, as previously described (45).

**Results**

**5 weeks of diabetes**

Diabetes increased sciatic nerve endoneurial blood flow by 57%, VAP, 83%, and NADH/NAD⁺c, ~80% (Table 1). Retinal blood flow and VAP were increased 14% and 83%, respectively, and aortic VAP was increased 90% (from 98 ± 16 μg plasma/g wet wt/min in controls to 191 ± 52; p < 0.001). SDI and ARI prevented all of these changes.

Diabetes increased sciatic nerve sorbitol and fructose levels by 12-fold and sevenfold, respectively, but decreased myo-inositol levels 30%. The SDI increased sorbitol levels another 5.4-fold, whereas the ARI normalized them; nevertheless, both inhibitors decreased fructose levels ~80%.

Nerve sorbitol levels in SDI-treated controls equaled those in untreated diabetic subjects, whereas in ARI-treated controls, sorbitol levels were decreased to 22% of controls; fructose levels in SDI- and ARI-treated controls were significantly decreased versus controls; blood flow, VAP, and myo-inositol levels in SDI- and ARI-treated controls did not differ from controls.

Plasma glucose levels in untreated diabetic rats were 25.7 ± 2.7 mM vs. 6.2 ± 0.7 in controls (p < 0.001); glucose levels in SDI- and ARI-treated diabetics were 26.0 ± 4.0 and 27.0 ± 3.6 mM, respectively, and p < 0.001 vs. controls for both groups. HbA₁c levels in SDI-treated diabetics were 11.1 ± 2.0% in untreated diabetics vs. 3.7 ± 1.3% in controls (p < 0.001); HbA₁c levels in SDI- and ARI-treated diabetics were 10.6 ± 1.4% and 10.8 ± 1.1%, respectively; p < 0.001 vs. controls. Plasma glucose and HbA₁c levels in SDI- and ARI-treated diabetics did not differ (p > 0.05) from those in untreated diabetics.

Mean arterial blood pressure (MABP) was 128 ± 9 mm Hg in diabetics vs. 144 ± 14 in controls (p < 0.01). Initial body weights were 240 ± 12 g in diabetics vs. 259 ± 17 in controls (p < 0.02). Body weights increased 9.0 ± 14% in diabetics vs. 33 ± 11% in controls (p < 0.001). MABP and body weights in SDI- and ARI-treated diabetics did not differ from those in untreated diabetics (p > 0.05).

**18 weeks of diabetes**

Diabetes decreased MNCV 11.5% in the distal tibial nerve (Fig. 1) and 10.4% in the peroneal nerve (from 65.3 ± 4.2 m/s to 58.5 ± 5.1; p < 0.01). Both inhibitors prevented these MNCV deficits. Diabetes increased sciatic nerve NADH/NAD⁺c and NADH/NAD⁺m by 80% and 60%, respectively (Fig. 1); both inhibitors prevented the increase in NADH/NAD⁺c, but not the increase in NADH/NAD⁺m.

Diabetes also increased sciatic nerve NADPH/NAD⁺c ratios ~80%, which also were normalized by the SDI (Table 2). The ARI partially attenuated the increase in NADPH/NAD⁺c (which did not differ significantly from controls or diabetics) but did not affect malate/pyruvate ratios.

Plasma glucose levels in diabetic rats were increased ~sixfold (39.7 ± 3.6 mM vs. 6.9 ± 0.5 in controls; p ≤ 0.001);
Neither the SDI nor the ARI significantly attenuated NEFA or treated diabetics.

HbA1c levels were increased by ~3.3-fold (14.7 ± 0.6% vs. 4.5 ± 0.2% in controls; p < 0.001). Plasma glucose and HbA1c levels in ARI- and SDI-treated diabetic rats did not differ from those in untreated diabetics (Table 2).

Nerve sorbitol and fructose levels were increased by diabetes by 13.4- and 5.7-fold, respectively (Table 2). The SDI reduced sorbitol and fructose levels to levels lower than those in controls (p < 0.001). Myoinositol levels were decreased ~37% by diabetes and were normalized by the ARI but unaffected by the SDI (p < 0.0001 vs. SDI-treated diabetics).

Plasma levels of NEFA were 69 ± 17 μEq/dl in diabetics versus 42 ± 8 in controls (p < 0.004); plasma β-HBA levels were 102 ± 51 μM in diabetics vs. 27 ± 3 in controls (p < 0.001). Neither the SDI nor the ARI significantly attenuated NEFA or β-HBA levels in diabetics. Initial body weights were similar in controls and diabetics; however, diabetics gained significantly less weight than did controls (p < 0.05).

Reversal of MNCV deficits and NADH/NAD⁺c by SDI and ARI

Reversal Study 1. After 6 weeks of untreated diabetes, the MNCV was decreased ~13–14% in all three groups of diabetic rats (Fig. 2, left panel). After 8 weeks of untreated diabetes, MNCV was decreased 17% (Fig. 2, middle panel). SDI and ARI administered during the last 2 weeks attenuated (but did not normalize) MNCV deficits to 11% and 4.6%, respectively. After 10 weeks of untreated diabetes, MNCV was decreased 14% and was statistically normalized by SDI and by ARI administered during the last 4 weeks (Fig. 2, right panel).

Diabetes increased sciatic nerve NADH/NAD⁺c and NADH/NAD⁺m about twofold (Table 3). The ARI normal-

<table>
<thead>
<tr>
<th>Blood flow</th>
<th>VAP</th>
<th>NADH/NAD⁺c</th>
<th>Sorbitol</th>
<th>Fructose</th>
<th>Myoinositol</th>
<th>Blood flow</th>
<th>VAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>65 ± 16</td>
<td>76 ± 13</td>
<td>1.1 ± 0.4</td>
<td>159 ± 55</td>
<td>814 ± 197</td>
<td>374 ± 15</td>
<td>75 ± 12</td>
</tr>
<tr>
<td>+SDI</td>
<td>64 ± 17</td>
<td>75 ± 13</td>
<td>2.050 ± 697*</td>
<td>524 ± 201*</td>
<td>3,451 ± 1290</td>
<td>360 ± 20</td>
<td>71 ± 8</td>
</tr>
<tr>
<td>+ARI</td>
<td>68 ± 30</td>
<td>76 ± 18</td>
<td>24 ± 11*</td>
<td>179 ± 60*</td>
<td>4,283 ± 938</td>
<td>376 ± 16</td>
<td>74 ± 19</td>
</tr>
<tr>
<td>Diabetic</td>
<td>102 ± 17*</td>
<td>139 ± 16*</td>
<td>1.9 ± 0.4*</td>
<td>1,863 ± 623*</td>
<td>5,815 ± 1,711*</td>
<td>4,26 ± 21*</td>
<td>137 ± 16*</td>
</tr>
<tr>
<td>+SDI</td>
<td>67 ± 26II</td>
<td>89 ± 25III</td>
<td>1.3 ± 0.4*</td>
<td>9,975 ± 4397II</td>
<td>1,001 ± 519II</td>
<td>2,844 ± 793II</td>
<td>380 ± 45II</td>
</tr>
<tr>
<td>+ARI</td>
<td>75 ± 24II</td>
<td>94 ± 3II</td>
<td>1.2 ± 0.4*</td>
<td>170 ± 85II</td>
<td>1,217 ± 253III</td>
<td>3,521 ± 1,229</td>
<td>382 ± 33III</td>
</tr>
</tbody>
</table>

BF; blood flow; VAP, vascular albumin permeation. Data are expressed as mean ± SD for 10–12 animals. Blood flow is expressed as μl/g wet wt/min; vascular albumin permeation is expressed as μg plasma/g wet wt/min; sorbitol, fructose, and myoinositol levels are expressed as nmol/g wet wt; see Research Design and Methods for calculation of free NADH/NAD⁺c × 10⁻³.

Different from Controls:  * p ≤ 0.001,  p ≤ 0.005,  p < 0.04.
Different from Diabetics:  p < 0.001,  p < 0.01.

FIG. 1. Eighteen weeks of diabetes. Effects of SDI (200 mg/kg bwt/day) and ARI (sorbinil, 50 mg/kg bwt/day) on distal tibial MNCV and on sciatic nerve NADH/NAD⁺c and NADH/NAD⁺m in rats with diabetes of 18 weeks’ duration. Mean ± SD; n = Nine to 12 rats for all parameters. Different from controls:  * p < 0.001,  p = 0.005,  p < 0.02. Different from diabetics:  p < 0.02,  p = 0.05.
SDI failed to normalize NADH with the substantially larger variance in NADH pharmacokinetics of SDI, as discussed earlier (31, 33, 45).

Diabetes increased NEFA and β-HBA levels by 12-fold and sevenfold, respectively (Table 4). The SDI increased sorbitol levels an additional fivefold (61x controls) while normalizing fructose levels. The ARI decreased sorbitol and fructose levels to 36 and 76%, respectively, of controls; and fructose levels were 35% lower than those in SDI diabetics (p < 0.02). Myoinositol levels were decreased 57% by diabetes and were unaffected by the SDI; however, the ARI normalized myoinositol levels, which were 1.7-fold higher than those in untreated diabetics (p < 0.01) and ~1.9-fold higher than those in SDI diabetics (p < 0.001).

Diabetes increased plasma glucose levels 4.5-fold (36.2 mM vs. 3.8 vs. 8.0 ± 1.8, p < 0.001) and increased HbA1c levels 3.8-fold (14.1 ± 1.0% vs. 3.7 ± 0.4%; p < 0.001). Plasma glucose levels and HbA1c levels in diabetic rats treated with SDI and ARI did not differ from those in untreated diabetics (Table 3).

Diabetes increased NEFA and β-HBA levels by about two- and fourfold; p ≤ 0.001. All three groups of diabetics failed to gain weight in contrast to controls. These parameters were unaffected by the SDI or ARI.

Reversal Study 2. After 6 weeks of untreated diabetes, MNCV deficits ranged from 9.3 and 14.3% before administration of SDI and ARI (Table 4). After 8 weeks of untreated diabetes, the MNCV deficit was 10.7% and was not attenuated by SDI or ARI treatment during the last 2 weeks. After 10 weeks of untreated diabetes, the MNCV deficit was 10.4% and was normalized by SDI and by ARI administered during the last 4 weeks. Four weeks of ARI treatment also normalized the associated 19% reduction in nerve blood flow; however, blood flow in SDI-treated diabetics was not significantly different from that in controls or untreated diabetics. Neither inhibitor attenuated the associated 2.5-fold increase in VAP. MABP was 132 ± 18 mm Hg in diabetics versus 148 ± 5 mm Hg in controls (p < 0.01) and was unaffected by the SDI or ARI.

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Diabetes tended to increase NEFA (58 ± 21 μEq/dl vs. 42 ± 14 μEq/dl in controls); however, the difference was not significant (p > 0.05). NEFA levels in SDI- and ARI-treated diabetics were slightly higher than those in untreated diabetics (71 ± 39 and 77 ± 26 μEq/dl, respectively; p < 0.01 vs. controls for both groups) but did not differ from those in untreated diabetics (p > 0.05). Diabetes increased β-HBA levels by ~5.4-fold to 113 ± 84 μM vs. 21 ± 4 in controls (p < 0.001); β-HBA levels in SDI- and ARI-treated diabetics were 71 ± 39 and 77 ± 26, respectively (p < 0.01 vs. controls and p > 0.05 vs. untreated diabetics). Initial body weights were 417 ± 11 g in controls and diabetics; final body weights increased 35 ± 7% in controls vs. a final weight loss of −9 ± 9% in untreated diabetics (p < 0.001). Weight losses in SDI- and ARI-treated diabetics were −6 ± 4% and −6 ± 10%, respectively (p > 0.05 vs. untreated diabetics).

Effects of diabetes, SDI, and ARI on sciatic nerve: (a) malate levels and malate/pyruvate ratios and (b) lactate/pyruvate ratios manifested by changes in lactate and/or pyruvate levels Table 5

Effects of untreated diabetes. Lactate/pyruvate ratios were increased by 1.5- to twofold in all three studies and were attributable largely to increased lactate levels, except in the 18-week study in which lactate levels were identical to controls, whereas pyruvate levels were decreased 43% (Table 5). In the 10-week study, the increased lactate/pyruvate ratio also was contributed to by a 19% decrease in pyruvate levels that approached statistical significance (p = 0.05–0.1). Malate levels did not differ from controls, whereas malate/pyruvate ratios were increased ~80% (p < 0.001 vs. controls).

Effects of SDI treatment. The SDI normalized lactate/pyruvate ratios in the 5- and 18-week studies, but not in the 10-week reversal study. In the 5-week study, 87% normalization of lactate/pyruvate ratios was due largely to increased (33%) pyruvate levels, together with decreased (8%) lactate levels. In the 18-week study, 83% normalization was due entirely to increased pyruvate levels (77%). Malate levels did not differ from those in controls or diabetics, whereas malate/pyruvate ratios were normalized.

Effects of ARI treatment. The ARI normalized lactate/pyruvate ratios in all three studies. In the 5-week study, normalization was manifested by the combination of a marginally significant increase in pyruvate levels (28%; p = 0.05–0.01) and a 17% (p = 0.26) reduction in mean lactate levels. In the 10-week study, normalization was due to significantly increased pyruvate levels. In the 18-week study, normalization was due to significantly increased pyruvate levels and a statistically insignificant reduction in lactate levels. Malate levels and malate/pyruvate ratios were not significantly different from those in the other groups.
Table 5. Effects of Diabetes, SDI, and ARI on Sciatic Nerve: Lactate and Pyruvate Levels, and Lactate/Pyruvate (L/P) Ratios in Rats with Diabetes of 5-, 10-, and 18-Weeks Duration, and Malate and Malate/Pyruvate (M/P) Ratios in Rats with Diabetes of 18-Weeks Duration

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<tr>
<td>5 wk</td>
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Data are expressed as mean ± SD for lactate and pyruvate levels and mean L/P ratios for animals in each group. Numbers of animals for each duration of diabetes are shown in corresponding Tables 1 and 2 and Fig. 1. Lactate, pyruvate, and malate levels are expressed as nmol/kg/g DNA.

Discussion

The observations in these experiments support two fundamental conclusions: (a) increased sorbitol oxidation plays a critical role in mediating diabetes-induced early neural and vascular dysfunction that precede pathologic structural changes, and (b) neural and vascular dysfunction evoked by sorbitol oxidation are primarily sequelae of increased cytoplasmic NADH/NAD⁺c rather than elevated mitochondrial NADH/NAD⁺m. Notably, these are the first studies in which neural or vascular dysfunction or both, NADH/NAD⁺c or NADH/NAD⁺m or both have been assessed in the same animal. Previous investigators have assessed neural and vascular dysfunction but not NADH/NAD⁺c or NADH/NAD⁺m (6, 7), is discordant with the current observations and with the findings of Chang et al. (10), Ido et al. (21), and others addressed in OAS II-A.

Treatment of diabetic rats with SDI-157 (also named S-0773) in an earlier study by Obrosova et al. (34) failed to prevent increases in neutral NADH/NAD⁺c. That finding differs from observations in the current experiments in which treatment with CP-166,572 (also named SDI-158) normalized sciatic nerve NADH/NAD⁺c (Fig. 1, Table 1) and was as efficacious as ARI in preventing MNCV deficits in all three experiments in which MNCV was assessed (Figs. 1 and 2; Table 4). Chemical structures of SDIs shown in OAS V.)

SDI-157 is a prodrug that is activated in vivo in the liver to its lower in vivo potency versus theARI WAY-121,509 used by Cameron et al. Consistent with this notion, sciatic nerve fructose levels in WAY-121,509–treated diabetic rats were markedly reduced to only 16% of fructose levels in nondiabetic controls; in contrast, fructose levels in zopolrestat–treated diabetic rats were 76% of fructose levels in nondiabetic controls. The suggestion by Cameron et al. (6) that MNCV deficits in untreated diabetic rats are attributable to decreased blood flow (i.e., ischemia), even in diabetes of short duration (6, 7), is discordant with the current observations and with the findings of Chang et al. (10), Ido et al. (21), and others addressed in OAS II-A.

Table 4. Effects of Diabetes, SDI, and ARI on Sciatic Nerve: Lactate and Pyruvate Levels, and Lactate/Pyruvate (L/P) Ratios in Rats with Diabetes of 5-, 10-, and 18-Weeks Duration, and Malate and Malate/Pyruvate (M/P) Ratios in Rats with Diabetes of 18-Weeks Duration

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demonstrated for ARI (6). Thus, we hypothesize that the failure of SDI to normalize neural NADH/NAD$^+$ c in the earlier experiment (34) may be explained by the combined effects of (a) the 50% lower dose administered of the prodrug SDI-157 

versus the active drug SDI-158 in the present experiments, (b) the rapid build-up of sorbitol substrate for SDH caused by the SDI, and (c) the very short half-life of the active SDI.

Therefore, the suggestion that hypoxia-like metabolic changes [i.e., increases in NADH/NAD$^+$ c, in tissues of diabetic rats (55)] are sequelae of increased AR activity, independent of increased sorbitol oxidation (36) remains discordant with the present observations, as well as with previous findings demonstrating that exposure of normoxic normoglycemic cells and tissues to elevated sorbitol levels in vivo and in vitro evokes metabolic imbalances or vascular dysfunction or both, like those evoked by hyperglycemia (9, 12, 26, pages 9–10 in OAS IV-A of 30, 46, 47, 49, 53).

Likewise, the suggestion (4) that the increase in free NADH/NAD$^+$ c evoked by diabetes is attributable to a marked decrease in total NAD$^+$ t because of its consumption by poly(ADP-ribose) polymerase (PARP) is inconsistent with (a) increased levels of total NADHt with no change in NAD$^+$ t (NADHt plus NAD$^+$ t) (14) and increased levels of NADHt and NAD$^+$ t (18) in retinas of diabetic rats, (b) the insignificant decrease in free NADH c associated with a twofold increase in NADH/NAD$^+$ c discussed later, (c) the demonstration that hyperglycemia increases free NADH/NAD$^+$ c = lactate/pyruvate $\times K_{LDH}$ ratios in human erythrocytes (48) that lack nuclei and mitochondria, and (d) observations of other investigators addressed in OAS II-E. Interestingly, recent observations suggest that activated PARP may further contribute to oxidative-nitrosative stress (OAS II-E, 9).

The second conclusion (see earlier; i.e., the primacy of increased NADH/NAD$^+$ c vs. increased NADH/NAD$^+$ m in mediating diabetes-induced neural and vascular dysfunction) is supported by observations that (a) SDH and AR are known to be primarily cytoplasmic enzymes (31), and (b) inhibition of either enzyme normalized/attenuated increased sciatic nerve free NADH/NAD$^+$ c (Fig. 1; Table 1), impaired MNCV (Figs. 1 and 2; Table 4), and vascular dysfunction (Table 1), while having little or no impact on associated increases in free NADH/NAD$^+$ m (Fig. 1; Table 3). Whatever putative mitochondrial changes may have contributed to neural and vascular changes, independent of the increase in NADH/ NAD$^+$ m, the observations that ARI and SDI prevented vascular and neural dysfunction indicates that those putative changes also were prevented. The most likely explanation for the increases in NADH/NAD$^+$ m in these experiments is increased oxidation of fatty acids and/or ketones that were significantly increased in Reversal Study 1 (Table 3) and in the 18-week study (see Results) and were not attenuated by ARI or SDI.

Limitations of the current studies are that they do not directly address (a) potentially important increased superoxide production caused by hypoxic/ischemic vascular disease in chronic diabetes (OAS I-C2) or (b) mitochondrial superoxide production. The effects of the two types of inhibitors of cytoplasmic enzymes (AR and SDH) in the present studies do not exclude potentially important downstream enzymatic reactions/sequelae in the same or other cellular compartments. A positive control to normalize NADH/NAD$^+$ m, but not NADH/NAD$^+$ c, would be of interest for future studies.

The possible relationship between increased mitochondrial leakage, i.e., opening of MPTPs (1) and changes in NADH/ NAD$^+$ m also need further study. Because the effects of diabetes on NADH/NAD$^+$ c and NADH/NAD$^+$ m are complex and their roles in initiating complications of diabetes remain controversial (4–8, 10, 11, 21, 29, 34, 36, 56), they are discussed critically in more detail in the current OAS II and in the OAS in (30) for interested readers. Notably, observations and speculations thought to be inconsistent with the importance of sorbitol oxidation do not withstand scrutiny.

An additional limitation of the current studies is the question of the absolute specificity of the inhibitors used. Off-target effects are always possible for low-molecular-weight inhibitors. This is the reason that two structurally distinct inhibitors of AR were used (i.e., sorbinil and zopolrestat). That similar results were obtained with either ARI attests to the salutary effects being a result of inhibition of the intended target, AR. The result is further supported and refined by using the SDI, an inhibitor in the second step of the pathway. However, only one structural class of SDIs is active in vivo, the one used. SDI normalized impaired nerve conduction in diabetic rats, but paradoxically evoked ultrastructural changes in autonomic nerves (iliac mesenteric nerves) and in prevertebral sympathetic superior mesenteric ganglia in the same animals (OAS V-A in ref.30). The functional significance and mechanism of those ultrastructural changes remains unclear.

The fundamental issue raised by the current observations is the basic mechanism by which NADHc generated by oxidation of sorbitol causes (a) metabolic imbalances and associated neural and vascular dysfunction observed in early diabetes, and (b) late complications of diabetes that are now generally agreed to be linked to excess production of ROS (4, 5, 20, 29, 53). Equations 1 and 2 (see Methods) are the keys to understanding this mechanism and the pathophysiologic importance of sorbitol oxidation in generating relatively small molar increases in free NADH, uncoupled from subsequent downstream production of equimolar pyruvate.

As shown in Eq. 2, free NADH/NAD$^+$ c = (lactate/pyruvate) $\times K_{LDH}$ ($K_{LDH} = 1.11 \times 10^{-4}$ at pH = 7.0; i.e., $\sim 1/10,000$). Molar lactate/pyruvate ratios in a wide spectrum of normal cells and tissues range from $\sim 10$ to $20/1$ [e.g., liver (54) and nerve (Table 5)]. Therefore, based on Eq. 2, a lactate/pyruvate ratio of 10:1 corresponds to a free NADH/NAD$^+$ c ratio of $\sim 10/10,000$ or $\sim 1/1000$ or $\sim 0.001$ (i.e., $\sim 1$ mole of NADH per 1,000 moles of NAD$^+$ c). In light of this large difference in concentrations of NADHc and NAD$^+$ c, molar changes in NADHc have a far greater impact on NADH/NAD$^+$ c and lactate/pyruvate ratios than do equimolar changes in pyruvate, lactate, or NAD$^+$ c, in decreasing order of impact. For example, accumulation of just 1 additional mole of NADH per 1,000 moles of NAD$^+$ c will increase NADH levels by about twofold (from 1 to 2 moles of NADH per 1,000 moles of NAD$^+$ c). This will be manifested by a corresponding approximately twofold increase in NADH/ NAD$^+$ c, from $\sim 0.001$ to $\sim 0.002$, and in lactate/pyruvate ratios from $\sim 10$ to $\sim 20$, as observed (a) in the current studies in the 5-week experiment (Tables 1 and 5), the 10-week Reversal Study 1 (Tables 3 and 5), and the 18-week experiment (Fig. 1; Table 5); (b) in previous reports in retina, sciatic nerve, and liver of diabetic animals (17, 34–37, 41, 54), and (c) normal rat retinas and sciatic nerve endoneurium incubated in 30 versus 5 mM glucose that are prevented by ARI (30, and current OAS IV).
If the 1 additional mole of NADHc per 1,000 moles of NAD\(^+\)c were generated by reduction of 1 mole of NAD\(^+\)c to NADHc via oxidation of sorbitol, then SDI or ARI should prevent the increase in NADH/NAD\(^+\)c, as observed in these experiments (Fig. 1; Table 1, and by the ARI in Table 3). In contrast, if the increases in NADH/NAD\(^+\)c were due to increased NADHc formation independent of sorbitol oxidation (e.g., by hypoxia), ARI and SDI would not be expected to affect it. This prediction has been tested by Nyengaard et al. (30) on the additive effects of hyperglycemia and hypoxia on free NADH/NAD\(^+\)c (manifested by increases in lactate/pyruvate ratios) in incubated normal rat retinas and sciatic nerve.
endoneurium (OAS IV). Hyperglycemia and hypoxia additively increased lactate/pyruvate ratios, triose phosphates, and G3P; the increases evoked by hyperglycemia were prevented by ARI, which did not affect the associated increases evoked by hypoxia.

The evidence that accumulation of just 1 mole of free NADH per 1,000 moles of free NAD c doubles the concentration of free neural NADHc and is associated with increased neural blood flow suggests that (a) the level of free NADHc is an exquisitely sensitive blood flow sensor needed for “hyperglycemic pseudohypoxia” (55), as well as true hypoxia, manifested by increases in NADH/NAD+ c and (b) increased levels of NADHc fuel signaling pathways that augment blood flow, (e.g., Table 1) (22, 23, 27, 51). This interpretation is consistent with observations (46) that exposure of granulation tissue to elevated levels of sorbitol (at normal glucose levels) or hyperglycemia increased blood flow and VAP (identical to those in nerve and retina in Table 1) that were prevented by SDI, SOD, or VEGF antibodies.

The near-equilibrium between free NADH/NAD+ c and lactate/pyruvate ratios maintained by LDH also plays a pivotal role in modulating energy metabolism, blood flow, and superoxide production in a spectrum of physiologic and pathologic conditions, including hypoxia and increased physiologic work in humans and animals addressed in OAS IC. Thus, infusion of lactate (which produces “prooxidant” NADHc when oxidized by LDH) (a) increases blood flow in sciatic nerve, retina, and other tissues in normal rats that are prevented by co-infusion of “antioxidant” pyruvate (23); and (b) augments the already increased blood flows evoked by increased physiologic work in normal humans and animals that also are prevented by co-infusion of pyruvate (22, 23, 27, 51, and OAS 1- C).

Observations in the 18-week study that diabetes increased sciatic nerve NADPH/NADP+ c (Table 2) and NADH/ NAD+ c by ~1.8-fold (Fig. 1), both of which were prevented by the SDI, are consistent with corresponding observations in an earlier report that were prevented by an ARI (35). Although the explanation for the increase in NADPH/ NADP+ c is not entirely clear, evidence that increased levels of NADHc generated by oxidation of sorbitol fuel superoxide production (46) and diacylglycerol synthesis (57) suggests that increased levels of NADHc might also fuel reduction of oxidized NADP+ c to NADPHc via the pyruvate/malate cycle (42), discussed in OAS I-B. This possibility is consistent with evidence that 40% of the NADPH + H+ used for fatty acid synthesis from glucose is provided by the pyruvate/malate cycle. These observations suggest that increased NADHc generated by oxidation of sorbitol may augment superoxide production by three mechanisms: (a) as substrate for NADH-driven oxidases, (b) as substrate for the pyruvate/malate cycle to increase NADPHc as fuel for NADPH-driven oxidases, and (c) by fuelling DAG synthesis, which activates the PKC that activates some isofoms of NADPH oxidase (Fig. 3). The third mechanism is supported by observations (57) that exposure of skin-chamber granulation tissue vessels to elevated glucose levels in nondiabetic rats (circumventing other metabolic and hormonal imbalances associated with the diabetic milieu) increased DAG levels and VAP, both of which were prevented by addition of pyruvate (which compensates for the uncoupling of pyruvate production when NADHc is produced by sorbitol oxidation rather than by glycolysis).

Taken together, observations in these experiments support an important role for increased “prooxidant” NADHc generated by sorbitol oxidation in fuelling superoxide production, metabolic imbalances, and neural and vascular dysfunction evoked by hyperglycemia. Uncoupling of prooxidant NADHc formed by sorbitol oxidation from equimolar formation of “antioxidant” pyruvate constrains reoxidation of NADHc to NAD+ c by LDH coupled to the reduction of pyruvate, which fuels synthesis of basal levels of diacylglycerol (DAG) and protein kinase C (PKC) activity. Both of these mechanisms of reoxidation of NADHc to NAD+ c maintain normal steady-state ratios of NADH/NAD+ c (42), discussed in OAS I-B. This possibility is consistent with evidence that 40% of the NADPH + H+ used for fatty acid synthesis from glucose is provided by the pyruvate/malate cycle. These observations suggest that increased NADHc generated by oxidation of sorbitol may augment superoxide production by three mechanisms: (a) as substrate for NADH-driven oxidases, (b) as substrate for the pyruvate/malate cycle to increase NADPHc as fuel for NADPH-driven oxidases, and (c) by fuelling DAG synthesis, which activates the PKC that activates some isofoms of NADPH oxidase (Fig. 3). The third mechanism is supported by observations (57) that exposure of skin-chamber granulation tissue vessels to elevated glucose levels in nondiabetic rats (circumventing other metabolic and hormonal imbalances associated with the diabetic milieu) increased DAG levels and VAP, both of which were prevented by addition of pyruvate (which compensates for the uncoupling of pyruvate production when NADHc is produced by sorbitol oxidation rather than by glycolysis).

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ruvate to lactate that diffuses out of cells (Fig. 3). Thus, NADHc levels increase (~1.5- to twofold) until their mass-action effect drives reoxidation of NADHc to NAD⁺c by (a) NADH-driven oxidases that produce superoxide, and (b) G3P-DHc that promotes de novo synthesis of DAG and activation of PKC (Fig. 3) that activates some isoforms of NADPH-driven oxidases.

Observations that ARI and SDI increased sciatic nerve pyruvate levels that were statistically significant or approached significance in five of six interventions (Table 5) were associated with (a) normalization of MNCV deficits, and (b) normalization/attenuation of associated increases in NADH/NAD⁺c and vascular dysfunction that are consistent with well-known antioxidant effects of pyruvate (OAS I). Pyruvate (a) stoichiometrically degrades H₂O₂ coupled to its own nonenzymatic oxidative decarboxylation, and (b) prevents superoxide production by NADH-driven oxidases by driving reoxidation of “prooxidant” NADHc to NAD⁺c by LDH, coupled to reduction of “antioxidant” pyruvate to lactate that diffuses out of cells (Fig. 3).

We suggest that the second mechanism may be especially important, because it also will attenuate product inhibition of GA3P-DH (by increased levels of NADHc) and associated metabolic imbalances (i.e., increased triose phosphate levels that (a) fuel synthesis of DAG that activates PKC that activates some isoforms of NADPH-driven oxidases, etc. (30-OAS III p. 7, 57, and current OAS I); and (b) undergo concentration-dependent degradation to methylglyoxal, a toxic and potent glycating agent (Fig. 3). GA3P-DH activity also can be inhibited by oxidative damage caused by superoxide; the present observations support the importance of increased sorbitol oxidation as the primary source of superoxide rather than increased mitochondrial oxidation of pyruvate (4, 5, 29).

Although we conclude that our current observations (together with evidence published by other investigators) are consistent with a key role for increased sorbitol oxidation in mediating diabetic complications, it is important that we also identify credible alternative interpretations of observations and caveats to various speculations in the literature. For interested readers, these are discussed in detail in OAS II.

Therapeutic implications of these experiments include the need to minimize metabolism of glucose via the sorbitol pathway by (a) maintaining glucose levels as close to normal as feasible, and (b) developing more-potent ARIs to prevent sorbitol oxidation and associated production of superoxide and metabolic imbalances that mediate complications of diabetes (31, 32). Recent observations in animal models of diabetes suggest that the disappointing efficacy of ARIs in normalizing neural and vascular dysfunction in past clinical trials may be largely attributable to inadequate inhibition of the sorbitol pathway (31, 32). Pyruvate supplementation in some form also merits further exploration in view of its extraordinary antioxidant effects in animal models of diabetes and hypoxic injury (OAS I-C).

Conclusions

Observations in these experiments support the importance of increased “prooxidant” NADHc generated by sorbitol oxidation in fueling superoxide production and associated metabolic imbalances, impaired nerve conduction, and vascular dysfunction evoked by hyperglycemia. Uncoupling of “prooxidant” NADHc formed by sorbitol oxidation from equimolar formation of “antioxidant” pyruvate constrains reoxidation of NADHc to NAD⁺c by LDH, which is coupled to the reduction of pyruvate to lactate that diffuses out of cells (Fig. 3). Increasing NADHc levels drive reoxidation of NADHc to NAD⁺c by (a) NADH-driven oxidases that produce superoxide, and (b) G3P-DHc that promotes synthesis of DAG, activation of PKC, and activation of NADPH-driven oxidases that further augment superoxide production (Fig. 3).

Attenuation of diabetes-induced vascular and neural dysfunction by ARI, SDI, and pyruvate supplementation is explained by (a) prevention of sorbitol formation by ARI, (b) inhibition of sorbitol oxidation by SDI, and (c) reoxidation of excess NADHc to NAD⁺c coupled to reduction of pyruvate to lactate by LDH rather than NADH- and NADH-driven oxidases and G3P-DHc.

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Author Disclosure Statement

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References

8. Ceriello A and Motz E. Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and
43. Scholz TD, Laughlin MR, Balabak RS, Kupriyanov VV, and Heineman FW. Effect of substrate on mitochondrial NADH,


