Issues Addressed

I. Interrelationships between retinal lactate levels and NADH/NAD$^+$c∝L/P ratios, lactate production, and lactate transport

A. -Independence of retinal lactate levels and lactate production

The observation that lactate levels were ~ twice as high in aerobic retinas incubated in 30 mM glucose-676 torr as in hypoxic retinas incubated in 5 mM glucose-36 torr appears to be inconsistent with the finding that lactate production was similar in both conditions (Table 1). It is well known, however, that intracellular levels of glycolytic metabolites such as lactate are not a reliable parameter of their rates of formation and turnover.

Tissue levels of lactate (and pyruvate) under steady state conditions are modulated by the near-equilibrium between NADH/NAD$^+$c and L/P ratios maintained by lactate-DH (Eq. 2 in the Main Text) and rates of lactate production and transport out of the cell. Steady state conditions in these experiments are attested to by observations noted in the Main Text that in aerobic retinas lactate levels and L/P ratios and rates of lactate production were stable during incubations of 1, 2, and 3 h duration under normoglycemic and hyperglycemic conditions and were unaffected by the addition of normal plasma levels of lactate and pyruvate to the medium.

The amount of lactate produced by hypoxic retinas in 5 mM glucose-36 torr in these experiments is equivalent to consumption of less than 4 % of available glucose, i.e., a negligible reduction of extracellular glucose levels from ~5 to 4.8 mM glucose. This finding, together with the observation that glucose levels did not differ in retinas incubated in 5 mM glucose at 676 and 36 torr (Table 2) supports the likelihood that glucose availability was not limiting to glycolysis/lactate production at 36 torr.

The 2-fold higher NADH/NAD$^+$c ∝L/P evoked by 30 mM glucose in retinas incubated at 676 torr *vs* 5 mM glucose-676 torr is accounted for by a ~3.6-fold increase in lactate levels *vs* only a ~1.9-fold increase in pyruvate levels. In contrast, the 16-fold higher NADH/NAD$^+$c∝L/P in retinas incubated in 5 mM glucose-36 torr *vs* 5 mM glucose-676 torr (and which was 8-fold higher than in retinas incubated in 30 mM glucose-676 torr) is accounted for largely by a 7-fold reduction in pyruvate levels *vs* only a 1.9-fold increase in lactate levels in the 5 mM glucose-36 torr retinas compared to controls (5 mM glucose-676 torr).

These data, however, do not explain the discordance in the observations that the sum of lactate + pyruvate was 2-fold higher in 30 mM glucose-676 torr retinas *vs* 5 mM glucose-36 torr retinas despite comparable rates of lactate production. This raises the possibility that the rate of lactate efflux was either augmented by hypoxia and/or decreased by hyperglycemia *via* changes in the activity of monocarboxylate transporters (MCTs).

In unilaterally stimulated brain, retina and skeletal muscle, intravenous injection of lactate increases blood flows in the stimulated tissue much more than in the contralateral unstimulated tissue (1-3). We suggest that these discordant effects of lactate injection on blood flow in stimulated *vs* resting tissues are somehow related to differences in MCT activity, which is rapidly augmented in stimulated tissues. These effects of stimulation on lactate-evoked blood flow changes are consistent with the demonstration by Lear and Kasliwal (4) that lactate transport rates are increased in regions of the brain activated by kainic acid.

How could MCT activities be acutely changed? This question has not been studied, but the observations in these different experimental paradigms suggest a possible common denominator. Increased physiological work (i.e. as with neural stimulation and muscle contraction) and hypoxia both decrease energy charge acutely; although ATP levels may not fall, ATP/ADP ratios decrease while AMP levels increase. Perhaps such a change in energy charge alone or together with an increase in NADH/NAD^+c∝L/P may acutely increase MCT activities which will facilitate lactate efflux out of cells, causing lactate levels drop.

On the other hand, as discussed below in Section I. *B*., the possibility that lactate efflux was reduced by hyperglycemia is consistent with evidence that expression of MCTs and lactate transport in skeletal muscle and adipose tissue are decreased in rats with streptozotocin diabetes (although the mechanism(s) that mediate effects of diabetes on MCT expression and lactate transport also are unknown).

Another interesting example of dissociation of tissue levels of lactate and lactate production is the report by Barron et al. (5). They observed that lactate production by incubated porcine carotid artery strips was decreased 63% by ouabain, which had no impact on tissue lactate levels or L/P ratios. Aminooxyacetate (an inhibitor of the malate-aspartate shuttle) prevented the decrease in lactate production evoked by ouabain, but increased lactate content 2.7-fold higher than controls, decreased pyruvate content by 53%, and increased L/P ratios 6-fold *vs* controls. These observations indicate that lactate production and MCT activity can vary independent of tissue lactate content and L/P ratios. (Barron et al. attributed the observation that ouabain had no impact on lactate levels and L/P ratios, while decreasing lactate production by 63%, to decreased activity of MCT B-H(+) transporter activity secondary to decreased availability of H(+) for cotransport.)

B.-Independence of hyperglycemia-evoked increases in retinal NADH/NAD^+c∝L/P vs increases in lactate production and lactate transport by monocarboxylate transporters

As discussed in the Main Text, data in Figs. 1-3 demonstrate that the increase in cytosolic NADH/NAD^+c∝L/P ratio evoked by hyperglycemia at 36 torr is independent of the associated increases in lactate production and transport under the conditions of these experiments. Figs. 1 and 3 demonstrate that inhibition of the sorbitol pathway by tolrestat selectively prevented increases in retinal NADH/NAD^+c∝L/P and triose phosphates evoked by 30 mM glucose at 36 torr, but had no impact on the associated increases in NADH/NAD^+c∝L/P and triose phosphates evoked by 36 torr *per se* at 5 or 30 mM glucose. (Increases in triose phosphates are attributable to inhibition of GA3P-DH by increases in NADH/NAD^+c caused by hyperglycemia and by hypoxia.) Fig. 2 demonstrates that tolrestat also had no impact on the increase in lactate production (and transport out of retinal cells) evoked by 30 mM glucose at 36 torr; nor did tolrestat impact on the increase in lactate production and transport out of retinal cells evoked by 36 torr in retinas incubated in 5 mM glucose. These findings are in accord with observations that inhibitors of the sorbitol pathway also prevent hyperglycemia-evoked increases in NADH/NAD^+c∝L/P in aerobic isolated glomeruli and retinas without impacting on increased lactate production (6,7).

The independence of increases in the retinal NADH/NAD^+c∝L/P and associated increases in glycolytic lactate production and transport evoked by hyperglycemia is further supported by observations shown in Fig. 6 (Section IV.*A)*. These observations demonstrate that addition of 25 mM sorbitol or galactose to incubation media containing 5 mM glucose increased retinal NADH/NAD^+c∝L/P and triose phosphates comparable to the increases evoked by 30 mM glucose; however, neither sorbitol nor galactose increased retinal lactate production-transport out of cells into the incubation medium. These findings, together with observations that inhibitors of aldose reductase and sorbitol-DH markedly attenuate or prevent increases in NADH/NAD^+c∝L/P in retina, peripheral nerve, and granulation tissue of diabetic rats (8-11), support the conclusion in the Main Text that the increase NADH/NAD^+c∝L/P evoked by hyperglycemia is independent of associated increases in glycolysis-lactate production (as well as perturbations in MCTs and associated changes in lactate transport in diabetic rats).

Expression of MCT 1 and MCT 4 and rates of lactate transport are decreased in heart and/or skeletal muscle and in adipocytes in rats with streptozotocin diabetes of 2-3 weeks duration (12-14). In adipocytes of rats with diabetes of only 4 days duration, MCT 1 expression is decreased and lactate transport capacity is reduced by 64% (12). The mechanism(s) that regulate MCT expression in streptozotocin-induced diabetes (and their potential to impact on cytosolic NADH) are not known (13). It is of interest, however, that pharmacological inhibitors of MCTs acutely decrease NADH/NAD^+c∝L/P under normoglycemic conditions in the perfused heart and in incubated tissues (see Section IV. *C.*-5 -Brownlee et al.)

II. Different enzymes oxidize NADHc generated by glycolysis and the sorbitol pathway; redox cycling of cytosolic NAD^+c⇌NADHc.

Each mole of free cytosolic(c) NADHc formed *via* glycolysis is followed by formation of equimolar pyruvate (15) (Fig. 5). Under steady state conditions NADHc and pyruvate are formed faster than they are utilized for ATP synthesis by oxidative phosphorylation in normoglycemic aerobic resting cells; lactate-DH catalyzes reoxidation of excess NADHc coupled to reduction of excess pyruvate to lactate that diffuses out of the cell. (Lactate levels in resting retina and skeletal muscle are several fold higher than in blood or plasma [1,2,16].) Since utilization of NADHc and pyruvate for oxidative phosphorylation by resting cells is limited by availability of ADP (i.e., State 4 respiration [15]) as depicted in Fig. 5, little if any of the additional NADHc formed *via* glycolysis in resting hyperglycemic retina is reoxidized by electron shuttles; nor is the additional pyruvate utilized by mitochondria for synthesis of ATP by oxidative phosphorylation. Instead, excess NADHc is reoxidized to NAD^+c largely by lactate-DH coupled to reduction of excess pyruvate to lactate that diffuses out of the cell (manifested by the increase in lactate production in Tables 1 and 2 and Fig. 2).

In contrast, metabolism of glucose to fructose (coupled to equimolar formation of NADHc) *via* the sorbitol pathway does not generate pyruvate requisite for reoxidation of NADHc to NAD^+_c by lactate-DH. Fructose is poorly metabolized by most cells and diffuses out *via* the facilitative sugar transporter GLUT5 (17,18). (To the extent that fructose is phosphorylated and

further metabolized *via* glycolysis, the pyruvate formed is sufficient only to reoxidize the equimolar increase in NADHc formed by GA3P-DH.) Thus, NADHc generated by the sorbitol pathway is reoxidized largely by enzymes other than lactate-DH.

Several observations support the conclusion that oxidation of sorbitol pathway-generated NADHc by lactate-DH is limited by availability of pyruvate. *1*. Addition of exogenous pyruvate or an ARI prevents glucose-induced: a) increases in triose phosphate levels in incubated human erythrocytes and in rat retina (7,19), and b) vascular dysfunction and accumulation of diacylglycerol in granulation tissue (20). *2*. Addition of exogenous pyruvate prevents accumulation of triose phosphates evoked by hyperglycemia in incubated glomeruli (6). *3*. Vascular albumin leakage in granulation tissue evoked by 1 mM sorbitol is prevented by coadministration of 1 mM pyruvate (21). *4.* Increased retinal and sciatic nerve blood flows evoked by acute hyperglycemia (produced by infusion of glucose for 5 h) in normal rats are prevented by co-infusion of pyruvate, by an ARI, and by an SODmimic (22,23, and unpublished observations by Ido Y, Chang K, Williamson JR).

Prevention of increased triose phosphate levels and diacylglycerol accumulation by exogenous pyruvate (20) is attributable to increased oxidation of sorbitol pathway-generated NADHc to NAD^+c coupled to reduction of pyruvate to lactate by lactate-DH. Otherwise the mass action effect of the increased NADHc will drive its oxidation to NAD^+c by G3P-DHc coupled to reduction of DHAP (a triose phosphate) to G3P (Fig. 5). Oxidation of NADHc by $G3P\text{-}DH_c$ is the first step in: *1*) one pathway for *de novo* synthesis of glycerolipids including diacylglycerol, which activates PKC, and *2*) the glycerol phosphate electron shuttle that transfers electrons and protons (E&P) from NADHc to mitochondrial FADm. E&P transferred to FADm can fuel mitochondrial superoxide production in addition to synthesis of ATP by oxidative phosphorylation.

Thus, inhibitors of aldose reductase and sorbitol-DH prevent reduction of NAD^+c to NADHc by sorbitol-DH, whereas the mass action effect of increased exogenous pyruvate levels drives reoxidation of NADHc to NAD^+c by lactate-DH, keeping pace with formation of NADHc by sorbitol-DH. Both interventions attenuate: 1) inhibition of GA3P-DH (by elevated NADHc levels), which increases levels of triose phosphates that, in turn, fuel *de novo* synthesis of diacylglycerol and activation of PKC and also promote formation of methylglyoxal, and 2) increased production of superoxide by extra-mitochondrial NAD(P)H oxidases coupled to oxidation of NADHc.

These observations and considerations suggest that a far greater inhibition of aldose reductase than was achieved in past clinical trials may be needed to prevent complications of diabetes fueled by *NADHc* generated by the sorbitol pathway.

Redox cycling of $NAD^+c \rightleftharpoons NADHc$ – Rapid reoxidation of NADHc is crucial for: *1*) maintaining high levels of NAD^+c needed to fuel increased ATP synthesis (*via* substrate and oxidative phosphorylation) during physiological work, and *2*) synthesis of ATP *via* substrate phosphorylation to sustain viability during hypoxia.

The retinal content of free NAD_c is unknown; however, the total (t) NADt content of normal rat retina (NAD^+t plus NADHt) is ~ 29 pmol/μg DNA [average of 3 published values (24-26)]. Thus, if oxidation of sorbitol to fructose were coupled to reduction of NADt, it would undergo redox cycling ($NAD^+t \rightleftharpoons$ NADHt) ~7 times to produce the ~200 pmol fructose/μg DNA/2h in the present experiments. However, since oxidation of sorbitol to fructose is coupled to reduction of free NAD^+c (which is only one of several pools of NADt that also include free NAD^+m, and enzyme bound NAD^+c and NAD^+m), the rate of redox cycling of free $NAD^+c \rightleftharpoons$ NADHc would necessarily be much higher than for NADt.

The observation that NADHc∝L/P was increased by 1 h of hyperglycemia and remained stable after 2 and 3 h of incubation indicates that increased steady state redox cycling of free $NAD^+c \rightleftharpoons$ NADHc was achieved by 1 h under the conditions of these experiments [contrary to the unfounded assertion of Obrosova et al. (27)]. Thus, exposure of the retina to hyperglycemia for no more than 1 h increased the concentration of free NADHc to levels that drove its reoxidation as fast as it was formed by oxidation of sorbitol to fructose. These observations support the conclusion that the increased rate of reduction of NAD^+c to NADHc coupled to oxidation of sorbitol to fructose evoked a corresponding increased rate of reoxidation of NADHc to NAD^+c (i.e. redox cycling of $NAD^+c \rightleftharpoons$ NADHc), albeit at a higher steady state concentration of NADHc than at normal glucose levels.

The observation that lactate production also was increased by 1 h of hyperglycemia and remained stable after 2 and 3 h of incubation (and was not attenuated by tolrestat) indicates that rates of reduction of free NAD^+c to NADHc by GA3P-DH and reoxidation of NADHc to NAD^+c by lactate-DH (i.e. redox cycling of $NAD^+c \rightleftharpoons$ NADHc *via* glycolysis) also were increased concurrently with (but independent of) sorbitol pathway-evoked increased redox cycling of $NAD^+c \rightleftharpoons$ NADHc. The finding that tolrestat prevented the increase in NADHc ∝L/P, but not the increase in lactate production evoked by hyperglycemia, supports the conclusion that different enzymes reoxidize NADHc generated by glycolysis and NADHc generated by the sorbitol pathway as discussed above.

Furthermore, the finding that the molar increase in lactate production (indicative of reduction of NAD^+c to NADHc by GA3P-DH and reoxidation of equimolar NADHc to NAD^+c by lactate-DH) evoked by hyperglycemia at 676 torr was 50-fold greater than the increase in fructose production (indicative of reduction of NAD^+c to NADHc by sorbitol-DH) (Figs. 2 and 4) indicates that the rate of redox cycling of $NAD^+c \rightleftharpoons$ NADHc *via* glycolysis was ~50-fold higher than the concurrent rate of redox cycling of $NAD^+c \rightleftharpoons$ NADHc coupled to the sorbitol pathway (*via* reduction of NAD^+c to NADHc by sorbitol-DH and reoxidation of NADHc to NAD^+c by enzymes in other metabolic pathways, e.g., G3P-DHc, malate-aspartate shuttle, and NAD(P)H oxidases (see Section III).

Since the equilibrium constants K_{LDH} and $K_{GL\text{-}DH}$ (Eqs. 2 and 3 in the Main Text) are pH sensitive, estimates of free NADH/NAD^+c and NADH/NAD^+m based on the assumption that intracellular pH is 7.0 could be biased by a decrease in pH due to increased lactate levels evoked by hyperglycemia and by hypoxia. As noted in the Main Text, extracellular pH did not change

during the course of these experiments, but measurements of intracellular pH were not performed. However, the increases in triose phosphates evoked by hyperglycemia and hypoxia shown in Table 2 and Fig. 3 indicate that the impact of any putative change in intracellular pH was insufficient to impact significantly on K_{LDH} and $K_{GL\text{-}DH}$ (under the conditions of these experiments) or alter the conclusions of these studies.

The direction and magnitude of changes in triose phosphates evoked by hyperglycemia and hypoxia correspond to the direction and magnitude of changes in estimates of NADH/NAD^+c based on the equilibrium constant for K_{LDH} of 1.11 $x10^{-4}$ at pH 7.0 in Eq. 2 in the Main Text. And, the increases in triose phosphate levels evoked by hyperglycemia and by hypoxia (like the estimates of increases in NADH/NAD^+c based on L/P ratios) were additive. It is important to note, however, that the increases in triose phosphates evoked by hyperglycemia and by hypoxia attest to inhibition of GA3P-DH *via* increases in cytosolic NADH/NAD^+c *per se* (independent of estimates of NADH/NAD^+c based on the L/P ratio that can be impacted by a pH change). The additive effects of hyperglycemia and hypoxia on triose phosphates are consistent with their additive effects on estimates of NADH/NAD^+c attributable to increased formation of NADHc by the sorbitol pathway and impaired reoxidation of NADHc *via* the malate-aspartate shuttle during hypoxia (which is consistent with the estimated increase in NADH/NAD^+m based on the equilibrium constant of 3.87 $x10^{-3}$ for GL-DH at pH 7.0 in Eq. 3 in the Main Text).

Furthermore, as shown in Figs. 1 and 3, tolrestat selectively prevented the increases in cytosolic NADH/NAD^+c$\propto$L/P and triose phosphates evoked by hyperglycemia without impacting on increases in cytosolic NADH/NAD^+c$\propto$L/P and triose phosphates evoked by hypoxia.

III. NADHc formed by the sorbitol pathway fuels synthesis of diacylglycerol, activates PKC, and fuels superoxide production by extra-mitochondrial NADH and NADPH oxidases

A growing body of evidence supports the importance of ROS generated by activated extra-mitochondrial NAD(P)H oxidases in mediating diabetic vascular disease as well as atherosclerotic and hypertensive vascular disease independent of diabetes (28-37). Increased superoxide production in 2 different animal models of hypercholesterolemia is mediated largely by activation of an NADH oxidase selectively fueled by NADH (28). Likewise, hyperglycemia-evoked superoxide production by porcine coronary artery segments is associated with activation of an NAD(P)H oxidase and is markedly increased by NADH (31). Increased superoxide production in the aorta of OLETF rats (a model of Type 2 diabetes) also is linked to increased expression of an NADH oxidase that is fueled selectively by NADH (33,35). And, increased superoxide production by arteries and veins from humans with diabetes is mediated by activation of an NAD(P)H oxidase fueled by NADH and by NADPH, and enzymatic uncoupling of eNOS (32).

Many of these reports implicate activation of PKC (by hyperglycemia, free fatty acids, diabetes, and hypertension) in mediating activation of NAD(P)H-dependent oxidases in vascular cells (and in a human monocyte cell line) (29,30,32,34,37); however, less attention has been

focused on: 1) the mechanism(s) of activation of PKC, and 2) the origin of the electrons carried by NADPHc and NADHc that fuel production of ROS.

Several lines of evidence (in addition to the effects of exogenous pyruvate discussed earlier in Section II.) support the conclusion that increased NADHc formed by the sorbitol pathway augments metabolic pathways that reoxidize NADHc to NAD^+c and: *1*) fuel *de novo* synthesis of diacylglycerol and activation of PKC, *2*) activate extra-mitochondrial NAD(P)H oxidases, *3*) fuel superoxide production by extra-mitochondrial NADH-dependent NADH oxidase, and *4*) mediate vascular and neural dysfunction evoked by diabetes. Thus:

A. Addition of sorbitol to normoglycemic incubation media (bypassing generation of sorbitol from glucose by aldose reductase and other effects of hyperglycemia) increases NADHc∝L/P and triose phosphates (that fuel *de novo* synthesis of diacylglycerol) in rat retina to the same extent as addition of equimolar glucose (Section IV.*A*.*Caveats*-- and Fig. 6).

B. An ARI prevents glucose-evoked increases in *de novo* synthesis of diacylglycerol and activation of PKC in explants of glomeruli from control rats (38).

C. Antibodies to VEGF, inhibitors of sorbitol-DH, and superoxide dismutase prevent vascular dysfunction evoked by addition of exogenous sorbitol (at normal glucose levels) (11).

D. Increased expression of sorbitol-DH markedly potentiates hyperglycemia-evoked increases in ROS production, VEGF mRNA levels, and impaired DNA synthesis in bovine retinal capillary pericytes (39). Predictably, overexpression of sorbitol-DH also markedly decreased sorbitol levels while increasing fructose levels *vs* control cells exposed to 30 mM glucose. The effects of sorbitol-DH overexpression on ROS, VEGF, and DNA synthesis were prevented by an antioxidant (N-Acetylcysteine) and by an ARI. (See caveats to effects of sorbitol-DH deficiency on nerve conduction in experimental diabetic mice in Section IV. *E. Caveats 5 and 6-Ng et al. and Ii et al.*)

E. Oxidation of sorbitol by sorbitol-DH and oxidation of lactate by lactate-DH are coupled to reduction of free NAD^+c to NADHc (40) as depicted in Fig. 5. And, reduction of NAD^+c to $NADH_c$ coupled to oxidation of sorbitol to fructose elevates the lactate/pyruvate ratio *via* the near-equilibrium between the NADHc/NAD^+c and lactate/pyruvate ratios (41). Since oxidation of lactate (but not oxidation of pyruvate) fuels production of superoxide by a fully active extra-mitochondrial NADH oxidase (42,43) as depicted in Fig. 5, it is likely that NADHc generated by oxidation of sorbitol also will fuel superoxide production by this same enzyme. The K_m of this NADH oxidase for NADH is only 8-9 μM, i.e. ~8% of total retinal NADHt (117 μmol NADHt/L of water) in normal rats (26). Thus, O_2^- production by this NADH oxidase must be fueled by a very small fraction of total NADHt (consistent with free NADHc); otherwise O_2^- production would be close to the maximum at normal glucose levels.

IV. Caveats in testing the role of free NADHc in mediating complications of diabetes

Virtually all published observations interpreted as discordant with a role for free NADHc in the pathogenesis of diabetic complications are inconclusive, i.e. the observations also are consistent with a role for NADHc. This is largely because free NADHc is impacted by many

factors independent of oxidation of sorbitol by sorbitol-DH. Free NADHc is increased or decreased by changes in the concentration of oxidized and reduced substrates of numerous enzymes (e.g. lactate-DH, GA3P-DH, G3P-DH, malate-DH, and galactose-DH) which, like sorbitol-DH, utilize free NAD^+(H)c as a cofactor for transferring E&P to and from their substrates (see below) as depicted in (Fig. 5). Changes in the concentration and/or catalytic activity of these enzymes also can effect changes in free NADHc.

A. Caveats to experimental galactosemia as a surrogate animal model of diabetes

Animals fed galactose-supplemented diets develop many early diabetes-like metabolic changes and complications (e.g. retinopathy, neuropathy, nephropathy, and cataracts). Like glucose, galactose is reduced by aldose reductase to its corresponding sugar alcohol (galactitol); and, elevated tissue galactitol levels in galactose-fed animals are comparable to sorbitol levels in diabetic animals. However, in contrast to sorbitol, galactitol is a very poor substrate for sorbitol-DH and is not further metabolized in tissues that develop diabetes-like complications. Nevertheless, early diabetes-like complications evoked by galactose-supplemented diets are markedly attenuated by ARI in association with reduction of galactitol levels. Largely for these reasons, despite the demonstration by Varma and Kinoshita 30 years ago that total lens NADH/NAD^+t was increased in galactose-fed as well as in diabetic rats (44), little attention has been focused on the possibility that galactosemia might increase free NADH/NAD^+c by an enzyme(s) other than sorbitol-DH that also is inhibited by nonspecific inhibitors of aldose reductase.

Recent studies have demonstrated that experimental galactosemia increases free NADHc in the same tissues in which NADHc is increased by hyperglycemia. Free NADH/NAD^+c∝L/P is increased in the lens and peripheral nerve of galactose-fed rats (8,45), in human erythrocytes (46,47), and in rat retinas incubated in galactose-supplemented media (Fig. 6). Elevated levels of sorbitol, but not galactitol, also increase retinal NADH/NAD^+c∝L/P; and, elevated levels of galactose, glucose, and sorbitol (but not galactitol) increase retinal triose phosphate levels similarly (Fig. 6). (An inhibitor of sorbitol-DH completely prevented the increase in triose phosphates evoked by elevated glucose levels but had no effect on the increase in triose phosphates evoked by elevated galactose levels [data not shown]) In contrast, while elevated glucose levels increase glycolysis, elevated levels of galactose, sorbitol, and galactitol do not augment glycolysis. The discordant effects of elevated galactose and glucose levels on retinal glycolysis may be linked to the discordant effects of diabetes and galactosemia on ATPase activity in rat sciatic nerve and dorsal root ganglia (48).

Observations of Berry et al. (47) support the likelihood that the increase in NADHc evoked by elevated galactose levels is mediated by oxidation of galactose to galactonate, possibly by galactose-DH. Incubation of human erythrocytes in 25 mM galactose increased NADH/NAD^+c∝L/P by 4-fold (from 13 to 57); and, 3-fold as much galactonate was formed as galactitol. Tolrestat (an ARI) markedly attenuated the increases in galactonate and galactitol and completely prevented the increase in NADH/NAD^+c∝L/P.

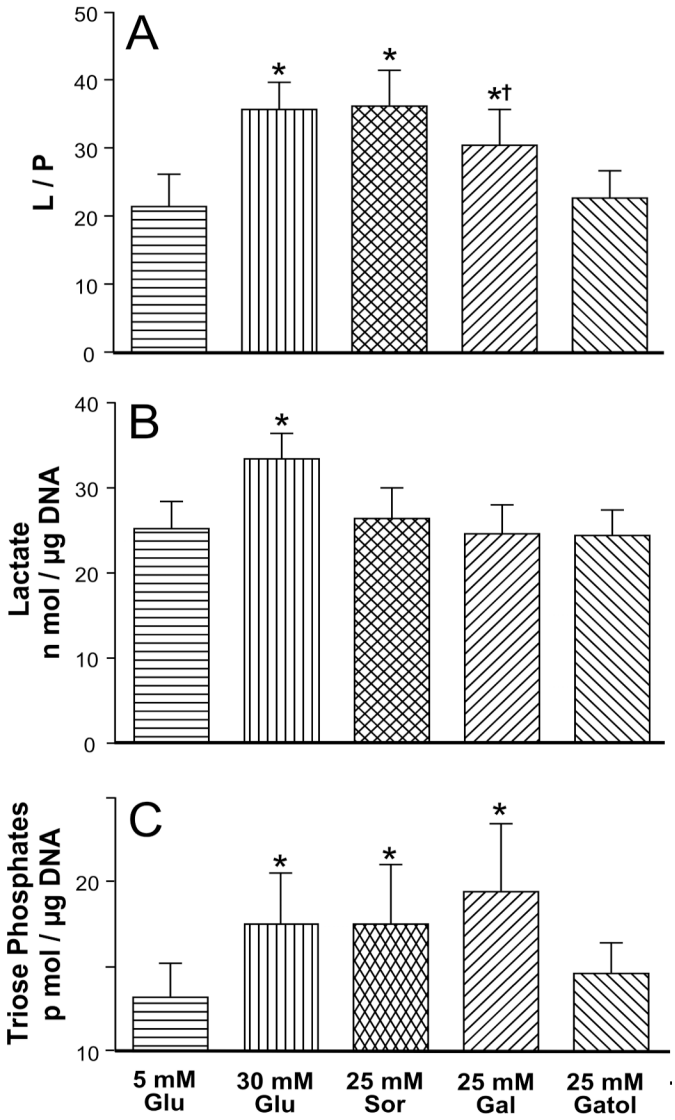

Figure 6 -Effects of elevated levels of glucose, sorbitol, galactose, and galactitol on retinal lactate/pyruvate (L/P) ratios, lactate production, and triose phosphate levels

Retinas from normal rats were incubated for 2 hours as described in the Main Text in 5 mM glucose (Glu) alone or 5 mM glucose supplemented with 25 mM glucose, 25 mM sorbitol (Sor), 25 mM galactose (Gal), or 25 mM galactitol (Gatol).

Panel A - L/P ratios were increased 65% by addition of 25 mM glucose, 67% by 25 mM sorbitol, and 41% by 25 mM galactose; L/P ratios were unaffected by 25 mM galactitol.

Panel B – Lactate production was increased 32% by addition of 25 mM glucose but was unaffected by equimolar additions of sorbitol, galactose, or galactitol.

Panel C – Triose phosphate levels were increased 33% by addition of 25 mM glucose and 25 mM sorbitol, and were increased 47% by 25 mM galactose; triose phosphates were unaffected by 25 mM galactitol.

* $P < 0.0001$ *vs.* 5 mM glucose, $^{\dagger}P < 0.01$ *vs*. 30 mM glucose and 25 mM sorbitol.

These observations demonstrate that: *1*) experimental galactosemia increases NADH/NAD^+c∝L/P in association with increased production of galactonate and galactitol, *2*) tolrestat prevents all 3 of these galactose-induced changes, and *3*) tolrestat (and very likely other ARI that prevent diabetes-like complications evoked by galactosemia) is not a specific inhibitor of aldose reductase. These findings support an important role for increased free NADHc in mediating diabetes-like complications evoked by galactosemia as well as complications of diabetes.

Galactose ingestion also increases urinary excretion of galactonate in normal subjects and in humans with galactosemia (49). Furthermore both galactose and glucose can be metabolized *via* the glucuronic acid pathway (50-53) that generates 2 moles of NADHc per mole of glucose metabolized to D-glucuronate (*vs* only 1 mole of NADHc produced by oxidation of sorbitol to fructose).

B. General caveats to cell culture and tissue incubation paradigms of diabetic complications

In vitro paradigms for assessing increased transfer of E&P to free NAD^+c (reducing it to NADHc) evoked by hyperglycemia are subject to at least 3 important caveats that are seldom acknowledged much less addressed.

1. Most cell culture studies are carried out in a toxic 'pro-oxidant' environment (i.e. culture media equilibrated with 20% oxygen or higher), which impairs growth of porcine retinal pigment epithelial cells and suppresses endogenous superoxide dismutase activity. Thus cells are more susceptible to damage by ROS (54) generated by hyperglycemia (see references in Introduction of Main Text). Likewise, proliferation of retinal pericytes and retinal microvascular endothelial cells is impaired when they are maintained at 20 *vs* 5% oxygen (55). Addition of SOD and catalase to the medium stimulates growth of cells exposed to 20% oxygen (54).

2. Hui et al. (56) have called attention to the potential of metal-ion impurities in phosphate and bicarbonate buffered media that mimic effects of advanced glycation endproducts (AGE) on permeability of cultured cell monolayers, especially in serum-free media. They concluded that *in vitro* effects of elevated glucose levels on oxidative stress, even in short duration experiments, can be confounded by metal-ion contaminants and the absence of endogenous antioxidants present in serum.

3. Accumulation of lactate in the incubation medium is a major confounding factor in interpreting metabolic effects of elevated glucose levels on cells and tissues *in vitro*.

As discussed earlier in Section II, the rate of aerobic glycolysis in resting cells generates free NADHc and pyruvate faster than they are used for ATP synthesis by oxidative phosphorylation. Excess NADHc is reoxidized to NAD^+c by lactate-DH coupled to reduction of excess pyruvate to lactate that diffuses out and is carried away by the vasculature. (Most of this excess lactate is utilized for gluconeogenesis in the liver *via* the Cori cycle (15); some is utilized by other tissues for energy metabolism (the lactate shuttle) (57).)

In contrast, lactate diffusing out of cells and tissues *in vitro* is confined to the volume of incubation medium. Accumulation of lactate in the extracellular medium will increase

intracellular lactate (*via* monocarboxylate transporters), which will restrain oxidation of NADHc to NAD^+c (*via* product inhibition) by lactate-DH (as depicted in Fig. 5) with the result that free $NADH/NAD^+c \propto L/P$ will increase. The increase in NADHc, in turn, inhibits transfer of E&P from GA3P to NAD^+c by GA3P-DH resulting in accumulation of GA3P, DHAP, and F1,6 BP (triose phosphates) that are in near-equilibrium with each other (Fig. 5).

Tilton et al. (58) reported that: *1*) the L/P ratio of a suspension of human erythrocytes (33 % hematocrit) increased 6-fold (from 9 at baseline to 57) after incubation for 3 h in 3 ml of TES buffer ('closed system') containing 2.5 mM glucose, and *2*) triose phosphate levels rose over 10-fold (from 36 to 538 μmol/L/gm hemoglobin). The 6-fold increase in the L/P ratio is indicative of corresponding increases in free NADHc and $NADH/NAD^+c \propto L/P$. (Plasma and tissue L/P ratios are generally in the range of 10 to 25 under steady state resting conditions, although intracellular concentrations of lactate and pyruvate are much higher than in plasma.)

To simulate *in vivo* dilution of lactate diffusing out of erythrocytes into plasma and subsequent removal by the liver, the same 3 ml volume of erythrocyte suspension was placed in a dialysis bag suspended in 30 ml of buffer ('open system'), i.e. the concentration of lactate in erythrocytes and the dialysis bag would be diluted 10-fold *vs* the closed system. *In marked contrast to the closed system, after incubation for 3 h in this 'open system', erythrocyte L/P ratios and triose phosphate levels did not differ from baseline.*

In addition to the increase in free $NADH/NAD^+c \propto L/P$ ratio in the 'closed system', the ratio of total(t) $NADH/NAD^+t$ increased 2.4-fold in the 'closed system', but not in the 'open system'. And, Tilton et al. demonstrated that: *1*) intracellular triose phosphate levels correlated with L/P ratios independent of lactate and pyruvate concentrations in the incubation medium + suspended erythrocytes, and *2*) elevation of triose phosphate levels was minimal at L/P ratios below 25.

The magnitude of lactate accumulation in the incubation medium (and its impact on free NADHc and triose phosphate levels) can vary greatly depending on the ratio of the volume of incubated cells and tissues to the volume of incubation medium, frequency of medium change, and initial concentrations of glucose, lactate, and pyruvate in the medium. In most published studies no information is provided regarding initial or final levels of these important metabolites in the incubation medium or in cells and tissues; in the few studies in which such data are provided, the values are highly unphysiological.

To the extent that redox-coupled metabolic imbalances and signaling pathways implicated in the pathogenesis of diabetic complications (e.g. activation of PKC by *de novo* synthesis of diacylglycerol, increased production of superoxide, increased VEGF, AGE formation, etc.) are fueled by increases in NADHc evoked by hyperglycemia, they also will be fueled by accumulation of extracellular lactate sufficient to increase L/P ratios in the incubation medium and $NADH/NAD^+c \propto L/P$ in cells. However, inhibitors of the sorbitol pathway, i.e. aldose reductase (ARI) and sorbitol-DH (SDI), will not prevent increases in NADHc and triose phosphates evoked by elevated lactate levels. Likewise, compounds such as benfotiamine (a derivative of thiamine) that lower triose phosphate levels by activating transketolase (59,60) will not decrease $NADH_c$ or superoxide production fueled by NADHc (42,43) formed by oxidation of

lactate or sorbitol. On the other hand (as discussed in Section II.) addition of sufficient pyruvate will reoxidize NADHc to NAD^+c, attenuating increases in NADHc and triose phosphates whether they are caused by increased oxidation of sorbitol, accumulation of lactate, or hypoxia as depicted in Fig. 5.

C. Caveats to published in vitro studies

1. Thomas et al. (61) reported that hyperglycemia did not increase NADHc∝L/P in a human retinal pigment epithelial cell line (selected for high aldose reductase activity) equilibrated with 95% O_2; nor was NADHc∝L/P affected by addition of 3 mM pyruvate to the culture medium. Remarkably, mean L/P ratios were 134 and 114 in retinal pigment epithelial cells exposed to 5 and 20 mM glucose, respectively. These L/P ratios are 10-fold higher than in freshly isolated retinal pigment epithelial cells from control rats and from rats with diabetes of up to 3 weeks duration (62), 5 to 8-fold higher than in normal rat retina (2,16,62,63), 4 to 6-fold higher than in retinas from diabetic rats (16,62,63), and 5 to 7-fold higher than in normal rat retinas incubated in 5 mM glucose-676 torr (ref. 7 and Fig. 1 and Table 1 in the Main Text); they are similar to L/P ratios in hypoxic retinas incubated in 5 mM glucose-36 torr (Fig. 1).

Since addition of 3 mM pyruvate to the culture medium had beneficial effects on *myo*-inositol uptake and phospholipid metabolism, but did not decrease L/P ratios, the authors concluded: "These results favor *myo*-inositol depletion rather than altered redox as the primary cause of glucose-induced aldose reductase-related defects in phospholipid metabolism….". The authors concluded that the failure of pyruvate to reduce cellular L/P ratios was not due to depletion of *medium* pyruvate levels since they were ~1 mM (*incubation medium* lactate levels were not reported) at the time *intracellular* L/P ratios were ~100. To the extent that intra-and extracellular L/P ratios were in near-equilibrium (which they should be under these experimental conditions), extracellular lactate levels would have been ~100 mM (i.e. 100-fold higher than normal plasma levels).

No information was provided regarding initial lactate and pyruvate levels or final glucose levels in the culture medium, sorbitol and fructose were not measured, nor were the effects of an ARI or SDI examined. In any case, the abnormal cytosolic redox-state of these RPE cells incubated in media containing normal glucose levels (and equilibrated with 95% O_2) indicates that the experimental conditions in this paradigm were unsuitable for investigating sorbitol pathway-linked redox changes evoked by elevated glucose levels.

2. At the other extreme, Gillies et al. (64) reported that L/P ratios in the incubation medium of bovine retinal capillary endothelial cells exposed to 5 and 30 mM glucose at room air (~20% O_2) were 0.009 and 0.008, respectively (normal L/P ratio = 10). The stock culture medium obtained from the manufacturer was specified to contain 1.2 mM pyruvate, but no lactate; these concentrations were not verified by the authors (personal communication from M. Gillies). Thus, it appears that the initial concentrations of lactate and pyruvate and L/P ratios in the stock culture medium were highly unphysiological. Furthermore, while medium lactate was measured at the end of incubations and determined to be ~ 6 mM (6-fold higher than normal), pyruvate was not

measured. Thus L/P ratios in the medium and in the cultured cells were unknown at the beginning or the end of incubations.

Ponalrestat (an ARI) decreased sorbitol levels substantially in cells exposed to 30 mM glucose; however, they remained elevated 2-fold *vs* control cells in 5 mM glucose. And, the ARI did not ameliorate the hyperglycemia-evoked decrease in electrical resistance or the increased paracellular inulin permeation across the endothelial cell monolayers. The authors concluded that the perturbations in endothelial barrier integrity and electrical resistance were mediated by hyperglycemia-evoked mechanisms independent of the sorbitol pathway. Since the ARI did not normalize sorbitol levels, and L/P ratios in the incubation medium in these experiments are unknown, the mechanism(s) that mediated the adverse effects of hyperglycemia on endothelial barrier function and electrical resistance also are unknown.

3. Xia et al. (65) reported that 100 μM sorbinil (an ARI) failed to prevent increased *de novo* synthesis of diacylglycerol evoked by elevated levels of glucose and galactose in cultured bovine retinal capillary endothelial cells and rat aortic endothelial and smooth muscle cells (effects of sorbinil on PKC activity were not reported). Sorbinil reduced a 2.2-fold increase in sorbitol levels in cells exposed to high glucose to 0.72% of the levels in cells incubated at normal glucose levels. Since lactate and pyruvate were not assessed in cells or culture medium, increased synthesis of diacylglycerol could be due to an increase in L/P∝ NADHc secondary to accumulation of lactate in the medium.

4. Lee et al. (66) reported that 10 μM sorbinil prevented increased sorbitol levels and impaired ouabain-sensitive Na^+, K^+-ATPase activity induced by hyperglycemia in bovine retinal capillary endothelial cells, but did not prevent increased PKC activity; paradoxically, a PKC agonist (phorbol myristate acetate) also attenuated the decrease in ouabain-sensitive Na^+, K^+-ATPase activity. Since lactate and pyruvate were not assessed in cells or medium, increased PKC activity could be secondary to an increase in L/P∝ NADHc due to accumulation of lactate in the medium.

In view of numerous reports that sorbinil and other ARI prevent a wide spectrum of retinal, neural, and renal changes in diabetic rats (67-70) the failure of sorbinil to prevent increased diacylglycerol synthesis and PKC activation by elevated glucose levels in the experiments of Xia et al. (65) and Lee et al. (66) implies that: *1*) increased diacylglycerol synthesis and PKC activation contribute little to the pathogenesis of vascular and neural changes induced by diabetes, or *2*) their culture cell conditions are unsuitable for investigating mechanisms that mediate increased diacylglycerol synthesis and PKC activation in diabetic animals. The latter view is supported by beneficial effects of PKC inhibitors in diabetic animals (71-75) as well as the prevention by ARI of increased diacylglycerol synthesis, activation of PKC and phospholipase A_2, and related changes induced by elevated glucose levels in cultured human and rat mesangial cells and rat glomerular explants (38,76,77).

5. Brownlee et al., in a series of recent publications (60,78-86), assert that "…. Hyperglycemia-induced ROS generation …arises exclusively from the mitochondrial electron transport chain…." (83). They contend that overproduction of superoxide in mitochondria is a

singular unifying mechanism for mediating a wide spectrum of hyperglycemia-evoked metabolic imbalances implicated in the pathogenesis diabetic complications. Metabolic imbalances they attribute to increased mitochondrial superoxide production include: *1*) activation of PKC, NF-κB, the hexosamine pathway, and poly(ADP-ribose) polymerase (PARP), *2*) inhibition of GA3P-DH, elevated triose phosphates, and methylglyoxal-derived glycation products, and *3*) accumulation of sorbitol, platelet dysfunction, increased PGE_2 synthesis, etc. Brownlee et al. attribute increased mitochondrial superoxide production to oxidation of pyruvate (fueled by increased production of pyruvate *via* glycolysis), which generates $NADH_m$ that fuels superoxide production by the electron transport chain.

In the initial report by Nishikawa et al. (78) this scenario was supported by evidence that: *1*) hyperglycemia increased glycolysis and oxidation of glycolysis-derived pyruvate by the TCA cycle ~ 2-fold and increased superoxide production ~ 3-fold, and *2*) the increase in superoxide production evoked by hyperglycemia was blocked by: (*a*) an inhibitor of pyruvate transport into mitochondria (4-OHCA:α-cyano-4-hydroxycyanocinnamic acid), (*b*) an inhibitor of Complex II of the electron transport chain (TTFA:thenoyltrifluoroacetone), (*c*) CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), an uncoupler of oxidative phosphorylation, (*d*) overexpression of uncoupling protein-1, and (*e*) overexpression of Mn-SOD, the mitochondrial isoform of SOD. In contrast, the increase in superoxide production was not prevented by: *1*) rotenone (an inhibitor of Complex I of the electron transport chain), or *2*) aminooxyacetate (an inhibitor of the malate-aspartate shuttle that transfers E&P from NADHc to NAD^+m).

Nevertheless, their conclusion that increased mitochondrial superoxide production evoked by hyperglycemia was fueled exclusively by electrons generated by mitochondrial oxidation of pyruvate appears to be untenable in light of: *1*) numerous important caveats to the interpretation of their data, and *2*) discordant observations of other investigators. Electrons generated by oxidation of glucose metabolites in the cytoplasm can fuel superoxide production in mitochondria as well as in the cytosol. Electrons generated in the cytosol that are carried by NADHc can: *1*) be transported into mitochondria by the malate-aspartate and glycerol phosphate shuttles for superoxide production by the electron transport chain, and *2*) fuel superoxide production in the cytosol by NADH and NADPH oxidases. Major caveats to the interpretation of the data of Nishikawa et al. include: *1*) metabolic imbalances evoked by the inhibitors of pyruvate transport and the malate-aspartate shuttle that were not addressed, *2*) limitations inherent to the *in vitro* experimental paradigm employed, and *3*) observations by other investigators and in other paradigms that are inconsonant with the conclusions of Nishikawa et al.

Before considering these caveats in detail, it is important to appreciate that pyruvate is a multifunctional metabolite. It functions as an anti-oxidant in the cytoplasm as well as a fuel for ATP synthesis by oxidative phosphorylation in mitochondria. In mitochondria E&P are transferred from pyruvate to NAD^+m and FADm, reducing them to NADH and $FADHm_2$, respectively, that fuel synthesis of ATP by oxidative phosphorylation. (Electrons carried by NADHm and $FADHm_2$ also can fuel mitochondrial superoxide production as emphasized by

Nishikawa et al.) In the cytosol E&P are transferred from NADHc to pyruvate coupled to oxidation of NADHc to NAD^+c and reduction of pyruvate to lactate in the reaction catalyzed by lactate-DH (15) as depicted in Fig. 5; this reaction is critical to support rapid synthesis of ATP by substrate phosphorylation needed for physiological work. ATP can be synthesized twice as fast by substrate phosphorylation as by oxidative phosphorylation, but requires high levels of NAD^+c (40); this reaction also is vital for cell survival during hypoxia/ischemia since reoxidation of NADHc to NAD^+c is essential for continuing glycolysis and associated synthesis of ATP *via* substrate phosphorylation (Fig. 5). In addition, reoxidation of NADHc to NAD^+c by lactate-DH deprives NAD(P)H oxidases of fuel (electrons carried by NADHc) for synthesis of superoxide from O_2 (see section III). Pyruvate also prevents decreases in myocardial NADPH/$NADP^+$m and glutathione antioxidant ratios of GSH/GSSH evoked by low flow ischemia/reperfusion (87). Lastly, pyruvate can protect cells from ROS damage by stoichiometric degradation of H_2O_2 coupled to nonenzymatic oxidative decarboxylation of pyruvate (88-90).

The observations that: *1*) blocking glycolysis-derived pyruvate transport into mitochondria by 4-OHCA prevented the ~3-fold increase in superoxide production evoked by hyperglycemia, and *2*) oxidation of glucose-derived pyruvate *via* the TCA cycle was increased ~2.2-fold by hyperglycemia were viewed by Nishikawa et al. as strong evidence that "…the TCA cycle is the source of increased ROS-generating substrate induced by hyperglycaemia". They propose that the high protonic gradient-electrochemical potential supported by NADHm (generated by oxidation of pyruvate via the TCA cycle) augments superoxide production by the electron transport chain.

However, inhibition of increased superoxide production by 4-OHCA also is consistent with the possibility that increased superoxide production evoked by hyperglycemia is generated largely in the cytoplasm by extra-mitochondrial NADH and/or NADPH oxidases as discussed in Section III. These oxidases can be fueled largely or in part by free NADHc, formation of which is increased by: *1*) accelerated oxidation of sorbitol (levels of which were increased ~3-fold) by sorbitol-DH, and *2*) accumulation of lactate (glycolysis was increased 2.3-fold by hyperglycemia). (See caveats to accumulation of lactate in Section IV.*B*.3)

A major caveat to interpreting the effects of 4-OHCA in the experiments of Nishikawa et al. is that blocking transport of pyruvate into mitochondria will increase cytosolic pyruvate levels. The mass action effect of increased cytosolic pyruvate levels will drive oxidation of NADHc to NAD^+c by lactate-DH (Fig. 5), decreasing levels of NADHc. This will attenuate inhibition of GA3P-DH by NADHc and hyperglycemia-augmented metabolic pathways fueled by NADHc (directly and/or indirectly) including: *1*) cytosolic superoxide production by NADH and NADPH oxidases, *2*) mitochondrial superoxide production from cytosolic electrons transferred from NADHc to Complexes I and II in mitochondria *via* the malate-aspartate and glycerol phosphate electron shuttles, respectively, (Fig. 5), *3*) activation of NF-κB, *4*) increased triose phosphate levels, *5*) formation of triose phosphate-derived methylglyoxal-AGE, and *6*) activation of PKC by *de novo* synthesis of diacylglycerol. Inhibition of pyruvate transport into mitochondria also will limit ATP synthesis by oxidative phosphorylation fueled by glucose; and, accumulation of

ADP will activate phosphofructokinase to increase glycolysis (and lactate production) and associated synthesis of ATP by substrate phosphorylation.

This alternative interpretation of the effects of 4-OHCA is supported by observations of Halestrap and Denton (91) and by Bünger and Mallet (92). Halestrap and Denton reported that 4-OHCA (at 50% of the concentration used by Nishikawa et al.) increased pyruvate release from incubated rat epididymal fat pads 6-fold; they also observed that a derivative of 4-OHCA (α-cyanocinnamate) increased myocardial pyruvate content 4-fold and reduced lactate content 45% (indicative of an ~ 87% decrease in myocardial NADH/NAD^+c∝L/P). Bünger and Mallet reported that 4-OHCA (at concentrations similar to those used by Nishikawa et al.): *1*) increased pyruvate efflux 20-fold and lactate efflux 3-fold from working guinea pig hearts (an 85% reduction in lactate/pyruvate ratio), *2*) increased intracellular myocardial concentrations of lactate and pyruvate 2-fold and 4-fold, respectively, indicative of a ~50% decrease in NADH/NAD^+c∝L/P, *3*) increased the extracellular concentration of pyruvate 16-fold, and *4*) decreased $^{14}CO_2$ production from [U-^{14}C]glucose and by [1-14C]pyruvate by 50% without decreasing O_2 consumption.

Thus, inhibition of pyruvate transport into mitochondria by 4-OHCA has profound effects on NADH/NAD^+c∝L/P that could markedly attenuate both cytosolic and mitochondrial superoxide production. In view of these caveats, it is noteworthy that Nishikawa et al. did not examine the effects of 4-OHCA on lactate and pyruvate levels, NADH/NAD^+c∝L/P, energy metabolism, or glycolysis. Under the conditions of their experiments, increased superoxide production evoked by hyperglycemia could be fueled entirely by electrons generated in the cytoplasm that are carried by NADHc, rather than by electrons generated in mitochondria by oxidation of pyruvate. Electrons carried by NADHc can fuel superoxide production in the cytoplasm by extra-mitochondrial NAD(P)H oxidase(s) as discussed in Section III; in addition, they can be transferred from NADHc into mitochondria by electron shuttles to fuel superoxide production by the electron transport chain independent of mitochondrial oxidation of pyruvate.

To the extent that increased mitochondrial superoxide production is mediated by oxidation of pyruvate (fueled by increased production of pyruvate *via* glycolysis) as proposed by Nishikawa et al., addition of exogenous pyruvate would be expected to augment superoxide production and exacerbate adverse sequelae of hyperglycemia. On the contrary (see Section II.), addition of pyruvate (or an ARI) to the medium of incubated rat retinas prevents hyperglycemia-evoked elevation of triose phosphates (substrate for methylglyoxal-AGE formation and for synthesis of diacylglycerol and activation of PKC) (7); addition of pyruvate to the medium of incubated glomeruli also prevents hyperglycemia-evoked elevation of triose phosphate levels (6). Likewise, elevation of pyruvate levels prevents hyperglycemia-induced vascular dysfunction and elevated diacylglycerol levels in granulation tissue (20); and, increasing pyruvate levels (or addition of an ARI) prevents vascular dysfunction in retina and sciatic nerve evoked by 5 h of acute hyperglycemia in nondiabetic rats (22).

Furthermore, addition of pyruvate (as well as an ARI) prevents hyperglycemia-evoked increases in sorbitol levels, free NADHc, triose phosphates, and methylglyoxal-AGE in human

erythrocytes that lack mitochondria as a source of superoxide to inhibit GA3P-DH (19,46,93,94). Lastly, aortic superoxide production evoked by addition of 10 mM lactate to the incubation medium is markedly attenuated, dose dependently, by addition of pyruvate; and, lactate-evoked increases in granulation tissue blood flow are prevented by coadministration of pyruvate as well as by a highly selective SODmimic (95). [The SODmimic used does not interact with $^{\bullet}NO$, H_2O_2, or peroxynitrite and does not block $O_2^{\bullet-}$ synthesis by neutrophils (see discussion in ref. 1).]

In addition**,** Bassenge et al. (96) reported that pyruvate dose-dependently inhibited over 50% of ROS production in homogenates of normal guinea pig aorta, heart, and liver; pyruvate also inhibited over 50% of the increase in ROS production in post-ischemic hearts. In contrast, lactate markedly increased ROS production prior to ischemia and in post-ischemic hearts (and the increase in ROS production by lactate was dose-dependently inhibited by pyruvate). Pyruvate did not inhibit ROS production by xanthine oxidase, which supports the conclusion that the effect of pyruvate on ROS production was mediated by enzymatic reoxidation of NADHc (that fueled ROS production by NADHoxidase) rather than by nonenzymatic degradation of ROS.

Another very important caveat to the conclusion that mitochondrial superoxide production was fueled by oxidation of pyruvate, rather than by electrons generated in the cytosol and transferred from NADHc into mitochondria by the malate-aspartate shuttle, is the observation by numerous investigators that inhibition of the malate-aspartate shuttle by aminooxyacetate markedly increases cytosolic NADH/NAD^+c∝L/P while decreasing mitochondrial NADH/NAD^+m, respiration, and energy metabolism (97-102). The failure of aminooxyacetate to prevent increased superoxide production is consistent with a shift in superoxide production from mitochondria *via* the electron transport chain (fueled by electrons transferred from NADHc to NADHm by the malate-aspartate shuttle) to the cytosol *via* extra-mitochondrial NADH and NADPH oxidases (fueled by NADHc). The increases in NADH/NAD^+c∝L/P evoked by aminooxyacetate in these experiments (97-102) indicate that electrons carried by NADHc (generated by glycolysis and by oxidation of sorbitol by sorbitol-DH) were not transferred to pyruvate by lactate-DH fast enough to maintain a normal NADH/NAD^+c ratio. The mass action effect of the resulting increase in NADHc augments its oxidation by additional enzymes including extra-mitochondrial NADH and NADPH oxidases to generate superoxide (see Section III).

Thus, Kauppinen et al. (99) reported that aminooxyacetate: *1*) increased free NADH/NAD^+c∝L/P ~7-fold while decreasing free NADH/NAD^+m ~7-fold, *2*) inhibited the increase in respiration evoked by an uncoupler of oxidative phosphorylation by 60%, *3*) lowered the mitochondrial membrane potential (which was prevented by addition of pyruvate), and *4*) decreased the ATP/ADP ratio in guinea pig cerebral cortex synaptosomes. Safer et al. (97) observed that aminooxyacetate attenuated oxidation of glucose and lactate and markedly increased NADH/NAD^+c∝L/P (~22-fold) in normal perfused rat heart. Cheeseman and Clark (100) reported that aminooxyacetate: *1*) decreased oxygen consumption ~42%**,** *2*) decreased glucose oxidation *via* the TCA 35%, *3*) increased NADH/NAD^+c∝L/P 2.2-fold, and *4*) inhibited acetylcholine synthesis by rat forebrain synaptosomes. Fitzpatrick et al (98) found that

aminooxyacetate: *1*) decreased oxygen consumption ~ 40%, *2*) impaired oxidation of glucose and pyruvate, *3*) increased NADH/NAD^+c∝L/P and lactate efflux ~3-fold, and *4*) decreased ATP, PCr, malate, citrate, and aspartate levels in rat cerebral cortex slices. Barron et al. (101) observed that aminooxyacetate: *1*) increased NADH/NAD^+c∝L/P ~3-fold and glycolysis 1.5-fold, *2*) decreased glucose oxidation and oxygen consumption 42% and 21%, respectively, *3*) decreased levels of PCr, citrate, malate, and α-Ketoglutarate, and *4*) impaired contractile function of porcine carotid arteries. And, Eto et al. (102) reported that aminooxyacetate attenuated glucose-induced increases in: *1*) oxidation of glucose by the TCA cycle (50%) and in NAD(P)H autofluorescence (which largely reflects NADHm), *2*) mitochondrial membrane potential, *3*) ATP levels, and *4*) insulin secretion by islets of Langerhans that are deficient in G3P-DHm .

An additional important observation of Barron et al. (101) was that octanoic acid prevented all of the adverse effects of inhibition of the malate-aspartate shuttle in porcine carotid arteries by aminooxyacetate. This finding indicates that: *1*) mitochondrial energy metabolism fueled by E&P generated by oxidation of fatty acids is independent of energy metabolism fueled by E&P generated in the cytoplasm and transported into mitochondria by the malate-aspartate shuttle, and *2*) increased energy metabolism fueled by oxidation of fatty acids compensates for inhibition of glucose-fueled energy metabolism resulting from inhibition of the malate-aspartate shuttle.

Barron et al. concluded that the adverse effects of inhibition of the malate-aspartate shuttle apply specifically to impaired reoxidation of NADHc generated by metabolism of glucose in the cytoplasm. Thus, the adverse effects of aminooxyacetate attest to the importance of reoxidation of NADHc in the cytosol specifically by electron shuttle(s) that transfer E&P from NADHc into mitochondria (that fuel ATP synthesis directly and also promote oxidation of glycolysis-derived pyruvate by the TCA cycle for ATP synthesis) *vs* reoxidation of NADHc by lactate-DH coupled to transfer of E&P to pyruvate for efflux out of the cell as lactate (Fig. 5). This interpretation is in agreement with the view of other investigators regarding the importance of reoxidation of NADHc by the malate-aspartate shuttle for energy metabolism (97-100,102).

Although the magnitude of the effects of aminooxyacetate on NADH/NAD^+c∝L/P and energy metabolism varied somewhat in the different paradigms (97-102), NADH/NAD^+c∝L/P was consistently elevated and utilization of glycolytic-pyruvate for oxidative phosphorylation was impaired. Overall, these observations were viewed as evidence that reoxidation of NADHc by electron shuttles is critical for fueling mitochondrial energy metabolism by NADHc and pyruvate. Thus a plausible explanation for the failure of aminooxyacetate to prevent superoxide production in the experiments of Nishikawa et al. is that electrons accumulating in NADHc fueled superoxide production largely by extra-mitochondrial NAD(P)H oxidases.

An important role for electrons carried by $FADHm_2$ in fueling increased mitochondrial superoxide production is supported by the observations of Nishikawa et al. that inhibition of Complex II (by thenoyltrifluoroacetone-TTFA) prevented superoxide production and associated metabolic changes including activation of PKC and NF-κB and increases in AGE. In contrast, superoxide production was unaffected by inhibition of Complex I by rotenone. Rotenone

increases superoxide production in numerous cells and tissues by augmenting electron leakage at a superoxide generating site upstream from the rotenone-binding site. And, production of superoxide in Complex I during physiological forward electron transport is fueled by electrons carried by NADHm, rather than by $FADHm_2$. Thus, superoxide production in Complex I is fueled by NADHm formed by oxidation of pyruvate *via* the TCA cycle as well as by NADHm generated by transfer of electrons from NADHc to mitochondrial NAD^+m by the malate aspartate shuttle.

Recent observations indicate that (in the absence of respiratory chain inhibitors) superoxide generation supported by the Complex II substrate succinate is fueled by reverse electron transfer from Complex II to Complex I rather than to Complex III (103,104). However, the precise site of superoxide production in Complex I under physiological conditions is not entirely clear. Observations of and Kushnareva et al. (103) and Genova et al (105) suggest it is an iron-sulfur complex. On the other hand, Lambert and Brand (106) suggest that the major site of high superoxide production in Complex I during physiological forward electron transport is a quinone-reducing site downstream from FMN and low potential FeS centers. And, Starkov and Fiskum (107) propose that: *1*) the superoxide generating site of Complex I is in redox equilibrium with mitochondrial pyridine nucleotides, *2*) the degree of reduction of the superoxide generating site is modulated by the $NADH/NAD^+$ ratio as well as by the membrane potential ($\Delta\Psi$) of mitochondria respiring on NADH-dependent substrates, and *3*) metabolism of NADH-linked substrates can result in the formation of any TCA cycle intermediate including succinate.

The failure of rotenone to impact on ROS generation in the experiments of Nishikawa et al. could (conceivably) be explained by recent observations supporting an important role for alpha-ketoglutarate dehydrogenase (α-KGDH) in generating mitochondrial ROS under physiological conditions (108,109). ROS generation by α-KGDH as well as by Complex I is closely regulated by the $NADH/NAD^+m$ ratio. Thus, α-KGDH-mediated ROS production is markedly increased when oxidation of NADHm is limited by: *1*) availability of ADP for oxidative phosphorylation, or *2*) inhibition of any segment of the electron transport chain. And, ROS generation by α-KGDH is independent of ROS production by the electron transport chain including Complex 1. Thus, to the extent that ROS generation by α-KGDH contributed to hyperglycemia-evoked increased mitochondrial superoxide generation in the experiments of Nishikawa et al., it would not be impacted by rotenone.

Nishikawa et al. concluded that the TCA cycle is the source of increased ROS-generating substrate induced by hyperglycemia based on their observations that: *1*) hyperglycemia-evoked increased superoxide production was unaffected by aminooxyacetate, a well characterized inhibitor of the malate-aspartate shuttle which transfers electrons carried by NADHc to NADHm, *2*) increased superoxide production was prevented by inhibition of glycolysis-derived pyruvate transport into mitochondria by 4-OHCA, and *3*) glycolysis and flux through the TCA cycle were increased ~ 2-fold.

Although Nishikawa et al. noted that mitochondrial NADH and $FADH_2$ are generated by the TCA cycle from cytosolic pyruvate, they did not address the important implications of discordant

effects of rotenone and TFFA for fueling superoxide production by NADHm *vs* $FADHm_2$. Since ~80% of the electrons derived from oxidation of pyruvate by the TCA cycle are transferred to NAD^+m, reducing it to NADHm (15), the failure of rotenone to affect superoxide production suggests that the increase in superoxide was not fueled by pyruvate-derived NADHm and was not generated by Complex I.

And, since only 20% of electrons derived from oxidation of pyruvate are transferred to $FADHm_2$ (15), it is questionable whether this source of electrons alone would be sufficient to account entirely for the increase in superoxide production evoked by hyperglycemia. An additional potentially important source of electrons carried by mitochondrial $FADHm_2$ is the glycerol phosphate electron shuttle. In the first step of this shuttle E&P are transferred from free NADHc to DHAP, reducing it to G3P coupled to oxidation of NADHc to NAD^+c by G3P-DHc (see Fig. 5). E&P carried by G3P are then transferred to mitochondrial FAD^+m, reducing it to $FADHm_2$, by mitochondrial G3P-DHm (Fig. 5). Although flux of E&P from NADHc to mitochondrial FAD^+m is limited by G3P-DHm activity (which is very low in some tissues such as the heart under physiological conditions) and by G3P levels, it may be substantial when NADHc is elevated as in hypoxia and work (97). And, (we suggest) it may also be substantial when NADHc is elevated due to: 1) increased oxidation of sorbitol by sorbitol-DH, and 2) accumulation of lactate in the culture medium as demonstrated by Tilton et al. in Section IV.*B*.3.

The finding that sorbitol accumulation was increased 2.6-fold by hyperglycemia suggests that the ~3-fold increase in superoxide production observed by Nishikawa et al. could indeed be fueled largely by electrons transferred from sorbitol to NAD^+c (reducing it to NADHc) by sorbitol-DH. Electrons carried by this NADHc can be transferred to Complex II by the glycerol phosphate electron shuttle and generate superoxide in mitochondria; they also can be transferred to O_2 by extra-mitochondrial NAD(P)H oxidases (42,43) to form superoxide in the cytosol. Although Nishikawa et al. reported that an ARI prevented accumulation of sorbitol, they did not assess its effects on fructose levels or on superoxide production.

The ~ 2.2-fold increases in glycolysis (from 2.39 nmol glucose/mg protein/min to 5.47 nmol) and oxidation of glucose by the TCA cycle (from 0.084 nmol glucose/mg protein/min to 0.183 nmol) evoked by 30 mM glucose indicate that pyruvate was produced *via* glycolysis at a rate 30-fold faster than it was oxidized in mitochondria (at both 5 and 30 mM glucose). Presumably, most of this excess pyruvate was reduced to lactate (by lactate-DH) that would accumulate in the medium ~2.2-fold faster (resulting in corresponding 2.2-fold higher medium lactate levels) for cells incubated in 30 mM glucose than for cells in 5 mM glucose. Since lactate and pyruvate levels were not determined, it is possible that accumulation of lactate (see Section IV. *B.* 3) inhibited oxidation of NADHc by lactate-DH causing a further increase in NADHc in addition to the increase in NADHc formed by oxidation of sorbitol.

The importance attributed by Brownlee et al. to mitochondria as the singular source of glucose-induced superoxide production in aorta (78) and glomerular mesangial cells (85) also is discordant with observations of other investigators. Hua et al. (110) reported that CCCP (an uncoupler of oxidative phosphorylation), which prevented glucose-induced superoxide

production in the experiments of Nishikawa et al. (78) and Kiritoshi et al. (85), did not prevent glucose-induced superoxide production by rat glomerular mesangial cells. (CCCP, like 2,4-dinitrophenol, carries protons across the inner mitochondrial membrane. This uncouples ATP synthesis from electron transport so that while transfer of electrons from $NADH_m$ to O_2 proceeds normally, ATP is not formed because the proton-motive force across the inner mitochondrial membrane is dissipated (15).

In contrast, Hua et al. (110) reported that: *1*) hyperglycemia increased expression of the p47phox mRNA oligonucleotide of NAD(P)H oxidase, and *2*) hyperglycemia-evoked ROS production was prevented by diphenyleneiodonium (an inhibitor of NAD(P)H oxidase), by p47phox antisense mRNA oligonucleotide, and by catalase. Interestingly, Venugopal et al. (34) reported that CCCP inhibited hyperglycemia-evoked ROS production by human aortic endothelial cells, but not by a human monocytic cell line. In contrast, the hyperglycemia-evoked increase in ROS production by monocytic cells was mediated by PKC-α activation of extra-mitochondrial NAD(P)H oxidase activity.

Observations of Kim et al. (33,35) also support the greater importance of an extra-mitochondrial NAD(P)H oxidase (preferentially fueled by NADH) in aortic superoxide production in OLETF diabetic rats. And, as discussed in Section III, a growing body of evidence supports the importance of extra-mitochondrial NAD(P)H-dependent oxidases (and dysfunctional NO synthase) in fueling increased superoxide production in animals and humans with diabetes (28-37). Furthermore, increased ratios of cytosolic free $NADPH/NADP^+c$ and $NADH/NAD^+c$ in peripheral nerve early after the onset of diabetes (8,10) and evidence that inhibitors of NAD(P)H oxidase attenuate neural dysfunction in diabetic rats (111) are consistent with potentially important roles for both $NADPH_c$ and NADHc in fueling cytosolic superoxide production by NAD(P)H oxidases.

The observation that inhibitors of superoxide production prevented sorbitol accumulation in the experiments of Nishikawa et al. is surprising and the explanation remains enigmatic. The authors' explanation that superoxide production stimulated aldose reductase activity to overcome downregulation of aldose reductase activity by nitric oxide [based on observations of Chandra et al. (112)] is discordant with observations of other investigators (113-115). Barnett et al. (113) demonstrated that increased sorbitol production by rat lenses exposed to elevated glucose levels was inhibited by oxidative stress induced by hydrogen peroxide. Seo et al. (114) reported that nitric oxide donors upregulate aldose reductase activity and mRNA levels in rat aortic smooth muscle cells; in addition, cytokine-induced expression of aldose reductase in the 264.7 cell line of murine macrophages was dependent on increased nitric oxide production. And, Stevens et al. (115) reported that the antioxidant DL-a-lipoic acid increased sorbitol and fructose levels in peripheral nerve of diabetic rats.

Clearly, numerous additional studies are needed to clarify the source(s) and mechanism(s) of superoxide production and associated metabolic imbalances evoked by hyperglycemia in the experimental paradigm of Nishikawa et al. These include assessment of effects of: *1*) 4-OHCA on glycolysis and on oxidation of pyruvate by the TCA cycle, *2*) 4-OHCA on lactate and

pyruvate levels, triose phosphates, and the adverse sequelae evoked by hyperglycemia, *3*) hyperglycemia on lactate and pyruvate levels/production in cultured cells and culture medium, and *4*) aldose reductase inhibitors and addition of exogenous pyruvate on superoxide production and the other adverse sequelae of hyperglycemia observed. The numerous caveats to the interpretation of observations reported by Nishikawa et al. attest to the need for caution in extrapolation of their observations and conclusions beyond the experimental conditions of their *in vitro* paradigm. Similar concerns have been raised by other investigators re: "…. limitations of the *in vitro* model system used…. that represents an imperfect model for studying the complex systemic metabolic derangements characteristic of diabetes *in vivo*." (116).

Lastly, the greater importance attributed by Brownlee et al. to mitochondrial redox changes and superoxide production *vs* cytosolic redox changes and superoxide production in mediating hyperglycemic damage is discordant with observations in nerve, aorta, and kidney. Impaired electrophysiological nerve function in rats with diabetes of 4 months duration is prevented by inhibitors of the sorbitol pathway (an inhibitor of sorbitol-DH as well as an inhibitor of aldose reductase) that also prevent the associated increase in NADH/NAD^+c, but not the associated increase in mitochondrial NADH/NAD^+m (10). Gupta et al. (117) reported that an inhibitor of aldose reductase completely prevented endothelium-dependent increased superoxide production as well as superoxide-mediated inhibition of Na^+-K^+-ATPase activity evoked by hyperglycemia in rabbit aortic rings. In contrast, increased superoxide production was greatly augmented by an inhibitor of Cu,Zn SOD, the cytosolic isoform of SOD. And, renal changes evoked by diabetes of 4 and 8 months duration were substantially attenuated in transgenic mice overexpressing Cu,Zn SOD (118,119).

Du et al. (84) - Based on their evidence that hyperglycemia-evoked increased superoxide production inhibited GA3P-DH activity (79) and that the inhibition of GA3P-DH activity was normalized by an inhibitor of the hexosamine pathway, Du et al. performed additional experiments to elucidate the mechanism(s) of inhibition of GA3P-DH and the consequences of inhibition of the enzyme. In bovine aortic endothelial cells incubated in 5 mM glucose, GAP-DH antisense oligonucleotides activated PKC, NF-κB, hexosamine pathway flux, and AGE formation to the same extent as 30 mM glucose. These changes were interpreted as evidence that corresponding changes evoked by hyperglycemia are mediated by inhibition of GA3P-DH (which they attributed to increased superoxide generated in mitochondria based on the report by Nishikawa et al. (78)).

PJ34 (an inhibitor of poly(ADP-ribose) polymerase), increased expression of MnSOD, and increased expression of uncoupling protein-1 all prevented 30 mM glucose-evoked: *1*) decreased GA3P-DH activity, and *2*) increases in GA3P-DH ribosylation, intracellular AGEs, activation of PKC, NF-κB, and hexosamine pathway activity. Increased expression of MnSOD and increased expression of uncoupling protein-1 also prevented hyperglycemia-evoked increases in DNA strand breaks and incorporation of radiolabeled NAD^+ into protein. Inhibition of GA3P-DH by 30 mM glucose was attributed to poly(ADP-ribosyl)ation of GA3P-DH by PARP *via* DNA strand breaks caused by overproduction of superoxide in mitochondria. Caveats regarding the

proposed role of mitochondrial superoxide production in meditating these changes have been addressed above.

GA3P-DH activity can be inhibited by a number of covalent modifications, as noted by Du et al., as well as by GAP-DH antisense oligonucleotides. In addition, GA3P-DH activity is dependent on the redox state of its cofactor, free cytosolic NADc; an increase in the ratio of free NADH/NAD^+c inhibits GA3P-DH activity. This metabolic inhibition (manifested by an increase in triose phosphate levels) is rapidly reversed by addition of pyruvate and/or by blocking reduction of NAD^+c to NADHc by sorbitol-DH (see Section II). Du et al. elected not to investigate the sorbitol pathway stating: "Because aldose reductase activity is extremely low in aortic endothelial cells, this pathway was not investigated." No reference was cited in support this unfounded assertion. On the contrary, Nishikawa et al. (78) reported that sorbitol levels in the same cell line used by Du et al. were increased 2.6-fold by 30 mM glucose as discussed above. Metabolite levels (e.g. sorbitol) are not a reliable parameter of their rates of synthesis and further metabolism. Thus, rates of reduction of glucose to sorbitol by aldose reductase and subsequent reduction of NAD^+c to NADHc by sorbitol-DH could be increased much more than 2.6-fold. Nishikawa et al. did not measure sorbitol or fructose (a more reliable parameter of reduction of NAD^+c to NADHc) in the incubation medium (78).

Brownlee (80) asserted that the hyperglycemia-evoked increase in the ratio of cytosolic free NADH/NAD^+c: "…reflects a marked decrease in the absolute concentration of NAD^+ as a result of consumption by activated PARP, rather than reduction of NAD^+ to NADH". In support of this assertion, Brownlee cited the observations of Soriano et al. (120) on the impact of hyperglycemia on total NAD^+t (which includes bound and free NAD^+ in the cytosol and in mitochondria), rather than on free cytosolic NADH/NAD^+c, in cultured cells.

There are numerous caveats to this assertion, including published reports discordant with the observations of Soriano et al. Before addressing these caveats, however, it is important to point out that assessment of cytosolic free NADH/NAD^+c with the redox metabolite indicator method is based on the near-equilibrium between the ratios of free NADH/NAD^+c and L/P maintained by lactate-DH (41) as depicted in equation 1 in the Main Text. (Free NADH/NAD^+c$\propto$L/P in a wide spectrum of normal cells and tissues is in the range of ~1 to $2x10^{-3}$ [which corresponds to free NAD^+/NADHc$\propto$P/L ratios of 500 to 1000]. In contrast, the ratio of total cytosolic NADH/NAD^+t in liver is ~100-fold higher, i.e. $139x10^{-3}$ [41]. And, the ratios of free and total mitochondrial NADH/NAD^+m in liver are ~$132x10^{-3}$ and $454x10^{-3}$, respectively.)

At a free NAD^+/NADHc ratio of 500 (500 moles of NAD^+/1 mole of NADHc), oxidation of only 2 moles of NAD^+c to NADHc will decrease the molar ratio of free NAD^+/NADHc to 166 (498/3); and, the decrease in NAD^+ from 500 to 498 is too small to measure directly. The ~3-fold decrease in NAD^+/NADHc from 500 to 166 corresponds to a 3-fold increase in free NADH/NAD^+c from 0.002 to 0.006. The magnitude of these changes in free NADH/NAD^+c corresponds to changes observed in diabetic animals.

While increased consumption of NAD by activation of PARP could contribute to an increase in free NADH/NAD^+c$\propto$L/P, free NADH/NAD^+c also is increased (independent of total NAD) by

limiting levels of oxidized substrates (e.g. pyruvate, DHAP, and oxygen) of enzymes (lactate-DH , G3P-DH, and NAD(P)H oxidases, respectively) that oxidize NADHc to NAD^+c coupled to reduction of pyruvate to lactate, DHAP to G3P, and oxygen to superoxide (see Sections II and III). And, observations discussed in Section III support the possibility that NADHc (generated by oxidation of sorbitol by sorbitol-DH) fuels the increased superoxide production (in mitochondria and by extra-mitochondrial NAD(P)H oxidases) that damages DNA to activate PARP.

Soriano et al. (120) reported that incubation of mouse pulmonary endothelial cells in 30 mM glucose for 24 h decreased concentrations of: *1*) total NAD^+t and total $NADP^+t$ by ~50%, *2*) total NADHt by ~28%, *3*) total NADPHt by 62%, and *4*) ATP levels by ~40%. 30 mM glucose increased PARP activity 2-fold (quantified by consumption of NAD^+) in mouse cells and in human umbilical vein endothelial cells (HUVEC); 30 mM glucose also activated NF-κB, but decreased eNOS activity by 40% in mouse cells. An inhibitor of PARP: *1*) substantially attenuated all of these effects of 30 mM glucose (with the exception of the decrease in ATP levels and activation of NF-κB) in mouse cells, and *2*) prevented the increased consumption of NAD^+ by HUVEC. The effects of 30 mM glucose on activation of PARP also were substantially attenuated: *1*) by SOD and inhibitors of nitric oxide synthase, and *2*) in cells from PARP-deficient mice. Soriano et al. (121) have reported similar changes in pyridine nucleotide and ATP levels in thoracic aorta of mice with streptozotocin-induced diabetes of 4 weeks duration after incubation in vitro for 1 hour.

In marked contrast, Asahina et al. (122) reported that total NAD^+t, NADHt, NADPHt, and ATP levels in HUVEC incubated in 33 mM glucose for 4 to 6 days did not differ from values in control cells incubated in 5 mM glucose. ($NADP^+t$ levels were not assessed). Lactate production (which is dependent on recycling of $NAD^+c \rightleftharpoons$ NADHc *via* glycolysis as discussed in Section II) was unaffected by 33 mM glucose. Asahina et al. also demonstrated that exposure of cells to 200 μmol H_2O_2 /l for 1 h markedly decreased the levels of all of these nucleotides and also decreased lactate production. (Lactate production was not assessed by Soriano et al.). Total NADH/NAD^+t was unaffected by 33 mM glucose; however, the ratio was increased by H_2O_2 (much more so in cells incubated in 33 mM glucose [2.5-fold] than in 5 mM glucose [1.5-fold]).

NADH/NAD^+t in cells incubated in media containing ~5 mM glucose was similar in the experiments of Soriano et al. and Asahina et al. (~14 and 8.8, respectively). However, in cells incubated in high glucose the ratio decreased 40% in the experiments of Soriano et al. (due to the 50% decrease in NAD^+t ($P < 0.05$), but only 8% (ns) in the experiments of Asahino et al.

The marked decreases in pyridine nucleotide and ATP levels evoked by high glucose levels observed by Soriano et al. in cultured cells and in incubated aorta may be attributable (at least in part) to increased free radical production catalyzed by metal ions (including Cu, Zn, and Fe) that is exacerbated by elevated glucose levels as discussed in Section IV.*B*.2. (The incubation medium used by Soriano et al. contained equal parts F12 [which is known to contain substantial amounts of the above metals] and DMEM; the medium used by Asahina et al. was MEM which does not contain substantial amounts of these metals.)

Another important difference in the experiments of Soriano et al. and Asahina et al. is revealed when NAD^+t levels are converted to the same units (either pmol NAD^+t/μg DNA or pmol NAD^+t/mg protein) using conversion factors given in the Main Text. Control cells of Asahina et al. contain over 200-fold more NAD^+t than control cells of Soriano et al. (i.e. 78.4 pmol NAD^+t/μg DNA *vs* 0.36 pmol NAD^+t/μg DNA, respectively). This raises more questions regarding the reliability of methods of measurement of NAD^+, suitability of cells utilized as in vitro paradigms of diabetes, etc. Total NAD^+t levels in normal rat retinas reported by Graymore and Towlson (25) contained ~50-fold more NAD^+t than the control mouse pulmonary endothelial cells of Soriano et al.

The much lower NAD^+ levels in mouse pulmonary endothelial cells *vs* HUVEC and whole retina, and the high rates of NAD^+ consumption by mouse pulmonary endothelial cells, raise concerns re: the suitability of their paradigm for addressing the impact of hyperglycemia on PARP activation. NAD^+ was consumed at a rate of ~ 40 pmol NAD^+/min/mg protein in control cells and was increased to ~ 90 pmol NAD^+/min/mg protein by 30 mM glucose. Since the total content of NAD^+ was 99.1 pmol NAD^+/mg protein in control cells and was decreased to ~ 50% to 48.4 pmol NAD^+/mg protein by 30 mM glucose, the entire content of NAD^+ was consumed (and synthesized) every ~2.5 min and every ~0.6 min in control cells and high glucose cells, respectively. The finding that the total NAD^+t was decreased by ~50% in cells exposed to 30 mM glucose indicates that the cells were unable to synthesize NAD^+ fast enough to maintain 'normal NAD^+ levels'. Whether or not the 50% reduction in total NADt by 30 mM glucose would limit reoxidation of free NADHc to NAD^+c would depend on availability of oxidized substrates (e.g. pyruvate, oxygen, DHAP, of enzymes that reoxidize NADHc to NAD^+c as discussed above. Interestingly, the rates of NAD^+ consumption observed by Soriano et al. for mouse pulmonary endothelial cells were ~6-fold and 7-fold higher than for HUVEC at 5 and 30 mM glucose, respectively; and, the NAD^+ content of HUVEC was not decreased by 30 mM glucose in the experiments of Asahina et al.

The observations of Soriano et al. that hyperglycemia markedly decreased NAD^+t and ATP levels also are inconsistent with numerous observations in diabetic rats: *1*) Heath et al. (16) reported that both total retinal NAD^+t and NADHt were increased in diabetic rats while total $NADP^+t$ was unchanged and total NADPHt was decreased by only 21%, *2*) Graymore and Towlson (25) reported that total retinal NAD^+t and $NADP^+t$ were unchanged in rats with diabetes of 10 weeks duration; total NADHt was increased ~54%, and total NADPHt was decreased 21%, *3*) Kern et al. (123) reported that ATP levels were not decreased in retinas of rats with diabetes of 2 months duration, *4*) Low et al. (124) reported that ATP levels were not decreased in sciatic nerve of rats with diabetes of 1 or 4 months duration, and *5*) Greene and Winegrad (125) reported that: (*a*) ATP levels did not differ in sciatic nerve fascicles and endoneurial preparations from control rabbits and from diabetic rabbits (14 days duration) incubated in 20 mM glucose and 5 mM glucose, respectively, for a 10 min equilibration period or for 120 min, and (*b*) lactate production was increased 1.9-fold at 20 mM glucose and 1.5-fold at 5 mM glucose in preparations from diabetic rabbits *vs* controls.

Furthermore, hyperglycemia increases free NADH/NAD^+c in human erythrocytes (19,46) that lack nuclei (increased nuclear DNA strand breaks caused by increased free radicals are proposed to activate PARP that consumes NAD^+) and mitochondria (the site proposed by Brownlee as the exclusive source of increased superoxide formation evoked by hyperglycemia as discussed in Section IV.*C*.5). And, inhibition of aldose reductase prevents increases in erythrocyte NADHc/NAD^+c evoked by hyperglycemia (19).

The discordant observations regarding changes in pyridine nucleotide and ATP levels evoked by hyperglycemia *in vitro* and by experimental diabetes in animals may reflect species differences (mice, rats, rabbits, humans), differences in severity of diabetes, cell types etc. Additional experiments are needed to elucidate the relationships between PARP activation and increased flux of glucose *via* the sorbitol pathway. It would be important to: *1*) ascertain the impact of PARP activation on the ratio of free NADH/NAD^+c$\propto$L/P in cultured cells and in diabetic mice, *2*) assess the impact of inhibitors of aldose reductase and sorbitol-DH on PARP activation, and *3*) examine the possible impact of accumulation of lactate in the incubation medium on pyridine nucleotides and ATP levels in cultured cells.

ATP synthesis may not be compromised by a substantial decrease in free NAD^+/NADHc$\propto$P/L (corresponding to an increase in free NADH/NAD^+c$\propto$L/P) *per se*, since ATP can be synthesized twice as fast by substrate phosphorylation (coupled to redox cycling of free NAD^+c$\rightleftharpoons$NADHc *via* glycolysis) *vs* oxidative phosphorylation (40). This possibility is supported by the data in Tables 1 and 2 as discussed in Section II. ATP levels were increased 1.6-fold by 30 mM glucose (Table 2) despite a 2-fold increase in NADH/NAD^+c$\propto$L/P (Table 1).

Lastly, data presented in the Main Text and discussed in Section II, support the conclusion that the hyperglycemia-evoked increase in free NADH/NAD^+c *via* the sorbitol pathway is not the consequence of a putative decrease in free NAD^+c. The increase in NADH/NAD^+c evoked by hyperglycemia at 36 torr in Fig. 1 was prevented by an aldose reductase inhibitor that had no impact on the associated increases in lactate production indicative of reduction of ~100x more NAD^+c (to NADHc) *via* glycolysis than was reduced by sorbitol-DH (Fig. 2). As discussed above and in Section II, the reason the 100-fold smaller amount of NADHc generated by oxidation of sorbitol by sorbitol-DH increases free NADH/NAD^+c$\propto$L/P is that the sorbitol pathway (in contrast to glycolysis) does not produce pyruvate needed for oxidation of NADHc to NAD^+c by lactate-DH.

Hammes et al. (60) reported that activation of transketolase by benfotiamine (a lipid-soluble derivative of thiamine) prevented hyperglycemia-evoked increases in intracellular AGE and hexosamine formation as well as activation of the diacylglycerol-PKC pathway and NF-κB in bovine aortic endothelial cells. All of these changes evoked by hyperglycemia were attributed to increased levels of glycolytic metabolites (triose phosphates and F6P, see Fig. 5) caused by inhibition of GA3P-DH by superoxide produced in mitochondria. Activation of transketolase, which converts GA3P and F6P into pentose phosphates and other sugars, was postulated to reduce the levels of triose phosphates and F6P. None of these metabolites, however, was measured. Nor did the authors measure lactate and pyruvate or assess effects of ARI or SDI to

address the possibility that inhibition of GA3P-DH was caused by an increase in NADHc generated by the sorbitol pathway or by lactate accumulation, rather than by superoxide generated in mitochondria. At the present time suppression of glucose-evoked accumulation of triose phosphates by thiamine apparently has been demonstrated only in human erythrocytes that lack mitochondria (59).

Regardless of whether GA3P-DH is inhibited by NADHc *per se* or by superoxide, the beneficial effects of lowering triose phosphates by activating transketolase are consistent with evidence that elevated levels of triose phosphates fuel production of AGE and *de novo* synthesis of diacylglycerol to activate PKC. On the other hand, reduction of triose phosphate levels by activation of transketolase would not be expected to prevent: *1*) increases in free NADHc that fuel production of superoxide by NADH oxidase (42,43) in the cytosol, or *2*) increased production of superoxide in mitochondria. In contrast, ARIs prevent both the increase in NADHc and the accumulation of triose phosphates evoked by hyperglycemia in human erythrocytes and in normoxic and hypoxic rat retina (7,19; Figs. 1 and 3 in Main Text).

Observations of Hammes et al. (60), coupled with those of La Selva et al. (126), raise additional doubts regarding the proposed role of increased mitochondrial superoxide production for inhibition of GA3P-DH. Hammes et al. reported that benfotiamine did not attenuate increased superoxide production or inhibition of GA3P-DH evoked by hyperglycemia. La Selva et al. (126) reported that thiamine largely abolished hyperglycemia-evoked 1.5 to 2-fold increases in lactate production by cultured bovine retinal endothelial cells and human umbilical vein endothelial cells. Since both benfotiamine and thiamine activate transketolase, benfotiamine also would be expected to inhibit glycolysis. This would attenuate increased mitochondrial superoxide production fueled by glycolysis-derived pyruvate as well as inhibition of GA3P-DH. The failure of benfotiamine to block superoxide production and inhibition of GA3P-DH suggests that increased NADHc formed by the sorbitol pathway is largely responsible for inhibition of GA3P-DH and associated adverse sequelae of hyperglycemia as discussed in the Main Text.

The marked decrease in glycolysis evoked by thiamine suggests another potentially important adverse effect of chronic transketolase activation, i.e. decreased glycolysis-coupled synthesis of ATP *via* substrate phosphorylation and decreased production of pyruvate for ATP synthesis by oxidative phosphorylation. Hammes et al. did not assess the effects of benfotiamine on glycolysis or on energy metabolism *in vivo* or *in vitro*. Nor did they examine whether inhibitors of the sorbitol pathway or addition of pyruvate could prevent hyperglycemia-evoked increases in hexosamine activity.

While the reduction in frequency of acellular capillary segments observed by Hammes et al. in retinas of diabetic rats given benfotiamine could be important, the significance of this observation is unclear. The staining characteristics of capillaries in untreated diabetic rats and those given benfotiamine appear to be similar; however, their appearance differs somewhat from that of control rats. This suggests potentially adverse chemical changes in capillary structural components mediated by: *1*) metabolic imbalances that were unaffected by benfotiamine

treatment, e.g. persistent elevation of NADHc formed *via* the sorbitol pathway and increased production of ROS, and/or *2*) impaired energy metabolism due to impaired glycolysis.

6. Winkler et al. (127) conducted experiments to independently test the hypothesis of Van den Enden (8) that elevated glucose levels cause a hypoxia-like redox imbalance (an increase in $NADH/NAD^+c \propto L/P$) in incubated retinas. They concluded: "… elevation of glucose levels in incubation media neither mimics the effects of hypoxia (in incubated retinas) nor represents initiating mechanisms of the complications of diabetic retinopathy".

Paradoxically, while the stated intent of their experiments was to test the hypothesis of Van den Enden et al., Winkler et al. did not measure any of the key metabolites required to test the hypothesis. They did not measure retinal pyruvate levels requisite to estimate $NADH/NAD^+c \propto L/P$, nor did they measure fructose, or assess the effects of an inhibitor of aldose reductase. And, they were unable to detect sorbitol in retinas incubated in 5 or 30 mM glucose. These and other caveats to their experiments and conclusions are addressed in the Main Text and in Letters to the Editor (128,129).

D. General caveats to in vivo paradigms of diabetic complications

In vivo, like *in vitro*, paradigms for assessing the role of free NADHc in mediating complications of diabetes also are fraught with caveats. Discordant observations from different laboratories may relate to: *1*) differences in duration and severity of diabetes, *2*) caveats inherent to methods for assessing blood flow, *3*) limitations of pharmacological interventions due to unfavorable pharmacokinetics, low potency, lack of specificity, compensatory reactions, etc., and *4*) tissue differences in metabolic imbalances that mediate complications of diabetes.

Discordant reports regarding whether blood flow is increased or decreased in retina and peripheral nerve appear to be related largely to methodological issues. When peripheral nerve blood flows are assessed by methods that require surgical exposure or manipulation of the nerve, flows are almost invariably observed to be decreased ~ 50% regardless of the duration of diabetes (even after acute hyperglycemia of a few hours duration in nondiabetic rats) and are interpreted as evidence of hypoxia/ischemia that could increase NADHc. In contrast, when assessed with radiolabeled particulate microspheres or plasma soluble tracers that do not require exposure of the nerve, blood flows are typically increased early after the onset of diabetes. (Caveats to surgical exposure of nerves for blood flow measurements as well as the use of particulate microspheres are addressed in refs. [130,131].) Likewise, most reports of decreased retinal blood flows early after the onset of diabetes are based on indirect methods such as fluorescein angiography (see caveats in ref. 132), whereas retinal blood flows are typically increased when assessed with radiolabeled particulate microspheres or plasma soluble tracers.

Reports of diabetes-induced impairment of vascular responses to vasoactive agents are often based on comparison of percent changes in blood flow or in vessel diameters in diabetics *vs* controls without any volumetric determination of baseline flows. To the extent that baseline flows/vessel diameters are increased closer to their maximum in the diabetic, the percent increase in response to a vasodilating drug will be attenuated ("impaired") *vs* controls.

E. **Caveats to published *in vivo* studies**

1. Obrosova et al. (63) reported that increases in retinal free NADH/NAD^+c and NADH/NAD^+m in rats with diabetes of 3 weeks duration were not prevented by treatment with SDI-157 (an inhibitor of sorbitol-DH) initiated at the onset of diabetes. (SDI-157 is a biologically inactive pro-drug that is hydroxylated *in vivo* to form the active compound SDI-158 [133].) In contrast, administration of sorbinil (an ARI) for 2 weeks, initiated after 4 weeks of untreated diabetes, reversed increases in retinal free NADH/NAD^+c and NADH/NAD^+m. There are numerous caveats to the authors' conclusion that "Hypoxia-like metabolic changes in the diabetic retina originate from aldose reductase, but not sorbitol-DH activity."

Important caveats to this conclusion of Obrosova et al. are: *1*) marked differences in the experimental protocols for assessing the effects of SDI-157 and sorbinil, *2*) the assumption that retinas in diabetic rats were hypoxic due to decreased blood flow (blood flows were not measured; and, see interventions with the vasodilators prazosin and *DL*-α-lipoic acid below), *3*) the very short half-lifes of SDI-157 and SDI-158 *vs* sorbinil (< 0.5 h *vs* 5 to 6 h) (ref. 133 and personal communication from PJ Oates), and *4*) evidence from other investigations that the dose of SDI-157 used (100 mg/kg body wt/day) was too small to ensure continuous inhibition of sorbitol-DH despite normalization of retinal fructose levels at the time retinas were removed (9,133,134). Since fructose readily diffuses out of cells, the normalization of *in vivo* retinal fructose levels observed by Obrosova et al. cannot be equated with normalization of flux of glucose to fructose and associated formation of NADHc (although the observation supports the likelihood that flux is decreased).

Neither sorbinil nor SDI-157 affected the ~5-fold increase in retinal glucose levels (substrate for aldose reductase) in diabetic rats. In contrast, sorbinil prevented the ~11-fold increase in sorbitol levels (substrate for sorbitol-DH) observed in untreated diabetic rats whereas SDI-157 increased sorbitol levels another 2.6-fold. Thus, retinal sorbitol levels in SDI-157-treated diabetics were ~30-fold higher than in sorbinil-treated diabetics. If SDI-157 levels fell intermittently (due to it's very short half-life) to concentrations that did not completely inhibit sorbitol-DH, the markedly elevated sorbitol levels observed when sorbitol-DH was more completely inhibited by high levels of SDI-157 could drive oxidation of sorbitol to fructose coupled to formation of free NADHc even faster than in untreated diabetics.

Transient increases in NADHc formation during intermittent increased oxidation of sorbitol to fructose are analogous to the effects of intermittent hyperglycemia (e.g. postprandial hyperglycemia) or hypoxia; intermittent increases in NADHc may be more deleterious than constantly elevated rates of NADHc formation associated with stable hyperglycemia. This possibility is supported by evidence that the adverse effects of intermittent hyperglycemia are more pronounced than stable hyperglycemia on production of ROS, activation of PKC, increased expression of NAD(P)H oxidase, and apoptosis in cultured human umbilical vein endothelial cells (36). And, brief periods of hypoxia (1 to 4 h/day) increase proliferation of cultured endothelial cells and reduce structural vascular resistance of retinal vessels almost as effectively as continuous hypoxia (135). Thus, great care must be taken when using drugs with a very short

half-life (i.e. SDI-157 and SDI-158) to ensure that: *1*) both food and water are available at all hours of the day and night to ensure continuous availability of the drug, and *2*) the drug is administered in sufficient amounts to achieve a very high degree of uninterrupted inhibition of sorbitol-DH.

These considerations explain why (in initial experiments by Tilton et al. [9]) SDI-157 (the prodrug) *increased* retinal blood flow in control rats when given at the same dose administered to diabetic rats by Obrosova et al. (100 mg/kg/day); in contrast, elevated retinal blood flows in diabetic rats (given 50 or 150 mg of SDI-157/kg body wt/day) were reduced by 50% and increased albumin leakage was reduced by 70% (9). In subsequent experiments, a higher dose of the active form of SDI-157 (200 mg SDI-158/kg/day for 6 weeks) prevented the increased vascular albumin permeation in diabetic rats (despite a 4-fold increase in retinal sorbitol levels) as well as an ARI, but did not increase retinal albumin permeation in control rats (136). Corresponding dose-dependent efficacy of SDI and ARI is observed for prevention of peripheral nerve electrophysiological dysfunction in diabetic rats (133,134).

Obrosova et al. included 2 additional experiments to assess effects of a vasodilator drug (prazosin) and an antioxidant (*DL*-α-lipoic acid) on diabetes-induced increases in retinal free NADH/NAD^+c and NADH/NAD^+m that might be caused by decreased retinal blood flow/ischemia. Diabetic rats were treated from the onset of diabetes with prazosin for 3 weeks or with *DL*-α-lipoic acid for 6 weeks at which time redox parameters were assessed.

Prazosin ameliorated, and *DL*-α-lipoic acid prevented, increases in retinal free NADH/NAD^+c∝L/P and NADH/NAD^+m in diabetic rats. However, since retinal blood flow was not measured, these observations do not demonstrate that the beneficial effects of prazosin and α-lipoic acid on redox changes were mediated by increased blood flow and amelioration of hypoxia. The finding that lipoic acid prevented mitochondrial and cytosolic redox changes is not surprising since *D*-α-lipoate is reduced to dihydrolipoate by dihydrolipoamide-DH coupled to oxidation of NADHm to NAD^+m *in vitro* independent of blood flow (137,138).

Prevention of increases in free NADH/NAD^+c and NADH/NAD^+m by prazosin also could be mediated by mechanisms independent of blood flow and tissue oxygenation, i.e. prazosin prevents increased lipid peroxidation and impaired energy metabolism evoked by free radicals and it attenuates oxidation of free fatty acids which increases NADH/NAD^+m (see discussion and refs. cited in refs. 68,70). Prazosin also attenuates increased oxidation of [1-^{14}C] palmitate to CO_2 evoked by norepinephrine in cultured rat hepatocytes (139,140). And, prazosin ameliorates increases in myocardial malondialdehyde levels and decreased levels of ATP and ADP (while increasing AMP and CrP levels) evoked by H_2O_2 in perfused rat hearts (141). In addition, prazosin attenuates impaired myocardial SOD activity and increased levels of peroxidized lipids (142) when given *in vivo* to rats with myocardial infarcts.

Furthermore, the likelihood that increases in retinal NADH/NAD^+m and in NADH/NAD^+c in the 3 week preventative study with SDI-157 are mediated by increased oxidation of *β*-hydroxybutyrate (and/or fatty acids), which increases free NADH/NAD^+m (68,70), is supported by evidence that: *1*) sciatic nerve *β*-hydroxybutyrate levels were increased 7-fold in a

corresponding 3 week study by Obrosova et al. of the effects of SDI-157 on redox changes in sciatic nerve of diabetic rats (143) (see below), *2*) retinal glucose uptake is impaired by elevated plasma ketone levels (consistent with increased oxidation of ketones) (144), and *3*) retinas from diabetic rats oxidize radiolabeled palmitate at a rate ~1.7-fold higher than control rats (144).

2. Obrosova et al. (143) reported that the prodrug SDI-157, given at the same low dose as in the previous study in the retina (63), had no impact on increases in sciatic nerve NADH/NAD^+c and NADH/NAD^+m in rats with diabetes of 3 weeks duration. All of the caveats addressed in the retinal study (most importantly the low dose and the very short half-life of SDI-157) also apply to these observations in sciatic nerve. In addition, although retinal fructose levels in diabetic rats given SDI-157 were not significantly different from those in nondiabetic controls in the previous study (63), fructose levels in sciatic nerve of diabetic rats given SDI-157 were 1.4-fold higher than in controls ($P < 0.05$); this implies at least an equimolar 1.4-fold higher rate of NADHc formation (*vs* controls) *via* oxidation of sorbitol by sorbitol-DH.

In addition to assessing mitochondrial matrix free NADH/NAD^+m based on the ratio of glutamate/α-ketoglutarate as in the retinal study (63), sciatic nerve mitochondrial cristae NADH/NAD^+m also was assessed based on the ratio of β-hydroxybutyrate/acetoacetate. Both ratios were increased ~ 40% and neural concentrations of β-hydroxybutyrate and acetoacetate were increased 7-fold and 4.5-fold, respectively ($P < 0.01$ for both), in untreated diabetic rats *vs* controls. The 7-fold increase in β-hydroxybutyrate levels attests to significant ketosis and the ~40% increase in mitochondrial cristae NADH/NAD^+m is consistent with increased oxidation of β-hydroxybutyrate coupled to reduction of NAD^+m to NADHm. The increase in NADH/NAD^+m may, like hypoxia, impair transfer of E&P from NADHc to NAD^+m *via* the malate-aspartate and glycerol phosphate electron shuttles causing NADH/NAD^+c to increase; and, SDI-157 would not impact on the redox change induced by oxidation of β-hydroxybutyrate. Thus, the increases in neural NADH/NAD^+m and NADH/NAD^+c in these diabetic rats may be mediated by increased oxidation of β-hydroxybutyrate and oxidation of sorbitol by sorbitol-DH, respectively, rather than by putative decreased blood flows (which were not measured).

The plausibility of this explanation is supported by observations that impaired nerve conduction in rats with diabetes of 4 months duration was associated with increases in cytosolic NADH/NAD^+c and mitochondrial NADH/NAD^+m. Inhibitors of the sorbitol pathway prevented impaired nerve conduction and the associated increase in cytosolic NADH/NAD^+c∝L/P but did not attenuate the associated increase in mitochondrial NADH/NAD^+m (10).

3. Cameron et al. (145) reported that a ~50% reduction in sciatic nerve blood flow and a ~20% reduction in motor nerve conduction velocity in rats with diabetes of 8 weeks duration were reversed by treatment during the last 2 weeks with an ARI (WAY-121509); in contrast, they were unaffected by an SDI (WAY-135706, also known as SDI-158, the active form of the prodrug SDI-157) given at a dose of 250 mg/kg body wt/day. Since both the ARI and the SDI markedly decreased neural fructose levels, the authors concluded "…ARIs have beneficial actions on nerve perfusion and conduction in experimental diabetes, whereas SDIs lack these effects." Although neural fructose levels were markedly decreased by SDI-158, they were still

elevated 2-fold *vs* the ARI-treated group ($P < 0.001$) attesting to incomplete inhibition of sorbitol-DH with a continuing 2-fold higher rate of reduction of NAD^+c to NADHc by sorbitol-DH. This finding is not surprising since nerve sorbitol levels (substrate for sorbitol-DH) were 320-fold higher in SDI than in ARI-treated rats, whereas nerve glucose levels (substrate for aldose reductase) were unaffected by either drug. In addition, the half-life of SDI-158 is less than 0.5 h *vs* 3-4 h for WAY-121509 in rats (133,146). Thus continuous inhibition of enzyme activity was much more likely achieved for aldose reductase than for sorbitol-DH.

Since we have observed that CP-166,572 (also known as SDI-158) and Sorbinil (an ARI) both prevented impaired nerve conduction in rats with diabetes of 4 months duration (10), we repeated the experiment of Cameron et al. using their identical protocol except that zopolrestat was used as the ARI. Two weeks after initiating treatment, the motor nerve conduction deficit was almost normalized by the ARI (it was decreased only 4.6% *vs* 17% in untreated diabetics) but remained decreased ~11% in the SDI-treated group. After 4 weeks of treatment nerve conduction was completely normalized by the SDI and the ARI. In contrast, while a 19% reduction in neural blood flow in untreated diabetic rats was normalized by the ARI, flow remained reduced 14% in SDI-treated rats (147). These observations demonstrate comparable efficacy of the SDI and ARI in reversing diabetes-induced motor nerve conduction deficits, albeit the reversal process is slower with the SDI. These findings also support the importance of NADHc generated by oxidation of sorbitol to fructose in mediating impaired neural-electrophysiological function evoked by diabetes. The slower rate of reversal of nerve conduction deficits with the SDI than with the ARI may relate to the dramatic difference in their impact on their respective substrate levels. The more marked reduction in neural blood flow observed by Cameron et al. in untreated diabetic rats may be more apparent than real (see caveats in refs. 130,131).

4. Thurston et al. (148) reported that sciatic nerve NADH/$NAD^+c \propto$L/P ratios in rats with alloxan-diabetes of 3, 6, and 26 weeks duration did not differ from controls. However, nerve L/P ratios in controls as well as in diabetic rats increased from ~25 at 3 weeks, to 45 at 6 weeks, and to 70 at 26 weeks; plasma glucose levels in control rats were ~10 mmol/l *vs* 30 to 40 mmol/l in diabetic rats at each time point.

An important caveat to these observations is that the "control" rats also had been injected with alloxan, but did not develop overt diabetes. It is noteworthy that vascular dysfunction is demonstrable in rats with very mild streptozotocin-induced diabetes of 3 months duration with non-fasting plasma glucose levels of only 7.4 ± 0.9 mmol/l *vs* 6.4 ± 0.6 (mean ± SD) in conventional control rats, $P < 0.0001$ (134); in these mildly diabetic rats sciatic nerve blood flow and vascular leakage of albumin were increased 1.5-fold and 3.3-fold, respectively, *vs* controls. And, nerve sorbitol levels were increased 1.5-fold ($P < 0.01$ *vs* controls) compared to 11-fold increased sorbitol levels in overtly diabetic rats (149).

The 3-fold increase in L/P ratios in the alloxan-injected "control" rats between 3 and 26 weeks may reflect gradual development of very mild diabetes. And, the increases in neural L/P

ratios in alloxan-injected controls and overt diabetics alike may reflect increases in NADH/NAD^+c∝L/P mediated by oxidation of sorbitol.

5. Ng et al. (150) reported that sciatic motor nerve conduction velocity in mice with streptozotocin diabetes was reduced to the same extent in spontaneously mutant mice lacking sorbitol-DH and in mice with normal sorbitol-DH activity. Fructose levels were undetectable in nondiabetic and diabetic sorbitol-DH deficient mice; in nondiabetic mice sorbitol levels were ~17-fold higher in sorbitol-DH deficient than in wild type controls; in diabetic mice sorbitol levels were 9-fold higher in sorbitol-DH deficient than in wild type controls; and, in wild type mice the ratio of fructose to sorbitol was ~20 in nondiabetics *vs* 11 in diabetics. Ng et al. concluded that sorbitol-DH deficient mice are a valid model for studying the role of the polyol pathway in diabetic neuropathy and suggested that oxidation of sorbitol by sorbitol-DH does not contribute to impaired motor nerve conduction velocity. An advantage of the sorbitol-DH deficient mouse is that it circumvents problems related to the very short half-life of currently available pharmacological inhibitors of sorbitol-DH. However, there are important caveats to the conclusions based on observations in sorbitol-DH deficient mice. Many of the caveats discussed above apply to this report, including the absence of lactate and pyruvate measurements for assessment of neural NADH/NAD^+c∝L/P.

Although the authors' observations support the conclusion that oxidation of sorbitol to fructose did not contribute to the nerve conduction deficit in sorbitol-DH deficient diabetic mice, the conclusion that the sorbitol-DH deficient mouse is a valid model for studying the role of the sorbitol pathway is questionable. Their observations do not rule out an important role for sorbitol-DH in mediating nerve conduction deficits in diabetic mice with normal sorbitol-DH activity. If sorbitol-DH activity has no role in mediating the nerve conduction deficit evoked by diabetes, then overexpression of sorbitol-DH also should have no impact on nerve conduction or associated metabolic imbalances implicated in the pathogenesis of diabetic complications (i.e. oxidative stress, increased VEGF expression). On the contrary, as discussed in Section III. *D*. NADHc formed--, Amano et al. (39) reported that overexpression of sorbitol-DH in cultured retinal capillary pericytes substantially increased hyperglycemia-evoked production of ROS and expression of VEGF mRNA, and exacerbated impaired DNA synthesis. Overexpression of sorbitol-DH might be anticipated to evoke corresponding adverse changes as well as impaired conduction velocity in nerve.

Other important caveats to this study are that the protocol did not include 2 important control groups, i.e. diabetic sorbitol-DH-deficient mice and wild type diabetic mice treated with an ARI. These controls are needed to support the conclusion that the nerve conduction deficit in sorbitol-DH deficient mice was mediated by the first step of the sorbitol pathway. And, while nondiabetic sorbitol-DH deficient mice gained weight normally, no mention was made regarding weight gain in diabetic sorbitol-DH deficient *vs* diabetic wild type mice. This information is important in view of evidence that the effects of diabetes on nerve conduction in diabetic rats is linked to age at induction of diabetes and the possibility that age-related maturation of the nerve may be delayed by diabetes-evoked poor nutrition and weight gain (151).

The absence of sorbitol-DH activity in mutant mice also may affect other metabolic pathways that could increase NADHc∝L/P when diabetes is induced. For example, elevated levels of glucose 6-phosphate evoked by hyperglycemia (Table 2 in Main Text) could increase metabolism of glucose *via* the glucuronic acid pathway as discussed in Section IV. *A*.-Caveats to galactosemia--. Measurement of sciatic nerve L/P ratios in sorbitol-DH deficient diabetic mice are needed to determine whether neural free NADHc∝L/P is increased by a mechanism(s) independent of sorbitol-DH, as in the retina in galactosemic rats (Fig. 6 in Section IV. *A*.-Caveats to galactosemia--), or possibly by increased oxidation of ketones and fatty acids.

In a mouse model that over-expresses aldose reductase in the lens Chung and associates provide evidence that both enzymes of the sorbitol pathway contribute to oxidative stress in the lens of diabetic mice (152).

6. Ii et al. (153) crossed the same strain of sorbitol-DH (*SDH*) deficient mice used by Ng et al. (150) with mice expressing transgene-derived human aldose reductase (*hAR-Tg*). Diabetes was then induced in offspring with 4 different genotypes (*hAR-Tg, hAR-Tg:SDH* null, *SDH* null, and normal littermates. They reported that renal sorbitol levels were higher in the diabetic groups but did not correlate with the urinary albumin excretion rate (UAE) in mice with the 4 different genotypes. Urinary albumin excretion was increased similarly in all 4 groups of mice with diabetes of 1 and 4 weeks duration; and, epalrestat (an ARI) decreased, but did not normalize, albumin excretion similarly in mice with diabetes of 4 weeks duration. Renal sorbitol levels were increased in all 4 groups of diabetic mice but were ~2 fold higher in the 2 *SDH* null groups; epalrestat decreased sorbitol levels in all 4 groups, but to a lesser extent in the *SDH* null groups in which they remained ~ 2-fold higher than in the *hAR-Tg* and littermate groups. And, albumin excretion in the 4 groups of untreated diabetics and the 4 control groups combined did not correlate with renal sorbitol levels (correlation coefficients and P values were not provided). Renal fructose levels were not measured.

Diabetes of 4 weeks duration decreased glutathione levels ~32% and increased L/P ratios 3-5 fold in the *hAR-T*g and littermate groups. These changes in glutathione levels and L/P ratios were prevented by epalrestat in both groups. The authors chose not to measure glutathione levels and L/P ratios in the 2 *SDH* null groups because "… the SDH-deficiency did not improve the diabetic increase in UAE….".

Several of the caveats to the study reported by Ng et al. **(**150) also apply to the interpretation of observations and the conclusions of Ii et al. The reduction of albumin excretion by epalrestat in all 4 groups of diabetic rats and the failure of sorbitol levels to correlate with albumin excretion evoked by diabetes in the 4 groups (including the 2 *SDH* null groups) appear to support the authors' conclusion that "…our findings that SDH-deficiency did not improve the increased UAE in diabetes does not support the pseudohypoxia hypothesis." On the other hand, these observations also are consistent with the possibility increased albumin excretion in the diabetic *SDH* null groups is mediated by increased glucose metabolism by an enzyme other than sorbitol-DH that is, nevertheless, blocked by a pharmacological agent that inhibits aldose reductase (e.g. as in galactosemia, see Section IV.*A*.). Having made the important observations that the increase

in UAE in diabetic mice was associated with an increase in renal L/P ratios in diabetic *hAR-T*g and littermates, and that an inhibitor of aldose reductase attenuated both changes, it is unfortunate that renal L/P ratios were not measured in diabetic *SDH* null mice.

7. Kador et al. (154) reported that SDI-1 (i.e. SDI-157, the inactive pro-drug that is hydroxylated *in vivo* to form the active compound SDI-158) and SDI-2 (SDI-158) accelerated cataract development in diabetic rats but not in galactose-fed rats; in contrast, 2 different ARI inhibited cataract formation in diabetic and galactose-fed rats. To the extent that osmotic stress and associated depletion of anti-oxidant osmolytes play an important role in cataractogenesis, the observation that the SDIs did not impact on cataract development in galactose-fed rats is not surprising since inhibition of sorbitol-DH would not increase galactitol levels.

There are 2 important caveats to the conclusion that SDIs accelerated cataract development in the diabetic rats. The dosage of SDIs (70 to 100 mg/kg/day) was too low to ensure continuous inhibition of sorbitol-DH; this concern is supported by the fact that lens sorbitol levels were not significantly increased by SDIs. In addition, no statistical evidence was provided in support of the conclusion that SDIs significantly accelerated cataract development.

Paradoxically, in an earlier report from this same laboratory, the same dosage of SDI-1 (referred to as S-88-0773) was reported to delay cataract formation in galactose-fed as well as in streptozotocin-diabetic rats (155). Oates et al. reported that CP-166,572 (the active SDI-158) significantly retarded opacification of incubated rat lenses induced by high xylose levels *in vitro* but had no effect on opacification evoked by high galactose levels (156). CP-166572 markedly increased polyol levels in rat lenses and in the incubation medium of lenses incubated in xylose. And, we have observed that SDI-158, given a dosage of 250 mg/kg/day for 4 months appeared to slightly accelerate cataract development in diabetic rats; however, the trend was not statistically significant (unpublished observations). In the same diabetic rats the SDI prevented peripheral nerve electrophysiological dysfunction (see ref. 10 and discussion in Section IV. *E*.3. Cameron et al.).

V. Tissue differences in mechanisms of sorbitol pathway-linked diabetic complications

A. Schmidt et al. (157) observed that SDIs normalized peripheral nerve electrophysiological dysfunction in diabetic rats, while markedly exacerbating neuroaxonal dystrophy in autonomic nerves (iliac mesenteric nerves) and in prevertebral sympathetic superior mesenteric ganglia in the same rats; in contrast, ARI attenuated the neuroaxonal dystrophic changes as well as peripheral nerve electrophysiological dysfunction.

The explanation for these discordant effects of SDI *vs* ARI on autonomic neuroaxonal dystrophy, despite beneficial effects of both inhibitors on electrophysiological and vascular dysfunction in peripheral nerve remains to be elucidated. Although the association of neuroaxonal dystrophy with dysfunction is firmly established in many human conditions, the precise relationship of individual structural lesions with pathophysiology is not yet established and is the subject of ongoing investigation.

Effects of hyperglycemia on metabolism of peripheral nerve sensory and motor fibers also differ (158,159); and, diabetes-like complications evoked by galactosemia mimic the effects of diabetes in some, but not all, tissues (48).

B. Ramasamy et al. (160,161) have demonstrated that ARI attenuate myocardial ischemia-reperfusion injury and associated increases in NADH/NAD^+c∝L/P in isolated perfused hearts from normal and diabetic rats. More recently they reported that SDIs also attenuate myocardial ischemia-reperfusion injury and associated increases in NADH/NAD^+c∝L/P ratios in isolated perfused hearts from normal rats (162). In addition, they found that relatively brief periods of ischemia increased the activities of aldose reductase (~2-fold) and sorbitol-DH (~1.5-fold) without increasing enzyme protein content; and ARI and SDI inhibited the increased activities of their respective enzymes.

In contrast to the observations of Ramasamy et al. in the heart, as demonstrated in the Main Text, ARI selectively attenuated the increase in retinal NADH/NAD^+c∝L/P evoked by hyperglycemia without impacting on the increase in NADH/NAD^+c∝L/P evoked by hypoxia. (The duration of hypoxia in incubated retinas is comparable to the duration of ischemia in the perfused hearts of Ramasamy et al.) These discordant observations attest to additional potentially important tissue differences in sorbitol pathway-mediated metabolic imbalances evoked by hypoxia/ischemia independent of diabetes.

C. Kuruvilla and Eichberg (163) reported that in a human neurofibroma-derived Schwann cell line (NIFT), 30 mM glucose evoked a marked decrease in the proportion of 1 or more arachidonoyl-containing molecular species (ACMS) to more saturated shorter chain length molecular species in phospholipids (like that observed in peripheral nerve of diabetic rats). The depletion of ACMS was prevented by the ARI zopolrestat, but not by the active SDI.

The observations that ARI, but not SDI, attenuated the deficit in ACMS evoked by 30 mM glucose suggest that the deficit may be the consequence of impaired NADPH-dependent elongation of fatty acid precursors of ACMS rather than impaired NAD(P)H-dependent desaturase activity (15). This possible explanation is consistent with the role of NADPH as the major carrier of electrons for reductive biosyntheses. The failure of the SDI to impact on the deficit in ACMS may also be accounted for, in part, by accumulation of lactate in the incubation medium (Section IV. *B*.3-Accuulation of lactate) as discussed earlier. Lactate, pyruvate, and glucose levels in cells and incubation media were not reported; and, lactate production may be increased in this tumor cell line.

Surprisingly, 25 mM fructose mimicked the deficit in ACMS evoked by 30 mM glucose; and, in contrast to the deficit evoked 30 mM glucose, the deficit evoked by fructose was unaffected by the ARI or the SDI. The deficit in ACMS evoked by fructose may be linked to adverse effects of high fructose levels on energy metabolism (depletion of ATP) and increased lactate production as reported in the liver (due to unregulated uptake and phosphorylation of fructose to fructose 1-phosphate by fructokinase [164]). Fructose 1-phosphate levels are increased in sciatic nerve of diabetic rats (165).

VI. Concluding comments

Despite numerous caveats inherent to different experimental paradigms of diabetes, taken together, they provide strong support an important role for increased metabolism of glucose by the sorbitol pathway in mediating a wide spectrum of diabetic complications. On the other hand, paradoxical tissue differences in the effects of inhibitors of aldose reductase and sorbitol-DH on diabetic complications suggest important differences in the role of aldose reductase *vs* sorbitol-DH in mediating sorbitol pathway-linked complications in different tissues. While it is problematic whether or not inhibitors of sorbitol-DH may be useful alone or in combination with ARI for clinical use, elucidation of the mechanisms that mediate concordant and discordant effects of ARI and SDI on complications evoked by experimental diabetes and galactosemia promises to yield important new insights into the pathogenesis of diabetic complications.

The current state of knowledge supports an important role for the increase in free NADHc in initiating metabolic imbalances that cause complications of diabetes. An increase in NADHc inhibits GA3P-DH, causing triose phosphate levels to rise. And, independently, the increase in NADHc also accelerates metabolism of triose phosphates to diacylglycerol, which activates PKC that, in turn, activates NAD(P)H oxidases; and, since NADHc is the substrate for many of these oxidases, increased NADHc levels further augment superoxide production. Increased superoxide production and activation of PKC, in turn, augment additional metabolic imbalances and pathways implicated in the pathogenesis of diabetic complications, e.g. activation of NF-κB, PARP, and apoptosis. Lastly, increased triose phosphate levels accelerate concentration-dependent degradation of trioses to methylglyoxal (a toxic and potent intracellular glycating agent) and also fuel the hexosamine pathway.

The fact that free NADHc and redox cycling of free $NAD^+c \rightleftharpoons$ NADHc are modulated by the activity of numerous enzymes (and by the concentrations of their oxidized and reduced substrates) is consistent with the central role and importance of free $NAD^+(H)c$ in fueling and regulating: *1*) ATP synthesis in the cytosol and in mitochondria, and *2*) signaling pathways that coordinate blood flow with energy metabolism in a wide range of physiological and pathological conditions including physiological work, diabetes, galactosemia, and hypoxia.

Acknowledgement – We thank Peter J. Oates and Ronald J. Tilton for comments and suggestions based on reading an early draft of this appendix.

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