Interactions Between Hyperglycemia and Hypoxia
Implications for Diabetic Retinopathy

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The primary aim of these experiments was to assess in vitro effects of hyperglycemia (30 mmol/l glucose) and hypoxia (Po_2 = 36 torr) of 2-h duration, separately and in combination, on cytosolic and mitochondrial free NADH (NADHc and NADHm, respectively) in retinas from normal rats. NADH is the major carrier of electrons and protons that fuel ATP synthesis and several metabolic pathways linked to diabetic complications. Hyperglycemia and hypoxia increase free NADHc by different mechanisms that are additive. Hyperglycemia increases transfer of electrons and protons from sorbitol to NAD^+c, reducing it to NADHc, but does not increase NADHm. Hypoxia increases NADHm by inhibiting its oxidation. Electrons and protons accumulating in NADHm restrain transfer of electrons and protons from NADHc to NAD^+m via the malate-aspartate electron shuttle. Hyperglycemia and hypoxia also increase glycolysis by different mechanisms that are additive, and hyperglycemia increases ATP levels in hypoxic and in aerobic retinas. The additive effects of hyperglycemia and hypoxia on accumulation of electrons and protons in a common pool of free NADHc confirm the test hypothesis and the potential of a combination of these two risk factors to accelerate the onset and progression of diabetic retinopathy (and other complications of diabetes) by augmenting metabolic pathways fueled by free NADHc. *Diabetes* 53:2931–2938, 2004

The importance of duration and severity of hyperglycemia in the development of diabetic retinopathy was clearly established by the Diabetes Control and Complications Trial (1); however, the mechanisms that mediate early and late retinal vascular dysfunction and structural changes remain unclear. Proliferative retinopathy is widely attributed to increased production of vascular endothelial growth factor evoked by hypoxia/ischemia caused by capillary closure and nonperfusion that develop relatively early after the onset of diabetes (2).

The possibility that hyperglycemia and hypoxia may interact via a common metabolic imbalance(s) to initiate and/or exacerbate complications of diabetes is suggested by the correspondence of several redox, metabolic, and pathophysiological changes evoked by either condition alone. Examples include an increased ratio of reduced to oxidized free cytosolic NADc (3–16), increased production of free radicals (3,17–24) and vascular endothelial growth factor (17,19,25–28), accumulation of triose phosphates (7,9,10,13–15), activation of protein kinase C (PKC) (22,28–30), decreased Na^+/K^+-ATPase activity (3,30–33), early increases in blood flow (3,33–35), and paradoxical protective effects of diabetes and brief periods of hypoxia/ischemia (i.e., ischemic preconditioning) that attenuate dysfunction/injury evoked by subsequent more severe hypoxia/ischemia (3,29,36–38).

An increase in free NADH/NAD^+c appears to be the best candidate metabolic imbalance for mediating pathophysiological changes common to diabetes and hypoxia (3,38). This redox imbalance develops when electrons and protons are transferred to free oxidized NAD^+c (reducing it to NADHc) faster than electrons and protons carried by NADHc are used for ATP synthesis in mitochondria by oxidative phosphorylation. Hypoxia increases free NADHc because lack of O_2 impairs utilization of electrons and protons carried by mitochondrial NADHm for oxidative phosphorylation. The mass action effect of electrons and protons accumulating in NADHm restrains transfer of electrons and protons from free NADHc to NAD^+m by the malate-aspartate electron shuttle; thus, electrons and protons accumulate in, and elevate, free NADHc.

In cells for which glucose uptake is insulin-independent, hyperglycemia augments glucose uptake and metabolism via the sorbitol pathway. In the second step of the pathway, sorbitol is oxidized to fructose by sorbitol dehydrogenase, which catalyzes transfer of a hydride ion ($:H^-$) from sorbitol to free NAD^+c (reducing it to NADHc) and removal of a second hydrogen atom that is released into solution as a proton (H^+):

$$\text{sorbitol} + NAD^+c \xrightarrow{\text{sorbitol dehydrogenase}} \text{fructose} + \text{NADHc} + H^+$$

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ARI, aldose reductase inhibitor; DH, dehydrogenase; DHAP, dihydroxyacetone phosphate; F1,6-BP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; GA3P, glyceraldehyde 3-phosphate; G3P, glycerol 3-phosphate; LDH, lactate dehydrogenase; L/P, lactate/pyruvate ratio; PKC, protein kinase C; ROS, reactive oxygen species.

In such cells, increased electrons and protons carried by NADHc can fuel several metabolic pathways implicated in the pathogenesis of diabetic retinopathy (3,9,10,17,38).

We coined the term "hyperglycemic pseudohypoxia" to draw attention to numerous metabolic imbalances and pathophysiological changes common to hyperglycemia and hypoxia (3). The present studies were undertaken to determine whether increases in NADHc evoked by hyperglycemia and hypoxia are additive, which would have the potential to augment NADHc-fueled metabolic pathways implicated in the pathogenesis of diabetic retinopathy.

Our findings demonstrate that hyperglycemia and hypoxia increase NADHc and triose phosphate levels by different mechanisms that are additive, and inhibition of the sorbitol pathway prevents both of these increases evoked by hyperglycemia but not the increases evoked by hypoxia. They also demonstrate that hyperglycemia and hypoxia increase glycolysis (manifested by increased lactate production) by different mechanisms that are additive; however, the increases in glycolysis are independent of the sorbitol pathway.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats that weighed 200–250 g were purchased from Sasco (O'Fallon, MO) and housed and cared for in accordance with guidelines of the University Committee for the Humane Care of Laboratory Animals. Rats were housed one per cage in a room with a 12:12-h artificial light cycle at a temperature of 21 ± 2°C and a humidity of 55 ± 2%. They had free access to standard rat chow (Ralston Purina, Richmond, IN) and tap water.

Tissue preparation and incubations. Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body wt) or by CO_2 narcosis. The whole eye was quickly removed and placed in Krebs bicarbonate/HEPES buffer at pH 7.45 for further dissection, and the rats were exsanguinated. The buffer composition was 110 mmol/l NaCl, 4.5 mmol/l KCl, 0.5 mmol/l $MgCl_2 \cdot 6H_2O$, 1.0 mmol/l $CaCl_2 \cdot 2H_2O$, 1.5 mmol/l Na_2HPO_4, 30.0 mmol/l HEPES, 15 mmol/l $NaHCO_3$, 5.0 mmol/l D-glucose, 0.1 mmol/l L-arginine, and 0.05% BSA.

The cornea was incised with the aid of a dissecting microscope, the lens was rapidly removed, and retinas were gently separated from the choroid with tweezers. Retinas were then incubated for 2 h at 37°C (pH ~7.40) in the above buffer (10 ml per retina in all experiments) that contained 5 or 30 mmol/l D-glucose ± 70 μmol/l tolrestat (an aldose reductase inhibitor [ARI]; a gift from Wyeth-Ayerst. Retinas that were incubated in 70 μmol/l tolrestat were obtained from rats that were fed tolrestat in the diet to provide a daily dose of 0.2 mmol/kg body wt for 1 week before removal of the retina to achieve inhibitory concentrations of the ARI. One retina from each rat was incubated in 5 mmol/l glucose at 676 torr (normoglycemic aerobic condition) and the other in 30 mmol/l glucose at 676 torr (hyperglycemic aerobic condition) or one retina in 5 mmol/l glucose at 676 torr and the other in 5 mmol/l glucose at 36 torr (normoglycemic hypoxic condition), etc., to permit paired comparisons of effects of hyperglycemia and hypoxia.

The buffer was gassed for 2 h before adding the retinas (and throughout the incubation) with the following humidified gas mixtures that contained 5% CO_2 + various percentages of O_2 with the balance made up of nitrogen: *1*) 95% O_2 (676 torr), *2*) 50% O_2 (357 torr), *3*) 20% O_2 (143 torr), or *4*) 5% O_2 (36 torr). The pH and Po_2 of incubation media were measured at the beginning and end of incubations with a Corning 170 pH/Blood Gas Analyzer, and the osmolality (~290 mOsmol) was measured with a Wescor 5100 C Vapor Pressure Osmometer. None of these parameters changed during the incubation period. Incubations were terminated by removal of retinas for measurement of metabolites.

The time course of 30 mmol/l glucose-induced increases in glycolytic metabolites, free NADHc, and lactate production was assessed in retinas that were incubated for 1, 2, and 3 h at 676 torr. The possibility that the absence of physiological levels of lactate and pyruvate in the incubation medium might have an impact on the effects of elevated glucose levels on these parameters was evaluated by adding 1.0 and 0.1 mmol/l lactate and pyruvate (normal plasma levels), respectively, to the medium. Effects of tolrestat on sorbitol pathway and glycolytic metabolites and on free NADHc were assessed in hypoxic retinas. (Effects of an ARI on these parameters in aerobic retinas have been published [10].)

Metabolite assays. Glucose, pyruvate, lactate, glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), ATP, glutamate, 2-oxoglutarate, and ammonia in extracts of retina (and pyruvate and lactate in extracts of incubation medium) were quantified by standard enzymatic methods (10,39,40). Because the overall reaction catalyzed by aldolase and triose phosphate isomerase is in near equilibrium under steady-state conditions (7), fructose 1,6-bisphosphate (F1,6-BP) was converted to dihydroxyacetone phosphate (DHAP) by aldolase and measured together with endogenous DHAP and glyceraldehyde 3-phosphate (GA3P) as triose phosphates (7,10). Sorbitol in retinal extracts was quantified by gas chromatography/mass spectrometry as previously described (10). D-Fructose in the incubation medium was quantified by an enzymatic fluorometric method (41).

All metabolite data are reported as moles per microgram of DNA that was quantified as previously described (10). To compare metabolite levels in the present experiments with values from other laboratories reported as moles/tissue wet or dry weight, we determined conversion factors from 10 normal male Sprague-Dawley rats that weighed 336 ± 15 g. The wet weight of the retinas was 12.12 ± 0.98 mg, and the dry weight was 2.55 ± 0.22 mg. The conversion factors (mean ± SD) are *1*) 56 ± 6 μg DNA/mg dry wt, *2*) 202 ± 22 μg protein/mg dry wt, *3*) 42 ± 4 μg protein/mg wet wt, and *4*) 0.210 ± 0.008 mg dry wt/mg wet wt. Protein was quantified by the bicinchoninic acid method using the BCA Protein Assay Reagent obtained from Pierce Chemical Company and BSA as the standard.

Free NADHc and NADHm cannot be determined by direct measurement of NADH in whole tissue extracts that contain enzyme-bound as well as free NADHc and NADHm, and free NADm is more than an order of magnitude more reduced than free NADc (6). Free NADHc and NADHm can be estimated only by redox metabolite indicator methods based on near-equilibria between ratios of free NADH to NAD^+ and reduced to oxidized substrates of dehydrogenase enzymes restricted in distribution to cytosol or mitochondria. Unless otherwise stated, NADHc and NADHm in this report refer to free, not total, NADHc and NADHm.

Free NADHc/NAD^+c was assessed on the basis of the near equilibrium between free NADHc/NAD^+c and lactate/pyruvate (L/P) ratios (Eqs. 1 and 2) and an equilibrium constant of 1.11×10^{-4} (at pH = 7.0) for lactate dehydrogenase (LDH) (6):

$$\text{NADHc} + \text{H}^+ + \text{pyruvate}_c \underset{\text{LDH}}{\rightleftharpoons} \text{NAD}^+\text{c} + \text{lactate}_c \quad (1)$$

$$\text{NADHc/NAD}^+\text{c} = \text{lactate}_c/\text{pyruvate}_c \times K_{\text{LDH}} \quad (2)$$

Because total retinal NADH and NAD^+ (and the ratio of total NADH to NAD^+) are increased in diabetic rats (4,5), it is reasonable to assume that hyperglycemia is unlikely to change substantially total free NADc and total free NADm in these brief experiments. Thus, changes in free NADHc will evoke proportional changes in the ratios of free NADHc to NAD^+c and L/P; for clarity, changes in measured ratios of L/P are referred to as changes in NADHc∝L/P or NADHc.

Free NADHm/NAD^+m was estimated on the basis of the near equilibrium between the ratios of NADHm to NAD^+m and glutamate (GL) to 2-oxoglutarate established by glutamate dehydrogenase (6) and an equilibrium constant of 3.87×10^{-3} mmol/l (pH 7.0):

$$\text{NADHm/NAD}^+\text{m} = [\text{glutamate}]/[\text{2-oxoglutarate}]\,[\text{NH}_4^+] \times K_{\text{GL-DH}}. \quad (3)$$

Because (as for changes in free NADHc) changes in free NADHm/NAD^+m should be proportional to changes in free NADHm, they are referred to as such.

Statistical analyses. Multiple ANOVA of all parameters was performed with the SAS general linear models procedure as previously described (16) with significance set at $P < 0.05$. All results are reported as the mean ± SD ($n \geq 6$ rats) unless indicated otherwise. Representative results from two to four independent experiments ($n = \leq 6$ rats per group in each experiment) are shown in the figures and tables.

RESULTS

Effects of hyperglycemia and hypoxia on retinal NADHc∝L/P, NADHm, lactate, and pyruvate

NADHc∝L/P and NADHm. Hyperglycemia increased retinal L/P ratios 1.4- to 2-fold at all Po_2 (Fig. 1, Table 1). Decreasing Po_2 from 676 to 357 torr did not affect L/P (Fig. 1); however, further reduction of Po_2 to 143 and 36 torr increased L/P 1.7-fold ($P \leq 0.035$) and >6-fold ($P < 0.0001$), respectively, both in normoglycemic and hyperglycemic retinas. Tolrestat did not affect the hypoxia-

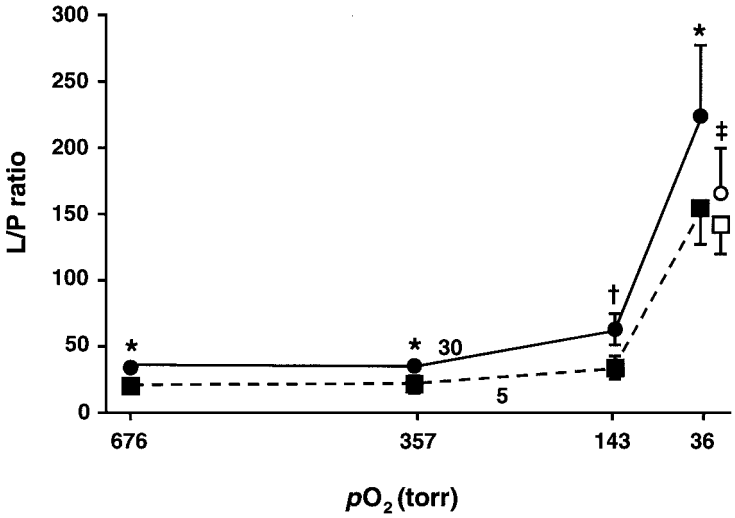

FIG. 1. Effect of Po_2 (in torr) on L/P ratios in retinas incubated in 5 mmol/l glucose (■), 30 mmol/l glucose (●), 5 mmol/l glucose + 70 μmol/l tolrestat (□), and 30 mmol/l glucose + 70 μmol/l tolrestat (○). Data are means ± SD, n = 6 for each data point. Different from 5 mmol/l glucose: *P < 0.001, †P < 0.02. Different from 30 mmol/l glucose at 36 torr: ‡P < 0.004.

induced increase in L/P in normoglycemic retinas but prevented the additional increase in L/P evoked by hyperglycemia (Fig. 1). NADHm was increased twofold by hypoxia but was unaffected by hyperglycemia in aerobic or hypoxic retinas (Table 1).

Lactate and pyruvate. Hyperglycemia and hypoxia increased retinal lactate levels ~3-fold and 1.7-fold, respectively, and the increases were additive (Table 1). In contrast, whereas hyperglycemia increased pyruvate levels approximately twofold, they were decreased ~90% by hypoxia in normoglycemic and hyperglycemic retinas.

Hyperglycemia and hypoxia increased glycolysis (based on the sum of lactate and pyruvate in retina and medium) and lactate production (sum of lactate in retina + medium) ~1.5-fold and 1.3- to 2-fold, respectively, and the increases were additive (Fig. 2, Tables 1 and 2). (See online appendix, section 1*A*, available at http://diabetes.diabetesjournals.org.) Increased lactate production evoked by hypoxia in normoglycemic and hyperglycemic retinas was unaffected by tolrestat. Pyruvate production (sum of pyruvate in retina + medium) by normoglycemic retinas was 2.3 ± 0.3 nmol · μg DNA^{-1} · 2 h^{-1} and was unaffected by hyperglycemia, hypoxia, or tolrestat (data not shown).

Lactate levels and L/P ratios in aerobic retinas and 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 (data not shown).

Effects of hyperglycemia and hypoxia on glycolytic metabolites and ATP

Glucose, G6P, F6P, and triose phosphates. Hyperglycemia increased glucose levels approximately eight times in aerobic and hypoxic retinas. G6P and F6P were increased approximately twofold by hyperglycemia but were reduced ~50% by hypoxia (Table 2); F6P was undetectable in normoglycemic-hypoxic retinas.

Hyperglycemia increased triose phosphates ~1.4-fold (Fig. 3, Table 2). Triose phosphates were unaffected by decreasing Po_2 from 676 to 143 torr (Fig. 3) but were increased ~1.7-fold ($P < 0.0001$) at 36 torr in normoglycemic and hyperglycemic retinas (Fig. 3, Table 2). Tolrestat had no effect on hypoxia-induced increases in triose phosphates in normoglycemic retinas but prevented the additional increase evoked by hyperglycemia (Fig. 3).

ATP. Hyperglycemia increased ATP levels 1.6-fold in aerobic retinas and 3.3-fold in hypoxic retinas. Hypoxia reduced ATP 66 and 28% in normoglycemic and hyperglycemic retinas, respectively (Table 2). Thus, ATP levels were the same in hypoxic-hyperglycemic retinas and in aerobic-normoglycemic retinas. Addition of plasma levels of lactate and pyruvate during incubation increased ATP levels twofold ($P < 0.01$ versus ATP levels in aerobic-

TABLE 1
Retinal lactate, pyruvate, L/P ratios, cytosolic and mitochondrial free $NADH/NAD^+$, and lactate production after incubation in 5 or 30 mmol/l glucose at 676 and 36 torr for 2 h

	Pyruvate	Lactate	Lactate/pyruvate	Cytosolic $NADH/NAD^+$ $\times 10^{-3}$	Mitochondrial $NADH/NAD^+$ $\times 10^{-3}$	Lactate production
5 mmol/l glucose						
676	14.4 ± 2.1	278 ± 60	19 ± 3	2.1 ± 0.3	66 ± 9	19.3 ± 3.7
36	2.0 ± 0.8*	526 ± 54*	304 ± 122†	29.8 ± 11.1†	150 ± 10†	25.7 ± 4.0‡
30 mmol/l glucose						
676	26.7 ± 2.0†§	1016 ± 256†‖	38 ± 10†‖	4.1 ± 0.9†‖	70 ± 17§	29.7 ± 4.0*
36	2.3 ± 0.8*#	1600 ± 254†¶**	754 ± 209†§#	78.8 ± 20.9†§#	137 ± 26†#	40.1 ± 2.8†¶**

Data are means ± SD, n = 6. Retinal levels of pyruvate and lactate are in pmol/μg DNA; lactate production (sum of lactate in retina + incubation medium) is in nmol · μg DNA^{-1} · 2 h^{-1}. See RESEARCH DESIGN AND METHODS for calculation of cytosolic and mitochondrial $NADH/NAD^+$. Different from 5 mmol/l glucose at 676 torr: *$P < 0.01$, †$P \leq 0.001$, ‡$P < 0.05$. Different from 5 mmol/l glucose at 36 torr: §$P \leq 0.001$, ‖$P \leq 0.03$, ¶$P < 0.005$. Different from 30 mmol/l glucose at 676 torr: #$P \leq 0.001$, **$P < 0.01$.

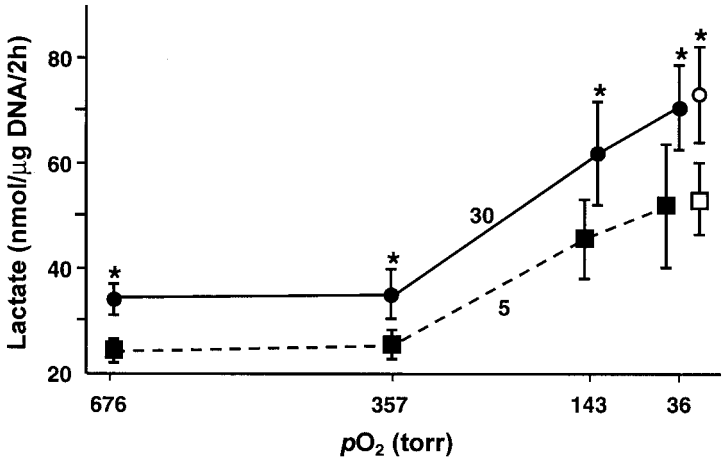

FIG. 2. Effect of Po_2 on total lactate production, i.e., sum of lactate in retina and medium by retinas incubated in 5 mmol/l glucose (■), 30 mmol/l glucose (●), 5 mmol/l glucose + 70 μmol/l tolrestat (□), and 30 mmol/l glucose + 70 μmol/l tolrestat (○). Data are means ± SD, $n = 6$ for each data point. Different from 5 mmol/l glucose: *$P \leq 0.003$.

normoglycemic and in aerobic-hyperglycemic retinas) (Table 2).

Effects of hyperglycemia and hypoxia on sorbitol pathway metabolism. Sorbitol levels in normoglycemic retinas ranged from 5 to 7 pmol/μg DNA and were increased 9- to 18-fold ($P < 0.001$ at all Po_2) by hyperglycemia (data not shown). Fructose production by normoglycemic retinas ranged from 1.3 to 3.6 pmol/μg DNA at all Po_2 (Fig. 4) and was increased 55- to 74-fold by hyperglycemia ($P < 0.001$ at all Po_2). Tolrestat reduced fructose production by 65 and 96% in hypoxic-normoglycemic and hypoxic-hyperglycemic retinas, respectively (Fig. 4).

DISCUSSION

These experiments demonstrate that *1*) hyperglycemia and hypoxia increase NADHc, triose phosphates, and glycolysis by different mechanisms that are additive, and *2*) hyperglycemia-evoked increases in NADHc∝L/P and triose phosphates are mediated largely by a small increase in glucose metabolism via the sorbitol pathway to fructose that is only ~2% of the increase in glucose metabolism via glycolysis. These observations have several important implications for the pathogenesis of diabetic complications. Increased ATP production by substrate phosphorylation evoked by hyperglycemia in oxygenated and hypoxic retinas may explain, in part, the transient worsening of retinopathy evoked by rapid normalization of plasma glucose levels.

Different mechanisms mediate increases in NADHc and glycolysis evoked by hyperglycemia. Increases in NADHc (manifested by an increase in the retinal L/P ratio) and in glycolysis (manifested by increased lactate production) are sequelae of two different mass action effects of hyperglycemia. The increase in NADHc∝L/P evoked by 30 mmol/l glucose at 676 and 36 torr is mediated entirely by increased flux of glucose via the sorbitol pathway to fructose (~200 pmol glucose · μg DNA^{-1} · 2 h^{-1} based on fructose production in oxygenated and hypoxic retinas; Fig. 4) and is independent of the associated increases in glycolysis (~5,000 pmol glucose · μg DNA^{-1} · 2 h^{-1} at 676 torr and ~10,000 pmol glucose at 36 torr) (Fig. 2). This conclusion is supported by the observations that tolrestat prevented increases in NADHc∝L/P and fructose production at 36 torr (Figs. 1 and 4) but not the associated increase in lactate production (Fig. 2). (See online appendix, section I*B*) NADHc∝L/P values in normoglycemic retinas and increases in NADHc∝L/P evoked by hyperglycemia agree closely with values in control and diabetic rats (8,11,12).

In contrast, increased glycolysis is mediated by suppression of product inhibition of hexokinase by G6P (42) (and, possibly, by increased phosphorylation of glucose by glucokinase [43]). Elevated levels of G6P and proportional increases in F6P (Table 2) support this conclusion. Increased levels of F6P drive its phosphorylation to F1,6-BP by phosphofructokinase, the rate-limiting step in glycolysis under most physiological conditions. These observations indicate a shift from transport to phosphorylation as the rate-limiting step in hexose uptake like that observed in rat cerebellum and red skeletal muscle under conditions of hyperglycemia and hyperinsulinemia (44).

TABLE 2
Retinal metabolite levels and lactate production after incubation in 5 or 30 mmol/l glucose at 676 and 36 torr for 2 h

	Glucose	G6P	F6P	TP	ATP	Lactate production
5 mmol/l glucose						
676	301 ± 53	3.58 ± 1.12	0.49 ± 0.33	11.8 ± 2.3	39 ± 3	16.5 ± 2.9
36	323 ± 163	1.84 ± 0.64*	BLD	19.4 ± 6.5†	14 ± 3*	24.5 ± 4.6*
30 mmol/l glucose						
676	2398 ± 480*‡	6.72 ± 1.28*‡	1.24 ± 0.36†	16.6 ± 2.5†	64 ± 4*	25.2 ± 1.4*
36	2468 ± 700*‡	3.48 ± 0.40‡§	0.68 ± 0.16‖	30.4 ± 3.3*‡§	46 ± 10‡§	32.5 ± 3.6*‡‖

Data are means ± SD, $n = 6$. Glucose, glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), triose phosphates (TP), and ATP levels are in pmol/μg DNA. Lactate production (sum of lactate in retina + incubation medium) is in nmol · μg DNA^{-1} · 2 h^{-1}. BLD, below limits of detection. Different from 5 mmol/l glucose at 676 torr: *$P \leq 0.001$, †$P < 0.01$. Different from 5 mmol/l glucose at 36 torr: ‡$P \leq 0.001$. Different from 30 mmol/l glucose at 676 torr: §$P < 0.001$, ‖$P < 0.004$.

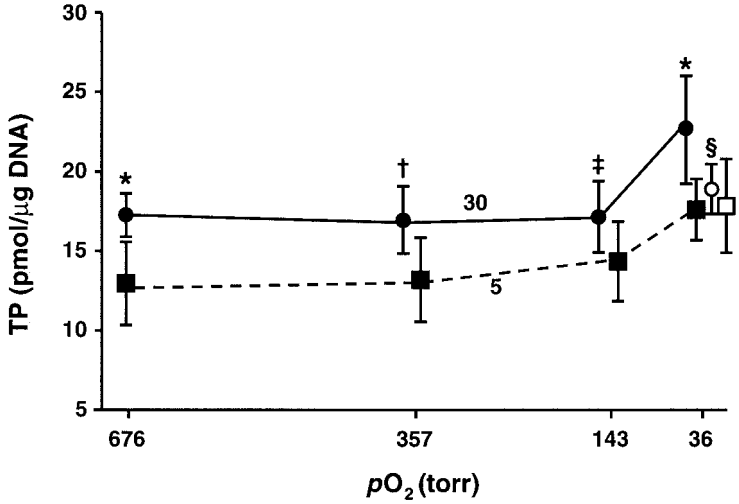

FIG. 3. Effect of Po_2 (in torr) on triose phosphates (TP) in retinas incubated in 5 mmol/l glucose (■), 30 mmol/l glucose (●), 5 mmol/l glucose + 70 μmol/l tolrestat (□), and 30 mmol/l glucose + 70 μmol/l tolrestat (○). Data are means ± SD, n = 6 for each data point. Different from 5 mmol/l glucose: *$P < 0.001$, †$P < 0.005$, ‡$P = 0.032$. Different from 30 mmol/l glucose at 36 torr: §$P < 0.028$.

Some investigators suggest that the increase in NADHc formed by the sorbitol pathway during hyperglycemia is so small (compared with the increase in NADHc formed by glycolysis) that it is unlikely to be of any pathophysiological significance (12,45). Winkler et al. (45) asserted that increased oxidation of sorbitol to fructose must account quantitatively for an increase in retinal lactate content for NADH/NAD^+c∝L/P ratios to be increased. Both of these notions are unfounded. The finding that tolrestat completely prevented increases in fructose production at 36 torr (which was only ~2% of the molar increase in flux of glucose via glycolysis) and in NADHc∝L/P evoked by hyperglycemia clearly demonstrates the greater impact of flux of glucose via the sorbitol pathway on NADHc∝L/P than flux of glucose via glycolysis.

Moreover, it is well known that several cytosolic enzymes in addition to LDH catalyze oxidation of NADHc to NAD^+c, e.g., G3P (glycerol 3-phosphate)-DHc, malate dehydrogenase, NADH oxidase, as depicted in Fig. 5. Lactate is formed only when NADHc is reoxidized by LDH.

Increased NADHc and pyruvate formed in resting cells (as a result of augmented glycolysis evoked by hyperglycemia) are oxidized to NAD^+c and reduced to lactate, respectively, by LDH. In contrast, because the sorbitol pathway does not form pyruvate, increased NADHc formed by the sorbitol pathway is reoxidized largely by enzymes other than LDH (some of which are implicated in mediating diabetic complications) coupled to reduction of substrates other than pyruvate. (See online appendix, section II.) Thus, a far greater inhibition of aldose reductase than was achieved in past clinical trials with ARI may be needed to prevent complication of diabetes fueled by NADHc formed by sorbitol dehydrogenase.

Increases in NADHc∝L/P and in glycolysis evoked by hypoxia are sequelae of impaired oxidative phosphorylation and increases in NADHm. In contrast to hyperglycemia, increases in NADHc and glycolysis evoked by hypoxia reflect impaired utilization of electrons and protons carried by NADHm for ATP synthesis by oxidative phosphorylation. The resulting increase in NADHm and decrease in NAD^+m limits *1*) reoxidation of NADHc to NAD^+c (which, in turn, increases NADHc) by the malate-aspartate electron shuttle, which transfers electrons and protons from NADHc to NAD^+m, and *2*) mitochondrial oxidation of glycolysis-derived pyruvate coupled to reduction of NAD^+m to NADHm by the Krebs cycle (46). Concurrent impaired utilization of ADP for synthesis of ATP by oxidative phosphorylation increases ADP and AMP levels, which activate phosphofructokinase (the key enzyme in regulation of glycolysis [46]), which mediates the hypoxia-evoked increase in glycolysis in normoglycemic and hyperglycemic retinas (Tables 1 and 2).

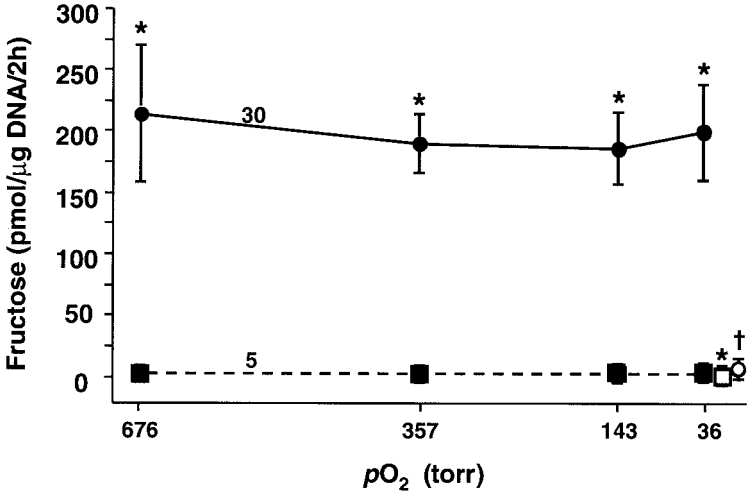

FIG. 4. Effect of Po_2 (in torr) on fructose production, i.e., fructose in incubation medium in retinas incubated in 5 mmol/l glucose (■), 30 mmol/l glucose (●), 5 mmol/l glucose + 70 μmol/l tolrestat (□), and 30 mmol/l glucose + 70 μmol/l tolrestat (○). Data are means ± SD, n = 6 for each data point. Different from 5 mmol/l glucose: *$P < 0.0001$. Different from 30 mmol/l glucose at 36 torr: †$P < 0.0001$.

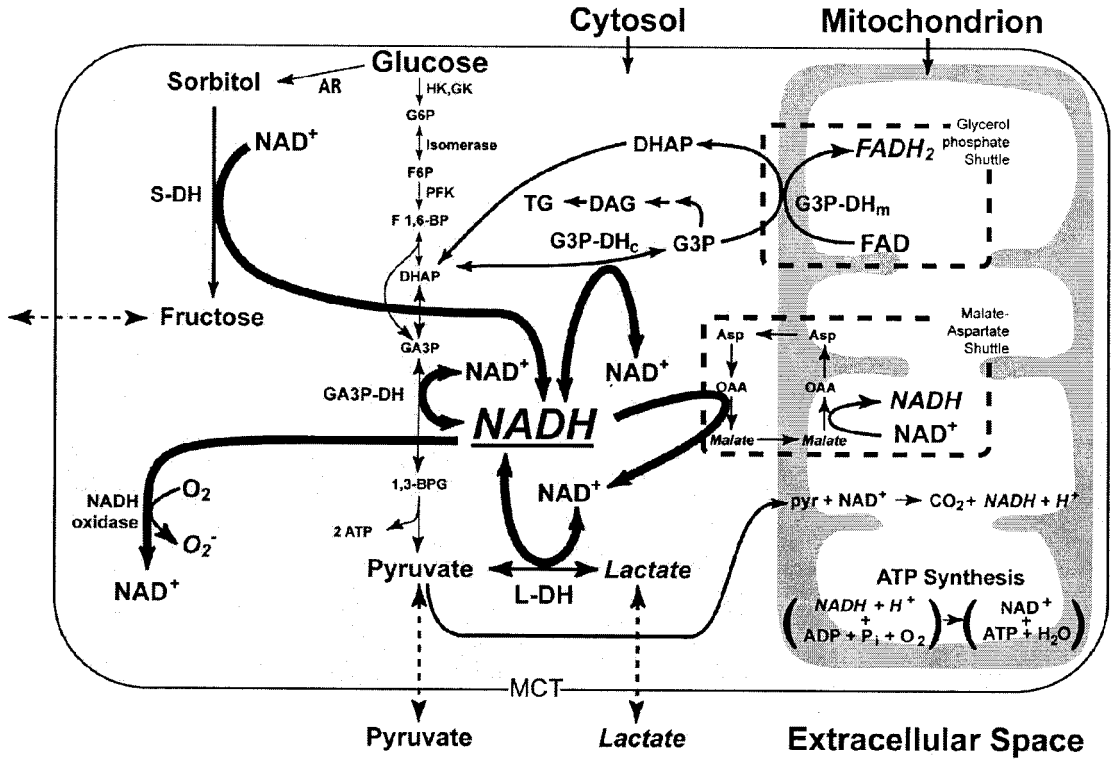

FIG. 5. Schematic diagram of a generic cell depicting effects of hyperglycemia and hypoxia on redox cycling of free cytosolic $NAD^+c \rightleftharpoons$ NADHc, ATP synthesis, and redox signaling pathways. In normoglycemic normoxic resting cells, electrons and protons are transferred from cytosolic metabolites of glucose (GA3P and sorbitol) to free NAD^+c, reducing it to NADHc. Some of these electrons and protons are transferred from free cytosolic NADHc to mitochondrial NAD^+m and FADm via the malate-aspartate and glycerol phosphate electron shuttles and fuel ATP synthesis by oxidative phosphorylation. However, electrons and protons are transferred to cytosolic free NAD^+c faster than they are used for ATP synthesis by oxidative phosphorylation. (Low ADP levels constrain utilization of electrons and protons carried by NADHc and NADHm for ATP synthesis by oxidative phosphorylation.) Some electrons and protons that are not used for oxidative phosphorylation are transferred from free NADHc to *1*) pyruvate (by LDH), which is reduced to lactate that exits the cell via monocarboxylate transporters in the plasma membrane, and *2*) DHAP (by G3P-DHc), reducing it to G3P, the first step in the glycerol phosphate electron shuttle and in one pathway for synthesis of glycerolipids including triglycerides and diacylglycerol that activates PKC. Electrons are also transferred to O_2 (by NADH oxidase), reducing it to O_2^-. All of these electron transfers are coupled to reoxidation of NADHc to NAD^+c, high levels of which are required to support continuing glycolysis and synthesis of ATP by substrate phosphorylation. Hyperglycemia increases the rates of transfer of electrons and protons from GA3P and sorbitol to free NADHc. The additional electrons and protons transferred to NADHc from GA3P via glycolysis are transferred largely to pyruvate that is not used for oxidative phosphorylation. Because the sorbitol pathway does not generate pyruvate (fructose is not further metabolized by most cells and diffuses out), the additional electrons transferred to NADHc from sorbitol are preferentially transferred to DHAP and O_2. Hypoxia limits transfer of electrons and protons carried by NADHm to O_2; electrons and protons accumulating in NADHm restrain transfer of electrons and protons from NADHc to NAD^+m via the malate-aspartate shuttle; electrons and protons accumulating in NADHc are transferred to DHAP and O_2 by G3P-DHc and NADH oxidase, respectively, and synthesis of ATP from ADP by oxidative phosphorylation is impaired. Decreased ATP and elevated levels of ADP and AMP activate phosphofructokinase (PFK), which increases glycolysis and ATP synthesis by substrate phosphorylation.

This scenario is supported by the observation that hypoxia augmented lactate production despite decreased retinal levels of G6P, F6P, and ATP (Table 2). These findings are not surprising but provide new information regarding retinal glucose metabolism. Decreased ATP levels in hypoxic retinas indicate that increased ATP synthesis via substrate phosphorylation did not keep pace with ATP synthesis by oxidative phosphorylation in aerobic retinas. The increased lactate production evoked by hypoxia in normoglycemic retinas is consistent with observations of Winkler et al. (45) and demonstrates (contrary to their conclusion) that glycolysis in normoglycemic-aerobic retinas was not limited by availability of extracellular glucose.

Finally, identical 1.6-fold increases in NADHc evoked by 143 torr (mild hypoxia) in normoglycemic retinas and by hyperglycemia in aerobic retinas (676 and 357 torr; Fig. 1) indicate that relatively mild hypoxia alone increases NADHc to the same extent as hyperglycemia alone; the finding that NADHc at 143 torr was increased 1.8-fold by hyperglycemia demonstrates that the effects of even mild hypoxia on NADHc are additive to those of marked hyperglycemia (Fig. 1).

Impact of increased NADHc on diabetic retinopathy. An increase in NADHc, whatever the cause (e.g., increased sorbitol pathway metabolism, hypoxia, increased oxidation of fatty acids, ketones, lactate, galactose), will tend to inhibit reduction of NAD^+c to NADHc by some enzymes (e.g., GA3P-DH) and augment oxidation of NADHc by others (e.g., G3P-DHc and NADH oxidase) (Fig. 5). Because the triose phosphates (GA3P, DHAP, and F1,6-BP) are in near equilibrium, their levels will rise with inhibition of GA3P-DH, and the combined mass action effects of increased triose phosphates and NADHc will drive reduction of DHAP to G3P coupled to reoxidation of NADHc to NAD^+c by G3P-DHc (Fig. 5). This is the first step in one pathway for de novo synthesis of diacylglycerol and activation of PKC (Fig. 5) implicated in mediating several complications of diabetes (22). And, concentration-dependent degradation of triose phosphates to methylglyoxal (a

toxic and potent intracellular glycating agent [47]) will be increased. In addition, the mass action effect of an increase in NADHc will augment formation of superoxide by NADH-fueled extramitochondrial NADH oxidase (48).

A growing body of evidence supports an important role for generation of reactive oxygen species (ROS) by PKC-activated, extramitochondrial NAD(P)H oxidases in mediating vascular disease evoked by diabetes. Several lines of evidence indicate that NADHc formed by oxidation of sorbitol *1*) fuels de novo synthesis of diacylglycerol that activates PKC and *2*) donates electrons that fuel cytosolic superoxide production by extramitochondrial NAD(P)H oxidases. (See online appendix, sections III and IV*C*.)

In contrast, Brownlee and associates (23) maintain that hyperglycemia-induced ROS are generated exclusively in mitochondria. They attribute increased ROS production to increased oxidation of pyruvate (produced by increased glycolysis), which generates NADHm that promotes superoxide production by the electron transport chain. They propose that mitochondrial superoxide inhibits GA3P-DH, causing triose phosphate levels to rise.

This scenario appears to be largely untenable when viewed in the context of *1*) discordant observations of other investigators and *2*) important caveats to the interpretation of their data. (See online appendix, sections III and IV.) For example, their scenario suggests that addition of exogenous pyruvate should exacerbate adverse sequelae of hyperglycemia. On the contrary, addition of pyruvate markedly attenuates elevated diacylglycerol levels and associated vascular dysfunction evoked by hyperglycemia in vivo (49). In addition, ARIs prevent hyperglycemia-evoked increases in triose phosphates and NADHc in normoxic and hypoxic retinas (10) (Figs. 1 and 3) without attenuating the associated increase in glycolysis (10) (Fig. 2).

Furthermore, hyperglycemia increases triose phosphates and NADHc in human erythrocytes that lack mitochondria as a source of superoxide to inhibit GA3P-DH (7), and the increase in trioses is prevented by addition of pyruvate and by an ARI. These and other observations support the importance of NADHc formed by the sorbitol pathway (rather than, or in addition to, increased mitochondrial oxidation of pyruvate) in mediating inhibition of GA3P-DH, elevation of triose phosphate levels, and increased superoxide production evoked by hyperglycemia.

Other seemingly discordant reports regarding a role for NADHc in the pathogenesis of diabetic complications are attributable largely to *1*) important caveats inherent to experimental paradigms used, e.g., experimental galactosemia, and *2*) the short half-life of inhibitors of sorbitol dehydrogenase used to test the hypothesis. (See online appendix, section IV.)

Interactions between hyperglycemia and hypoxia on ATP synthesis: implications for worsening of retinopathy with improved glycemic control. Substantial increases in ATP synthesis via substrate phosphorylation evoked by hyperglycemia (in aerobic and hypoxic retinas) suggest an explanation for transient early worsening of diabetic retinopathy associated with rapid normalization of blood glucose levels in individuals with long-standing poor glycemic control (50). Namely, the mass action effect of elevated glucose levels that increases ATP synthesis by substrate phosphorylation will be attenuated. Adverse effects of lowering glucose levels will be more pronounced to the extent that *1*) the retina is hypoxic and more dependent on ATP synthesis by substrate phosphorylation and *2*) vascular disease restrains increased retinal blood flows (and associated delivery of glucose and oxygen) evoked by physiological visual stimulation and dark adaptation. (Increased retinal blood flow in response to dark adaptation is impaired in diabetic rats [51]. In normal human subjects, acute hyperglycemia impairs dilation of retinal vessels in response to flicker photostimulation [52].)

In conclusion, these observations attest to the potential of additive increases in NADHc evoked by hyperglycemia and hypoxia to promote the onset and progression of diabetic retinopathy via metabolic pathways fueled by NADHc. This conclusion and recent observations that increased retinal and visual cortex blood flows evoked by photostimulation are mediated by an increase in NADHc$\propto$L/P (53,54) are consistent with the central role of free NADc in fueling energy metabolism and signaling pathways that coordinate blood flow with energy metabolism in resting cells, during physiological work, and in pathophysiological states including diabetes and hypoxia.

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