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Forum Review

Pyridine Nucleotide Redox Abnormalities in Diabetes

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ABSTRACT

In addition to hyperglycemia, diabetes is associated with increased levels of circulating free fatty acids, lactate, and branched chain amino acids, all of which produce an excessive reduced form of pyridine nucleotides NADH (reductive stress) in the cytosol and mitochondria. Our studies suggest that cytosolic NADH reductive stress under high glucose is largely caused by increased flux of glucose through polyol (sorbitol) pathway consisting of aldose reductase and sorbitol dehydrogenase. Inhibition of aldose reductase that blocks the polyol pathway has been shown to ameliorate diabetic neuropathy in humans. Cytosolic NADH reductive stress is predicted to increase production of diglycerides, reactive oxygen species, and methylglyoxal. Recent studies indicate that increasing NADH affects gene expression through the NADH activating transcriptional co-repressor, C-terminal binding protein (CtBP). In addition, it has been shown that the NADH utilizing enzyme, glyceraldehyde-3-phosphate dehydrogenase, participates as transcriptional regulator. These findings testify to the importance of NADH redox balance in cell biology and pathogenesis of diabetes and its complications. For example, through CtBP, the high NADH to NAD^+ ratio decreases an expression of SirT1, the protein inducing longevity and anti-apoptosis. This review covers metabolic cascades causing reductive stress and oxidative stress in diabetes after a brief introduction of the redox concept. *Antioxid. Redox Signal.* 9, 931–942.

INTRODUCTION

CHEMICAL REACTIONS involving movement of electrons are called redox reactions (reduction and oxidation). This review will describe characteristics of redox abnormalities observed in diabetes. Diabetes affects a number of tissues and organs including liver, micro- and macrovasculature, heart, kidney, retina, lens, and peripheral nerves. Both the DCCT (Diabetes Control and Complications Trial) (89) and a UK prospective study (114) clearly demonstrated the importance of hyperglycemia as a causal role of these pathologies. However, a less appreciated fact is that metabolic abnormalities associated with diabetes are not limited to hyperglycemia; this review will begin with this subject. Substantial reports describe metabolic pathways to understand pyridine nucleotides redox couple abnormalities that are associated with diabetes (56, 118, 122). The sorbitol pathway will be discussed as the major causative pathway for redox imbalance in diabetes, in addition to recent reports of aldose reductase inhibitors. Finally, I review recent topics about roles of NAD and NADH redox signaling in gene expression.

Although thiol redox and thiol modifications are important topics in cellular regulation, specific topics about diabetes are scarce; therefore, this subject will not be covered in this review.

EXCESSIVE FUEL SUBSTRATES AS CHARACTERISTICS OF DIABETES

Redox abnormalities can be found in many diseases in many ways. The most notable changes happening in diabetes are possible abnormalities in the NAD^+–NADH redox couple (56, 118, 122). NADH is produced by substrate oxidation. By definition, plasma glucose levels are elevated in diabetes. Insulinopenia observed in type 1 diabetes increases the levels of plasma ketone bodies. In type 2 diabetes, elevated levels of plasma ketone bodies were reported (3). Less known facts are

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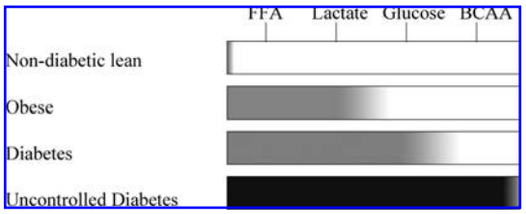

FIG. 1. Excessive substrates supplies found in obese and diabetes. From obese to diabetes, circulating fuel supplies increase. *Gray area* indicates increase the levels of particular fuel. For example, obese are frequently found with high levels of plasma free fatty acids and lactate but without hyperglycemia.

that plasma concentrations of free fatty acids (FFA) (11), lactate (4, 20, 53, 113), and branched chain amino acids (BCAA, including leucine, isoleucines, and valine) (107, 116) are also elevated in diabetes. Higher plasma levels of FFA and lactate are reported even in the obese population (107). These reports suggest that in the patients with obesity and type 1 and 2 diabetes, plasma levels of fuel substrates are generally increased (Fig. 1). These increasing levels of substrates could enhance insulin secretion from β-cells because they produce more ATP, but at the same time lead to redox imbalance and oxidant stress in other tissues. Potentially higher levels of glucose and lactate can increase NADH in cytosol (Fig. 2) whereas ketone bodies, FFA, and BCAA increase NADH in mitochondria (92) (Fig. 2).

In turn, such redox imbalance may further increase the levels of circulating substrates. Fatty acid synthesis requires cytosolic acetyl-CoA and cytosolic NADPH that are produced from citrate solely coming from mitochondria (92). This process is upregulated when isocitrate dehydrogenase is inhibited by the increased the levels of mitochondrial NADH (92) (Fig. 3). Glycerol-3-phosphate (glycerol-3-P), a precursor of diglyceride and triglyceride, is the product of glyceraldehyde-3-phosphate using cytosolic NADH, and its production can be upregulated by increased cytosolic NADH levels (92) (Figs. 2 and 3). Randle showed that glucose oxidation of mitochondria was inhibited by the presence of free fatty acids (88). Lactate, as well as fatty acids, have been postulated to produce insulin resistance (68, 119).

Oxidation of BCAA starts with cytosolic deamination by branched-chain amino acid transferase, and this step requires 2-oxolgutarate to produce glutamate, which is a part of the malate–aspartate shuttle (73, 92). Therefore, it could directly compete with cytosolic NADH shuttling activity to mitochondria and may contribute to increasing cytosolic NADH. In addition, the products of BCAA deamination directly go to mitochondria and produce NADH by branched-chain alpha-ketoacid dehydrogenase and enter the TCA cycle to produce acetyl-CoA (Fig. 2).

It is worth mentioning the peculiar nature of lactate. Whereas glucose, free fatty acids, and amino acids can also be used for anabolic reactions (*i.e.*, glycogen, triglyceride, and protein synthesis without producing NADH), there is no direct pathway for lactate to be used other than to be first converted to pyruvate by lactate dehydrogenase (73). Thus, extracellular lactate inevitably produces NADH in cytosol in the cells that metabolize it. In other words, lactate conveys electron signals from one cell that produces it to other cells that metabolize it, essentially working as an intercellular signaling molecule (44, 45). Standard redox potential suggests that converting lactate to pyruvate is not a favorable reaction (22). Therefore, textbooks only describe that circulating lactate is used for gluconeogenesis or lipid synthesis (Fig. 3) in the liver (92). However, during work such as exercise and neural activity in brain, skeletal muscle and neural glial cells produce high levels of lactate from glycolysis. The levels are high enough to be metabolized in nearby vascular cells to cause expected cytosolic NADH redox change, which subsequently induces vascular dilatation and increased blood flows (44, 45). Similar increased blood flow is observed by infusion of glucose or in diabetes. In this case, cytosolic NADH redox change is induced by sorbitol pathway as discussed later.

Taken together, the redox imbalance found in diabetes is characterized by excessive substrate levels that produce more NADH in cytosol and mitochondria. The imbalance may lead to fundamental biochemical changes and plays a significant role in the development of diabetes and its complications. In the next section, metabolic pathways producing pyridine nucleotides reducing equivalents (NADH and NADPH) will be discussed more detail.

SOURCES OF NADPH AND NADH

In the cells, total NAPDH levels are two–three-fold more abundant than total NADH (56, 118, 122) (Fig. 4). Because of high binding of NADH to the protein, free cytosolic NADPH levels are expected to be much higher than free NADH (56, 118, 122) (Fig. 4). Therefore, cytosolic reducing capability depends almost completely on NADPH (104) and the enzymes reducing oxidized-thiols employ exclusively NADPH as an electron donor. Two pathways produce cytosolic NADPH: pentose phosphate pathway (Figs. 2a and 3a) (92, 105) and pyruvate–malate/citrate shuttles (23, 63) (Figs. 2d and 3b). Whereas the pentose phosphate pathway oxidizes glucose to reduce NADP to NADPH (Figs. 2a and 3a) (92), the latter pathways use NADH as an electron donor to produce NADPH through a sequence of enzymatic reactions (23, 63). As net reactions, the pyruvate–malate shuttle converts mitochondrial NADH to cytosolic NADPH, which can be utilized to reduce oxidized glutathione or other thiol molecules (Fig. 2d) (63). In contrast, the pyruvate–citrate shuttle uses cytosolic NADH (Fig. 3b) (23, 92) to produce NADPH and acetyl-CoA in cytosol, and is the favored reaction for fatty acid synthesis in the liver (Fig. 3) (12, 92). Under ischemic conditions, the rat heart was demonstrated to produce cytosolic NADPH and pyruvate from aspartate via the cascade similar to the pyruvate–malate shuttle (84). Presence of these shuttles suggests that: a) cytosolic reducing power (NADPH) does not solely come from the cytosolic pentose phosphate pathway, and b) mitochondrial NADH produced by substrates oxidation can provide cytosolic NADPH. Consistent with these, in diabetes, the total NADPH levels are not decreased but increased in lens (55, 117), nerves (46, 80), and liver (31). Mitochondrial substrate oxidation also can produce mitochondrial NADPH by the reactions catalyzed by NADP-dependent isocitrate dehydrogenase and mitochondrial transhydrogenase (Fig. 2f) (38).

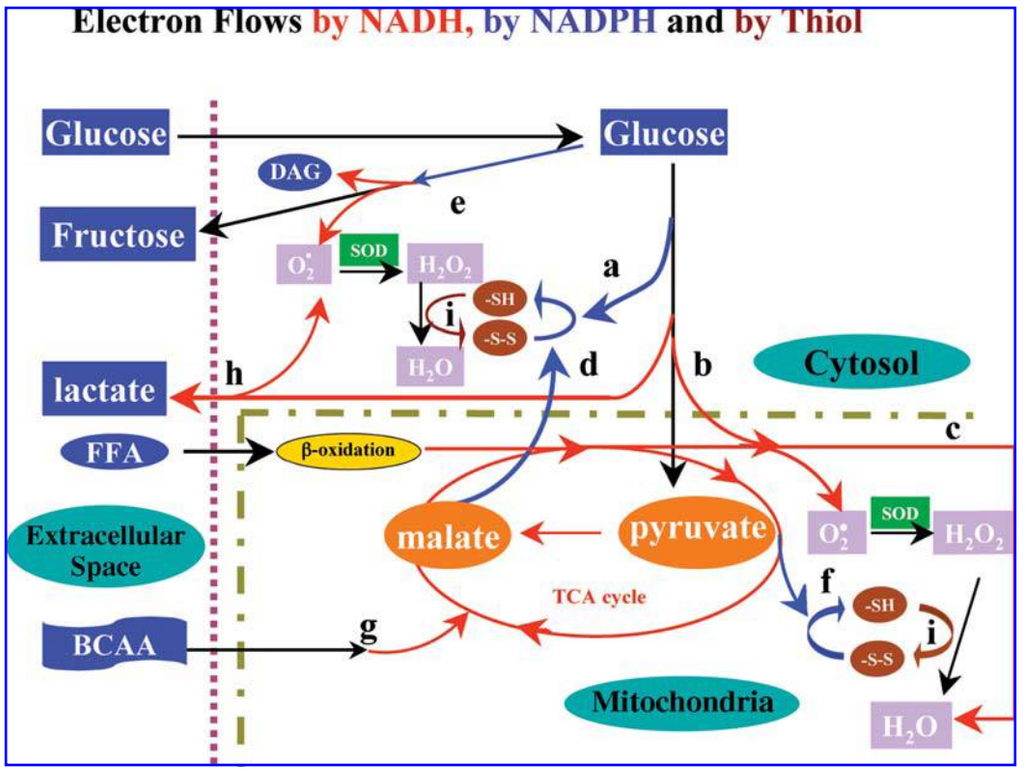

FIG. 2. Metabolic pathways (1). Each colored line represents electron flows produced by substrates oxidation. (**a**) Pentose phosphate pathway, (**b**) malate-aspartate shuttle and glycerol-3-phosphate shuttle, (**c**) electron transport system in mitochondria, (**d**) pyruvate-malate shuttle, (**e**) polyol (sorbitol) pathway, (**f**) isocitrate dehydrogenase and transhyrogenase reaction, (**g**) branched chain alpha-ketoacid dehydrogenase, (**h**) lactate dehydrogenase, (**i**) glutathione peroxidase.

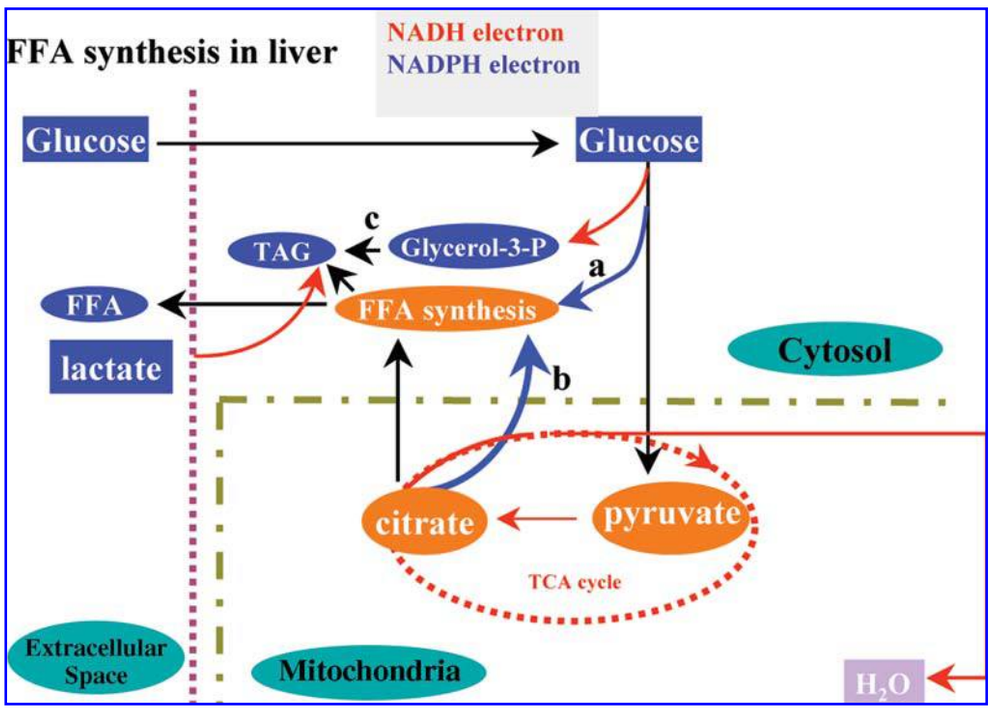

FIG. 3. Metabolic pathways (2). (**a**) Pentose phosphate pathway, (**b**) pyruvate-citrate cycle, (**c**) di- and triglyceride synthesis.

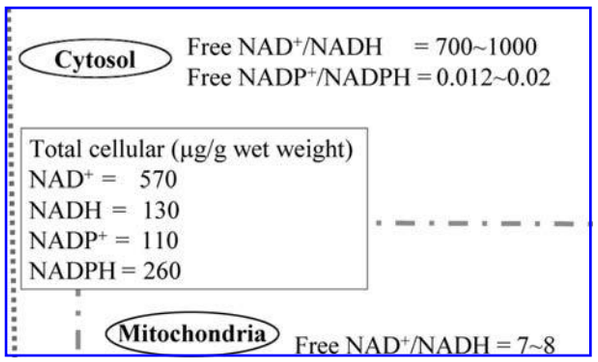

FIG. 4. Pyridine nucleotides distribution in the liver.

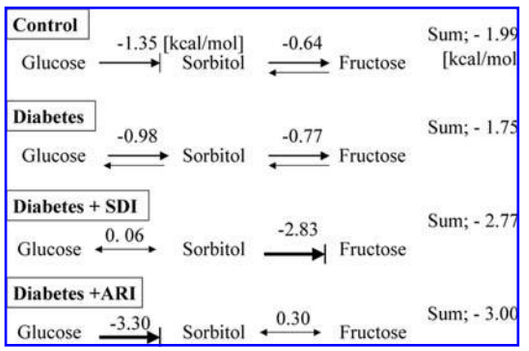

FIG. 6. Gibbs free energy (ΔG) through the sorbitol pathway.

Although the absolute levels of free NADH in cytosol is very low (<1 μ*M*) (130), fluctuation of cytosolic NADH can impact on the metabolic cascade. In physiological conditions, cytosolic free NAD^+/NADH ratios in various tissues are between 400–800, as calculated by metabolite indicator methods assuming near-equilibration of lactate dehydrogenase (Fig. 4) (31, 122–125). The total cellular NAD^+/NADH ratios are 2–4, suggesting that most (~99%) (16) of NADH is bound and is not readily available for reactions. Through glycolysis, two NADH molecules are formed in the cytosol, and if all pyruvate are converted to lactate by lactate dehydrogenase oxidizing NADH to NAD^+, which happens in red blood cells, a net production of NADH in cytosol equals to zero (Fig. 2h). Cells other than erythrocytes utilize pyruvate for oxidation in the TCA cycle in varying degrees, which makes the pyruvate amount short for reoxidation of cytosolic NADH (73). Thus, the net production of cytosolic NADH (mole) through glycolysis will be equal to two times the glucose (mole) minus pyruvate utilized in mitochondria (mole), which has to be reoxidized in mitochondria by the malate–aspartate and glycerol-3-P shuttles (Fig. 2b) (73, 92).

Another source of NADH produced in cytosol is the sorbitol pathway (Figs. 2e and 5), which consists of two enzymes: aldose reductase and sorbitol dehydrogenase (39, 112, 117). As a net reaction, one NADHP and glucose are converted to one NADH and fructose (Fig. 5). As mentioned before, free NADPH is far more abundant than free NADH, and the standard reduction of NADH and NADPH is close to equal (–0.315 vs –0.320 V) (22). Therefore, the rate of net flux could be higher, but actually glucose, the substrate of this reaction is not a good substrate for aldose reductase (Km = 95 m*M*; human aldose reductase (121). This is partly because aldose reductase only takes an aldehyde-open (not pyranose) form of glucose as a substrate (55).

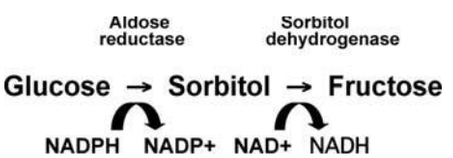

FIG. 5. Sorbitol pathway.

Figure 6 shows the thermodynamic aspect of this reaction (unpublished data). The Gibbs free energy (ΔG) at each step of this pathway (22) was calculated from the sciatic nerve data obtained from the experiment using the rats with 4 months duration of diabetes treated with inhibitors of aldose reductase (ARI) or sorbitol dehydrogenase (SDI), using published thermodynamics data of the enzymes (28). As shown in Fig. 6, even in nondiabetic rats, ΔG of the pathway is negative, indicating the presence of potential flux. In nondiabetic control rats, ΔG of aldose reductase gives –1.36 kcal/mol that can be judged as far from equilibrium (| ΔG| >1.0 (22, 104)); meaning that this is the rate limiting step in the pathway. In diabetes, | ΔG| of this step was decreased, which may indicate upregulation of activity (103) or expression of aldose reductase (52). Interestingly, the sorbitol dehydrogenase step does not become rate limiting unless addition of SDI, suggesting that sciatic nerve contains enough sorbitol dehydrogenase activity to catalyze this step even in diabetic conditions.

Measurement of absolute flux of the pathway has been hampered by the fact that fructose can be diffused out from the cells as well as metabolized in glycolysis (78). Nevertheless, *ex vivo* incubation studies done by us using the rat retina showed that fructose production was increased 55–74-fold under 30 m*M* glucose, which was >9–18-fold increase in sorbitol (78). Recent studies by the group of Ramasamy using the rat heart suggest that substantial flux which impacts on cytosolic NADH redox appears to exist at least during ischemic condition (43, 51, 87).

UTILIZATION OF NADH AND ITS ROLE IN DIABETIC METABOLISM

The cytosolic free NAD^+/NADH ratio is approximately 600~800 which can be reduced to 100–200 by minute increase of NADH from NAD^+:NADH = 600:1 to 600:3 ~600:6 (56, 118, 122). Such a small increase in NADH can affect many metabolites levels governed by the enzymes using NAD^+-NADH as cofactors. For example, the pyruvate to lactate ratio catalyzed by lactate dehydrogenase is changed proportionally to the NAD^+ to NADH ratio if pH is constant (56, 118, 122).

The excessive NADH produced in cytosol must be oxidized to maintain the glycolysis flux, since amounts of cofactors are far less than the flux. Two shuttling systems, glutamate–aspartate shuttle and glycerol-3-phoshate shuttle, transfer NADH to mitochondria (Fig. 2b). The mitochondrial free NAD^+/NADH ratio, however, is two orders of magnitude lower than cytosol because of substrate oxidation in mitochondria (31). Therefore, the glutamate–aspartate shuttle transfers cytosolic NADH against redox potential difference between cytosol and mitochondria. This is enabled by the fact that this shuttle is energetically coupled in the way to lower mitochondria membrane potential when each aspartate is transferred into mitochondria (13, 21, 122). In contrast, glycerol-3-phosphate shuttle transfers NADH to FAD^+ that is higher redox potential couple and does not require the same mechanism as glutamate–aspartate shuttle (73).

Although the shuttle activity keeps cytosolic NADH in the normal range, the activity cannot keep up if glycolysis and/or pyruvate utilization are rapidly increased. In addition, the activity could be compromised in diabetes when mitochondrial substrate oxidation is increased and cytosolic NADH production through sorbitol pathway is upregulated. This results in increased cytosolic NADH/NAD^+ ratio, which impacts on the levels of metabolites and fluxes in several ways (Fig. 8) (44–47, 123–125).

First, the flux from dihydroxyacetone phosphate to glycerol-3-phosphate will be increased. This reaction is a part of the glycerol-3-phosphate shuttle but is also shared with neutral lipids like diglyceride *de novo* synthesis pathway (Figs. 2 and 3c).

Second, the levels of glyceraldehyde-3-phosphate (GAP) will increase. GAP dehydrogenase (GAPDH) reduces NAD^+ and mass action ratio, although this enzyme is sensitive to the cytosolic NAD^+:NADH ratio. Therefore, increasing NADH to NAD^+ ratio increases G3P and dihydroxyacetone phosphate which are known to increase methylglyoxal synthesis (8). Similar to lactate dehydrogenase reaction, flux through GAPDH (glycolysis) is not be inhibited by increasing levels of NADH as far as NADH shuttling is functional.

Third, NAD(P)H oxidase preferentially utilizes cytosolic NADH as substrate (70, 126) (Fig. 2) to produce superoxide or hydrogen peroxide. As explained earlier, free NADPH levels are far more abundant than NADH. In classical NAD(P)H oxidase in macrophages, the protein gp90 is located on plasma membrane and uses NADPH for an electron donor to produce superoxide and/or hydrogen peroxide outside the cells (126). On the other hand, some NAD(P)H oxidase(s) found in other types of cells (endothelial, vascular smooth muscle, and fat cells) appear to be located in cytoplasmic space as well as membrane and produce reactive oxidants inside the cells.

Lastly, increasing cytosolic NADH levels affects gene expression. This subject will be discussed later.

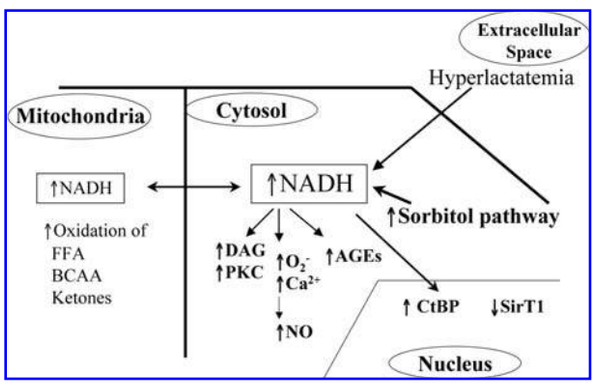

FIG. 8. Cytosolic NADH redox signaling pathway in diabetes. Under hyperglycemia, increased cytosolic NADH is mainly caused by activation of sorbitol pathway. Hyperlactatemia and oxidation of FFA, BCAA, and ketone bodies in mitochondria produce NADH in cytosol and mitochondria enhancing sorbitol pathway effect to increase cytosolic NADH. High levels of NADH upregulates diglyceride synthesis, superoxide production, and advanced glycation end (AGE) formation. In addition, a high level of NADH activates CtBP transcriptional co-repressor that suppresses an expression of proteins like SirT1.

CYTOSOLIC NADH REDUCTIVE STRESS HYPOTHESIS IN DIABETES

The assay of pyridine nucleotide became possible in the 1950s. In the late 1960s to 1970s, the metabolite indicator method which enables estimating free pyridine nucleotide ratios was developed and revealed that levels of free pyridine nucleotide, in particular NADH, are very different from total levels (56, 118, 122). The method is based on the assumption that if the enzyme activity is high enough compared to the flux, the mass action ratio through this enzyme becomes closer to the ratio predicted by the equilibrium constant measured in closed system (31).

For example, lactate dehydrogenase present in cytosol catalyzes the reaction (28, 122);

$$\text{Pyruvate} + \text{NADH} = \text{lactate} + \text{NAD+ H}^+ \ (\text{K} = 1.11 \times 10\text{^}4 \text{ at pH } 7.0)$$

This equilibrium constant, which is measured in a closed system *in vitro*, can be applied to calculate free cytosolic NAD^+/NADH ratio *in vivo*. Although pyruvate is present in mitochondria, and there is constant flow from pyruvate to lactate (or lactate to pyruvate in gluconeogenesis), the enzyme activity appears to be high enough to make this reaction near-equilibrium (56, 118, 122). The method was validated by the values calculated from other enzymes that use the same cytosolic NAD^+-NADH pool showed very similar values (56, 118, 122). Recently, more advanced methods employing two-photon-excitation microscopy and fluorescence lifetime imaging independently confirmed the validity of the method (59).

During developing the method, the researchers reported changes of reduced-to-oxidized ratios of pyridine nucleotides in the diabetic rat liver (31). Diabetes decreases cytosolic free NAD^+/NADH ratio from ~700 to ~350 and increases cytosolic NADPH/$NADP^+$ ratio from ~110 to ~170. It was also reported that there are substantial differences in the NAD^+/NADH ratio

between mitochondria and cytosol, as described earlier (31) (Fig. 4). Subsequent studies confirmed that such changes caused by diabetes are not unique to the liver but are observed in other tissues. For example, in the article published in 1974 describing the sorbitol pathway (117), Varma and Kinoshiata reported a similar decrease in NAD^+/NADH ratio and increase in NADPH/ $NADP^+$ in diabetic lens.

Based on these facts and results of studies examining the roles of sorbitol pathway in diabetes, Williamson's group in Washington University, St. Louis, including the author of this review, postulated (123) that: (a) the decrease in cytosolic NAD^+/NADH ratio (an increase in NADH in essence) in diabetes plays a major role in developing diabetic complications; (b) sorbitol dehydrogenase (SDH) in the sorbitol pathway is responsible for this redox change under hyperglycemic conditions; (c) beneficial effects of inhibition of aldose reductase can be explained by inhibition of flux through the pathway. Because decrease in cytosolic NAD^+/NADH is also observed in hypoxic condition, we named this as "pseudohypoxia" or reductive stress hypothesis (47, 111, 124, 125) (Fig. 8). Others and we have been testing these three aspects of this hypothesis for the past 10 years.

There is consensus that diabetes is associated with decreased cytosolic free NAD^+/NADH ratio in tissues or cells, including lens(117), retina (29), peripheral nerves (46), kidney (109), red blood cells (112), aorta (71), and the liver (118, 122).

Evidence shown by us suggests that the cytosolic NAD^+/NADH ratio plays a significant role in regulating regional blood flow. Hyperglycemia-induced increased blood flow was prevented by pyruvate, ARI, and SDI in the tissue chamber models, suggesting that involvement of NADH redox changes though sorbitol pathway (111). Similarly, in the rat, diabetes as well as infusion of glucose for 3–5 h increased blood flow in the retina, kidney, and sciatic nerves. We also observed that infusion of L-lactate but not D-lactate increased regional blood flow in various tissues (44, 45). From these results, we postulated that similar redox change mediates increases in regional blood flow under physiological conditions such as exercise or brain activity, since these conditions induce similar redox change. We demonstrated that increased brain blood flow associated with brain activities can be modulated by changing plasma lactate and pyruvate ratios, strongly indicating that decreasing NAD^+/NADH ratios induced by increasing glycolytic flux in neuronal glial cells regulates regional blood flow (44, 45). Similar mechanisms were found operational in humans (69). During this study, we also found the mechanism to increase blood flow that involves a small increase of superoxide production by NADH oxidase that subsequently activates nitric oxide (Fig. 8) (45). Although it was widely believed at that time that superoxide only attenuates nitric oxide action, there were ample examples to suggest that superoxide or oxidative stress can increase blood flow (26, 108, 120, 127). It was found later that glutathiolation of sarcoendoplamic reticulum Ca^{2+}-ATPase at cysteine 647 by peroxinitrite, a product of superoxide and nitric oxide (86), increases its activity to uptake Ca^{2+} and causes vasodilatation, essentially confirming our observations that both superoxide anion and nitric oxide are required for vasodilatation (1).

In streptozotocin diabetic rats, we demonstrated that inhibition of SDH as well as aldose reductase normalized the NAD^+/NADH ratio and prevented neural and vascular dysfunctions associated with diabetes (110). Geisen *et al.* first reported normalization of increased glomerular filtration rate and motor nerve conduction velocity deficit caused by diabetes (27). A later series of experiments were performed and all of them confirmed the results of this initial study. For example, as shown in Fig. 7, in this experiment, streptozotocin diabetic rats were treated with 200 mg/kg/day SDH inhibitor (SDI) CP-166,572 (Pfizer, Groton, CT) and 100 mg/kg/day Zopolrestat (ARI) for 4 months. Both ARI and SDI were equally effective to prevent motor nerve conduction velocity (MNCV) deficit and abnormalities in cytosolic ratios of NAD^+/NADH in sciatic nerve.

Consistent with these results, it was reported that a transgene of human aldose reductase (AR) augmented diabetes-induced

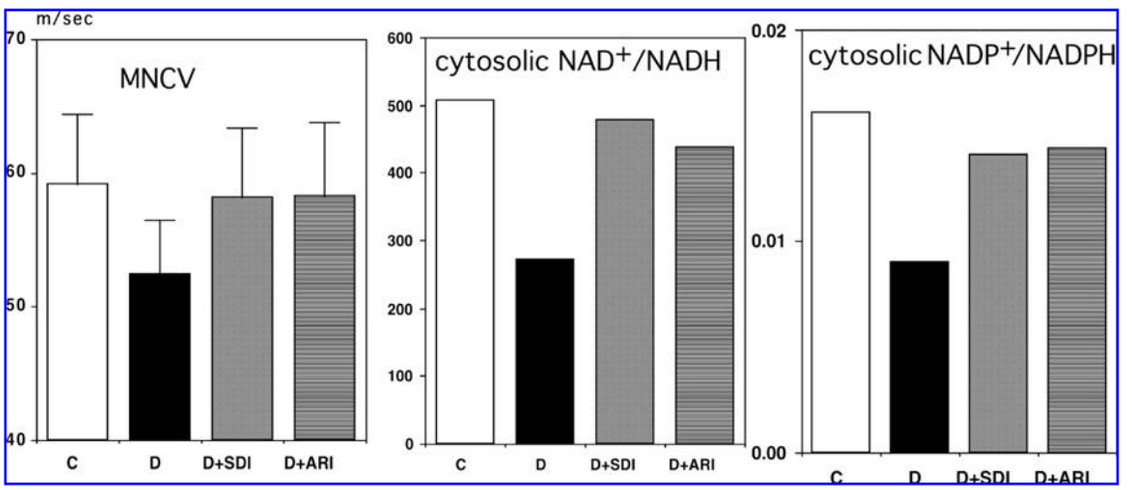

FIG. 7. Effects of aldose reductase inhibitor (ARI) and sorbitol dehydrogenase inhibitor (SDI).

NAD^+/NADH ratio change in the mouse kidney, and this effect was prevented by inhibition of aldose reductase (48). Depletion of SDH with AR-transgenic mice attenuated diabetes-induced oxidative stress (19, 60). Ramasami's group showed that SDH plays a major role in determining cytosolic NAD^+/NADH ratio in the heart, even in nondiabetic rats, and inhibition of aldose reductase or SDH attenuated ischemic injury in the heart (43, 51). Similar protecting effects of ARI in the heart were obtained in aldose reductase transgenic mice (49). However, deletion of aldose reductase but not sorbitol dehydrogenase was shown in mice to protect from ischemic injury in the brain (61). These observations, particularly in the rat heart, suggest that there is a substantial flux through the sorbitol pathway impacting on cytosolic NAD^+/NADH change during ischemia. This phenomenon may be related to the activation of aldose reductase by cysteine modification happening in ischemia (50). In cultured retinal pericytes, high glucose-induced apoptosis was increased by overexpression of SDH (2). This result suggests that, unlike rat sciatic nerve, normal bovine retinal pericytes may not contain enough SDH activity; that remains to be confirmed.

In contrast to these supportive data, Cameron *et al.* could not repeat the beneficial effects of SDH inhibition on neural dysfunction (17), although they used a different compound and the NAD^+/NADH ratio was not assessed. Similarly, Obrosova *et al.* found that SDH was ineffective to prevent diabetes-induced NAD^+/NADH redox change in the nerve and retina (82, 83). SDH-depleted mutant mice were found viable, and development of diabetic neural and renal dysfunction in these mice was not different from control mice (48, 75). However, since NAD^+/NADH ratios were not assessed in these animals, it is not possible to conclude whether redox changes were prevented by SDH depletion. Because of potential complications inherent with gene disruption, SDH-deficient mice may develop an alternative pathway to metabolize sorbitol which causes similar NAD^+/NADH change. Here is the reason to suspect this possibility. In galactose-fed rats, decreased NAD^+/NADH was observed in lens (79), that was partially prevented by ARI. Rat retina incubated with galactose causes a similar decline in the NAD^+/NADH ratio (78). These results are surprising because SDH does not take and convert galactitol to tagatose as it does sorbitol to fructose (78). Therefore, galactose should not decrease the NAD^+/NADH ratio. A similar observation was obtained from the experiment in which erythrocytes from the patients with galactose-1-phosphate uridylyltransferase deficiency were incubated with galactose (10). Nobody except us suspected that galactose produces similar redox change in NAD^+/NADH as glucose does. One possible explanation for this enigmatic phenomenon is that there may be an alternative pathway to produce the NADH redox change from galactitol. Therefore, it is necessary to assess NAD^+/NADH ratio in SDH-deficient mice to interpret the results.

Nevertheless, in order to halt the flux of sorbitol pathway, inhibition of sorbitol dehydrogenase may not be a better strategy than inhibition of aldose reductase. As shown in Fig. 6, when SDH inhibitor (SDI) was administered, the reaction through aldose reductase became near-equilibrium ($\Delta G \sim 0$), Similarly, when ARI was given, the sorbitol dehydrogenase reaction became near-equilibrium. These are expected changes because the flux stopped. However, to do that, SDI has to increase the sorbitol amount tremendously. In diabetic nerve, this corresponds to a 40- to 50-fold increase in sorbitol. We did not observe any pathology in nondiabetic SDI-treated animals, while tissue sorbitol levels in these animals were comparable to diabetic rats. However, under the stress condition as diabetes, such drastic increase in sorbitol may decrease other osmolytes, including taurine which may work as antioxidant (37) and may outweigh the benefit of stopping the flow through the sorbitol pathway in tissues such as autonomic nerves (94–96). This would be particularly true if SDH inhibitor's half life is short and the dose is not enough. Reported negative results using SDI-157 (82, 83) may fit this case since its active molecule's half life is only 30 min, and a dose of 100 mg/kg/day appears to be too small to inhibit continuously (78).

The publications reporting beneficial effects of aldose reductase inhibition in diabetic models are simply too many to be covered, therefore only recent topics are mentioned here. The result of a 3-year study to evaluate Epalrestat, clinically approved ARI in Japan, on diabetic polyneuropathy confirmed its efficacy observed in early studies (41), suggesting that this could be a final proof of ARI's efficacy in neuropathy (106). Similarly, a 60-week study of Ranirestat demonstrated its efficacy on diabetic polyneuropathy (14). Epalrestat treatment has also been shown to decrease erythrocyte *N*-(carboxymethyl)lysine and 3-deoxyglucosone levels in diabetic patients (36), supporting our hypothesis (Fig. 8). Aldose reductase-deficient mice was created and reported to be protected from nerve dysfunctions caused by diabetes (40), although the effects on morphological changes were modest.

Some investigators postulate that such redox changes are the results of hypoxia (81) or activation of PARP (poly-ADP ribosyl transferase) (101, 102). These factors could contribute similar redox changes mentioned above and would be important particularly in the late stage of diabetic complications in humans. Although these may happen in some animal models of diabetes, tissue hypoxia is usually associated with severe neuropathy or proliferative retinopathy in patients with long-term or severe diabetes. Our experiments showed that polyol pathway-mediated NAD^+/NADH changes occur very quickly within 1 h incubation with high glucose medium (78, 115) independent of hypoxia (78), suggesting this mechanism is operable without sustained hyperglycemia and could occur in the conditions such as postprandial hyperglycemia. Therefore, the polyol pathway-mediated redox change mechanism proposed by us likely contributes in the early stage of diabetes to develop diabetic complications.

MITOCHONDRIAL SUBSTRATE OXIDATION AND OXIDATIVE STRESS IN DIABETES

Mitochondria have been of recent interest as a source of oxidative stress, particularly in diabetes (15). The most important function of mitochondria is undoubtedly to produce ATP. This is done by transporting protons from inside to outside though the inner membrane, which is coupled with movement of electrons on the electron transport system (73, 92). The electron transport is the site where superoxide

radical is produced (15). Both complex I (30) and III are thought to be the major sites to generate superoxide radicals. However, there seems to be a difference between these two in nature; complex I appears to produce superoxide only toward inside (30), whereas complex III does toward both inside and outside at 1:1 ratio (72). It is also said that complex III generates superoxide in hypoxia (32, 33).

Recent cell biology studies have elucidated the mechanisms of mitochondrial genesis. Nuclear transcription cofactor PGC1α (peroxisome proliferators-activated receptor gamma coactivator 1 alpha) appears to play the most important role (42). Upregulation of PGC1α has been shown to be sufficient to increase the number of mitochondria in various cell types (42). Like other nuclear factors, the acetylation status of PGC1α regulates its binding to other nuclear factors, thereby, deacetylation of PGC1α by SirT1 (58, 74, 91), a NAD^+-dependent deacetylase (34) which will be mentioned later, increases PGC1α function and upregulates mitochondrial biogenesis. Expression of PGC1α is also upregulated by nitric oxide, more precisely, activation of cGMP-dependent protein kinase, therefore, nitric oxide production plays an important role in regulating mitochondria numbers (77).

Although increased mitochondrial substrate oxidation has been postulated to increase oxidative stress in diabetes (15), recent reports suggest a more complicated process. For example, caloric restriction, which induces PGC1α activation through SirT1, is associated with increased mitochondrial number (62) and metabolic rate (99) but decreased oxidative stress (62) . Type 2 diabetes and insulin resistance are associated with decreased but not increased mitochondrial oxidation function in skeletal muscle (7). Activation of AMP-activated protein kinase protects endothelial cells from high glucose-mediated apoptosis by increasing but not decreasing mitochondrial numbers through a PGC1α-mediated mechanism (57). These examples suggest that the number of mitochondria but not increased metabolism in the mitochondria is important in diabetes.

Another line of investigations in mitochondrial biology revealed that mitochondria could change their shape and number quickly by fission and fusion processes (18, 67). Apparently, increased ROS production by high glucose in endothelial cells, as shown by Brownlee *et al.* (76), requires mitochondrial fission (129). The fusion process of mitochondria is partly controlled by the protein mitofusin, and a recent publication showed that mitofusin 2 mRNA levels are decreased in the skeletal muscle from type 2 diabetes (5), which suggests that mitochondria in type 2 diabetes could be smaller in size, round in shape, and prone to produce superoxide (67). Collectively, recent results challenge the simple vision that substrate oxidation in mitochondria determines ROS production.

IMPACT ON REDOX CHANGES ON SIGNALING

Although the major function of NAD^+ is to accept electrons in enzymatic reactions, NAD^+ itself can be used as a substrate in cell signaling. Similarly, NADH has another face in regulating gene expression.

Sir2 (Silent Information Regulator 2)

Sir2 is an NAD+-dependent histone/protein deacetylase; the enzyme catalyzes removal of acetate from acetylated lysine in the target proteins. There are seven homologs in mammals (SirT1–SirT7), among them, SirT1 has been the most studied (100). A number of nuclear and cytosolic proteins are found acetylated, presumably by the enzymes acetylating enzymes such as p300/CBP, PC/AF, and GCN5 (65). Acetylated proteins are mostly found in the nuclear fraction but some, for example, tublin, are exclusively found in cytosol. In addition to phosphorylation, acetylation and deacetylation of nuclear transcription factors and cofactors play pivotal roles in their activity, stability, and in some case localization. For example, FOXO1 nuclear transcription factor is acetylated by CBP and deacetylated by SirT1 (66). Deacetylated FOXO1 loosens its binding to DNA and subsequently is phosphorylated by Akt which induces translocation to cytosol (66). Deacetylation of PGC1α by SirT1 results in activation, as mentioned before (6, 58, 91). Expression and activation of Sir2 has been linked to longevity and the health of mice fed with a high fat diet (7, 93).

In vitro Sir2 deacetylation activity absolutely depends on the presence of physiological levels of NAD^+. NAD^+ cannot be replaced by NADH, NADPH, or NADP, and these similar nucleotides had no effect on Sir2 activity at least *in vitro* (93). Sir2 deacetylation reaction breaks NAD^+ down to acetyl-ADP-ribose and nicotinamide. Nicotinamide can inhibit Sir2 *in vitro*. Mammalian cells salvages nicotinamide directly back to NAD^+ through two enzymes, NAMPT (nicotinamide phosphoribosyltransferase) and NMNAT (nicotinamide mononucleotide adenyltransferase) (90), which were identified only a few years ago. Overexpression of the rate-limiting enzyme NAMPT appears to increase NAD^+ and activate Sir2 (90). Therefore, in the cells expressing enough NAMPT, nicotinamide may actually work as an activator. By sensing fluctuation of the level of NAD^+, NAD^+/NADH ratio, Sir2 is considered as nutritional sensor.

It is not known how Sir2 activity can be modulated by post-translational modification. In expression levels, it has been shown in skeletal muscle of mice that streptozotocin-induced diabetes reduces the expression of mRNA of SirT1 and SirT3 (128). SirT1 expression levels are also upregulated by incubating with high levels of pyruvate, likely through increasing the NAD^+/NADH ratio (91). Similarly, skeletal muscle differentiation was found to be regulated by NAD^+/NADH ratio through SirT1 (25). Recently, a molecular connection between SirT1 expression and NAD^+/NADH ratio was revealed, and this subject will be discussed below. Although it is still not clear how the Sir2 cascade plays roles in diabetes or in development of diabetes, based on redox hypothesis, Sir2 functions are predicted to be downregulated by the conditions associated with diabetes.

Carboxy-terminal binding protein (CtBP)

Whereas SirT1 likely senses NAD^+, CtBP senses NADH (24, 130). The protein was originally isolated as the protein bound to the C-terminal domain of adenovirus E1A protein. Subsequent analysis revealed that the protein recognizes the PXDLS motif and that it associates with a number of nuclear

proteins and works as transcriptional corepressor (130). The protein sequence analysis revealed a significant homology to D2-hydroxyacid NAD dehydrogenase (130) and obviously it has a catalytic activity able to oxidize NADH with a substrate such as pyruvate . The protein works as transcriptional corepressor as coordinating histone modifications (98), interacting with acetyltransferase p300 (54) and Polycomb proteins (97), and plays essential roles in cell differentiation and survival (9).

As sensor of NADH, the protein is activated under hypoxia and regulates tumor migration (132). Recently, it was found that CtBP regulates SirT1 protein expression via forming a complex with HIC1 (hyperacetylated protein in cancer) transcriptional factor (131). Therefore, increased NADH negatively regulates SirT1 expression by CtBP:HIC1 complex. Finding the target proteins of CtBP will be very important for understanding redox-mediated gene regulation in diabetes.

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH) AS THE TRANSCRIPTIONAL REGULATOR

Because of the sequence homology to dehydrogenase, CtBP appears to be evolutionally derived from functional dehydrogenase or it can be a functional dehydrogenase whose true substrate is still not known. This link between dehydrogenase and transcriptional regulator is an interesting subject regarding gene regulation by redox (64). Indeed, similar to CtBP, GAPDH was found as an essential component in OCA-S coactivator complex regulating histone H2B expression (133). And interestingly LDH was also found in the same complex although its role is unclear. This coactivator activity of GAPDH was suppressed by binding to NADH, suggesting it is regulated by redox potential. Furthermore, GAPDH also directly interacts with general transcription factor OCT-1 (133), suggesting that this regulation may affect expression of a diverse array of genes. The recent work using chromatin immunoprecipitation analysis revealed that in yeast many metabolic enzymes work as transcriptional regulators directly binding to DNA (35). Similarly, LDH was also found to bind the AU-rich element region of mRNA, which indicated that LDH may work as a posttranscriptional regulator (85). These results indicate that there would be many other proteins like CtBP and GAPDH which directly involved in transcriptional and posttranscriptional regulation under redox change.

CONCLUSION

As mentioned in the Introduction, redox abnormality means abnormality of flows of electron, the energy for life. In cultured cells, hyperglycemia per se does not kill the cells, suggesting that the redox abnormalities that can be found in diabetes are rather subtle. That is why diabetes takes years to manifest to its effects and why finding the cure for diabetic complications is difficult. The polyol (sorbitol) pathway was first reported in 1959 (39); this pathway is still important today for understanding the redox abnormality found in diabetes. Recent advancement of our knowledge and techniques in cellular and molecular biology will reveal the secrets of redox balance not only diabetes, but also in cancer and aging.

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ABBREVIATIONS

ARI, aldose reductase inhibitor; BCAA, branched chain amino acids; CtBP, carboxy-terminal binding protein; FFA, free fatty acids; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDI, sorbitol dehydrogenase inhibitor; Sir2, Silent information regulator 2; ROS, reactive oxygen species.

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