NADH: sensor of blood flow need in brain, muscle, and other tissues

Yasuo Ido*[1], Katherine Chang[1], Thomas A. Woolsey[2], and Joseph R. Williamson[1]

*Diabetes and Metabolism Unit, Boston University Medical Center. Hospital, 88 East Newton St., Boston, MA; [1] Department of Pathology; [2]Department of Neurology and Neurological Surgery, Washington University School of Medicine, 660 South Euclid Ave., St Louis, MO

Corresponding author: Joseph R. Williamson, Department of Pathology, Box 8118, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110-1093. E-mail: jrw@PATHOLOGY.WUSTL.edu

ABSTRACT

The sensor for blood-flow need with neural activity and exercise is not known. We tested the hypothesis that accumulation of electrons in cytosolic free nicotinamide adenine dinucleotide (NAD) activates redox signaling pathways to augment blood flow. NAD is the primary carrier of electrons from glucose and lactate for ATP synthesis. Because increased glycolysis transfers electrons from glucose to NAD^+ faster than they are used for mitochondrial ATP synthesis, electrons accumulate in cytosolic NADH. Because cytosolic NADH and intra- and extracellular lactate/pyruvate (L/P) ratios are all in near-equilibrium, NADH can be increased or decreased by i.v. lactate or pyruvate. Here, we report that elevated plasma LJ`P in non-nal rats increases blood flow in numerous resting tissues and augments blood flow increases in activated somatosensory (barrel) cortex and contracting skeletal muscle. Increased flows are largely prevented by injection of pyruvate (to lower L/P), a superoxide dismutase mimic (to block vascular effects of superoxide), or an inhibitor of nitric oxide synthase (to block *NO vasodilatation). Electrons carried by. NADH, in addition to fueling ATP synthesis, also fuel redox

signaling pathways to augment blood flow in resting and working tissues. These novel findings are fundamental to understanding blood-flow physiology and pathology.

Key words: diabetes *glycolysis * NAD * nitric oxide * redox * superoxide * whisker barrels *work

The coupling of work to increased energy metabolism and augmented blood flow was recognized more than 100 years ago (1, 2). Increases in blood flow are widely viewed as a response to increased need for 02 and substrates (e.g., glucose, lactate, and lipids) for ATP synthesis and for removal of byproducts of energy metabolism. Recent studies show that brain blood-flow and glucose use with neural activity, surprisingly, exceed concomitant increases in 02 cons~rnption by as much as 10x despite normal or elevated 0_2 levels (3, 4). Furthermore, although increased blood flow is a hallmark of physiological work, enhanced flow is not required to support augmented glucose uptake for brief periods of neural activity (5-7). Mediators that increase blood flow are known, but the sensor(s) of blood-flow need has not been identified and the signaling cascade(s) that increase flow are not understood. Nerve and muscle work are fueled by hydrolysis of ATP to ADP, which activates glycolysis to replenish ATP. The electrons and protons that drive ATP synthesis are carried primarily by the cofactor NAD (8). With increased glycolysis, both the transfer of electrons and protons from glucose to cytosolic free NAD^+---reducing it to NADH---and the production of pyruvate exceed their use for mitochondrial ATP synthesis by oxidative Phosphorylation (OP). Excess electrons and protons accumulating in NADH drive reduction of pyruvate to lactate coupled to oxidation of NADH to NAD^+ by lactate-Dehydrogenase (DH) (Equation 1). This condition accounts for increased lactate production during aerobic work in brain and muscle (9-13).

Observations in diabetic animals first suggested to us that accumulation of electrons in NADH might augment blood flow. Increased flows in tissues affected by diabetic complications are linked to accelerated flux of glucose via the sorbitol pathway. Specifically, the oxidation of sorbitol to fructose is coupled to transfer of electrons and protons from sorbitol to NAD' increasing NADH as with increased glycolysis. This redox change is

independent of glycolysis, work, and oxygen tension (14-17); both redox. change and increased blood flows are prevented by inhibitors of the sorbitol pathway. When ATP synthesis by OP is limited by availability of oxygen (i.e., hypoxia), glycolysis is increased to augment ATP synthesis by substrate Phosphorylation (SP), electrons accumulate in NADH in the cytosol (as well as in mitochondria), and blood flow is increased (14, 17-20) just as with aerobic work. We introduced the term "hyperglycemic pseudohypoxia" to emphasize similarities between effects of hyperglycemia and hypoxia on blood flow and accumulation of electrons in NADH (14).

The coupling of increases in NADH and blood flow under such diverse metabolic and functional challenges as exercise, hyperglycernia, and hypoxia led to the hypothesis tested here: Accumulation of electrons in NADH signals blood-flow need and activates redox signaling pathways to augment flow.

MATERIALS AND METHODS

Strategy
Under steady-state conditions, near-equilibria exist between: 1) cytosolic-ftee $NADH/NAD^+$ and intracellular $_{(ic)}$ L/P ratios that are established by lactate-DH (L-DH) as shown in Equations I and 2, in which KL-DH = 1.11 x 10^{-4} at pH 7.0 (21,22); and 2) intracellular and extracellular$_{(ec)}$ L/P ratios (Equation 3) that are established by monocarboxylate transporters (MCT) (23).

Free cytosolic NADH and NAD^+ cannot be determined from measurements of NADH and NAD in whole tissue extracts that contain enzyme-bound as well as free cytosolic and mitochondrial NADH and NAD^+. Also, enzyme-bound NAD is ~2 orders-of-magnitude more reduced than free NAD (21). At present, free cytosolic $NADH/NAD^+$ can be evaluated only by the redox metabolite indicator method based on near-equilibrium between the ratios of free $NADH/NAD^+$ and reduced/oxidized substrates of cytosolic Dehydrogenase enzymes such as lactate-DH (Equations I and 2) (21)].

Here, $NAD^+(H)$ refers specifically to cytosolic free $NAD^+(H)$ VS. $NAD^+(H)$, which is enzyme-bound, and $NAD^+(H)$ in mitochondria.

Changes in pyruvate have a much greater impact than equimolar changes in lactate on L/P and NADH because the K_m of L-DH is higher for lactate than for pyruvate; in resting tissues and plasma, lactate levels are ~10x higher than pyruvate. We assume that total cytosolic free NAD is unlikely to change substantially in these brief experiments so that increases in $NADH/NAD^+$ indicate increases in NADH, and the terms are used interchangeably. Therefore if the hypothesis is correct, injecting lactate to increase plasma L/P will increase L/P_{ic} and drive reduction of NAD^+ to NADH (Equation 1), and increase NADH and blood flow; injection of pyruvate will have the opposite effect.

Paradigms
Regional blood flows were determined -in tissues at rest 5 min after intravenous (i.v.) bolus injections and after 5-14 continuous infusions of saline, lactate, and/or pyruvate. We evaluated direct effects of extra vascular lactate levels on blood flow by applying lactate solutions to granulation tissue in skin wounds covered by plastic chambers where potential systemic effects of metabolites and pharmacological agents on tissue blood flow are obviated. Skeletal muscle contraction was evoked by electrical stimulation of the sciatic nerve in one hind limb. Increased neural activity in rat somatosensory cortex was evoked by contralateral whisker stimulation. Signaling pathways of blood-flow changes were evaluated in all paradigms.

Preliminary studies were performed to optimize protocols for injecting lactate and pyruvate in each paradigm. Plasma UP is rapidly normalized by the Cori cycle (8) and the lactate shuttle (24) by conversion of lactate to pyruvate (and vice versa) in numerous tissues and cells via L-DH (Equation 1) Minimal effective doses also were determined for N^G-nitro-L-arginine methyl ester (L-NAME), a nonselective inhibitor of nitric oxide ('NO) synthase (NOS), and for SC-52608, a plasma-soluble ($M_r = 341$) Mn^{2+} caged superoxide (O_2) dismutase (SOD) mimic. (SC-52608 does not interact

with *NO, H_2O_2, or peroxynitrite. Also, it potentiates 'NO-induced vasodilatation but does not block neutrophil O_2 production (25, 26, and Dennis P. Riley, unpublished observations).

Animals

We used male Sprague-Dawley rats. Housing, care, and all experimental protocols met Washington University and National Institutes of Health guidelines.

Blood flows

We assessed blood flows by using 11.3 µm. [46]Sc microspheres or [3]H- or ^{125}I-desmethylimipramine (DMI) (16, 27). DMI, a plasma soluble tracer (M_r = 266), was injected i.v.

Resting tissues

Rats were anesthetized with thiopental, and microspheres were injected 5 min after bolus injection of Na L-lactate (1 mmol/kg) and/or Na pyruvate (0.05 mmol/kg) or Na D-lactate (I mmol/kg) or after 5-h infusion of Na L-lactate (1.35 mmol/kg/h) and/or Na pyruvate (0.027 mmol/kg/h). L-NAME was infused at 2.5 µmol/kg/min beginning 10 min before bolus lactate injection. Skin chamber granulation tissue was prepared 1 week before use (16). Test substances in HEPES buffer (1.5 ml, pH 7.4) were added to chambers 20 min before assessment of blood flows with microspheres. Concentrations of test substances were 10 or 20 mM Na L-lactate, 10 mM Na D-lactate, 1 mM Na pyruvate, I mM EGTA, 0.05 mM Dantrolene, 1 mM L-NAME, 0.3 rnM SOD_{mimic}, and 1000 U of Catalase/ml.

Muscle stimulation

Rats were anesthetized with thiopental, and carmulae were placed in a subclavian artery to measure blood pressure and to obtain blood samples, a carotid artery for the withdrawal pump, and in both distal femoral veins for injection of tracers and for infusion of test substances. One sciatic nerve

was stimulated in the sciatic notch at 10 Hz (10 V, 100 µs pulses). [3]H- or ^{125}I-DMI was injected I min before termination, and muscles were sampled for measurement of metabolites and blood flow.

Somatosensory cortex stimulation

Rats were anesthetized with urethane, and both iliac arteries and femoral veins were cannulated. One arterial cannula was used to monitor blood pressure and to obtain blood samples; the other, to measure blood flow. One femoral vein was used for injection of tracers; the other, for test substances. On one side of the face, ~20 whiskers were trimmed and fitted into a screen ˜8 mm from the skin attached to a mechanical device for rostrocaudal vibrissal deflection of 1.75 mm at 7.5 Hz (28). After 5 s of stimulation, ^{125}I-DMI was injected and the withdrawal pump was started. After 1 min, the great vessels were severed before we opened the skull and removed stimulated and unstimulated whisker barrel cortex (~3 mm in diameter and 1 rnm thick (29)), as well as olfactory bulbs and visual cortex. Cerebral blood flows with ^{125}I-DMI were comparable with flows with microspheres.

Metabolites

Lactate, pyruvate, and glucose were measured in extracts of arterial blood and muscle by standard enzymatic methods (15, 16). $NADH/NAD^+$ was calculated by using Equation 2.

Statistics

Differences in parameters between animals are significant at $P < 0.05$, based on the general linear model procedure with SAS (16, 27). Differences in parameters in the same rat were assessed by the paired t-test.

RESULTS

Resting tissues
Bolus injection or infusion of lactate increased blood flows in all tissues examined except heart and brain. Blood flows increased 50% in retina, 65% in sciatic nerve, 30% in epitrochlearis and gastrocnemius skeletal muscles, 86% in soleus muscle, and 40% in kidney. After lactate infusion for 5 h, blood-flow increases were 45% in retina, 2.4x in sciatic nerve, 3x in epitrochlearis muscle, 17% in diaphragm, and 77% in skin. Increased flows were prevented by injection of pyruvate with lactate; pyruvate alone did not affect flow. Bolus injection of D-lactate, which has the same isoelectric point as L-lactate, had no effect on regional blood flows. (This observation argues against a decrease in extracellular pH in mediating vasodilation by L-lactate.) Direct application of 10 or 20 MM L-lactate (but not D-lactate) trebled blood flows in skin chambers (P<0.0001). These increased flows were prevented when 1 mM pyruvate was coadministered with 20 mM lactate; pyruvate alone had no effect.

Plasma lactate levels doubled after 5 h of lactate infusion (from. 1.25 ± 0.46 to 2.61 ± 0.82 MM; mean $\pm$ SD), and blood pH increased (from 7.46 ± 0.05 to 7.54 ± 0.03). Confusion of pyruvate with lactate did not change plasma lactate levels or blood pH. Infusion of lactate with or without pyruvate did not affect mean arterial blood pressure (MAP; 127 ± 13 mmHg in controls). Lactate alone decreased peripheral vascular resistance and increased cardiac output by ~ 17% (P<0.05), reflecting widespread dilation -of resistance arterioles. These cardiovascular changes were completely prevented when pyruvate was confused with lactate.

Working muscle
Stimulation for 2 or 15 min increased blood flow in contracting adductor magnus, muscle 7x (Fig.1A) and in soleus muscle 3.5x. Infusion of lactate increased blood flows further in active muscle, whereas infusion of pyruvate decreased them (Fig.1A). Infusion of lactate or pymvate in this protocol did not affect blood flow to contralateral resting muscle. In contracting muscle, a strong positive relationship between blood flows and plasma L/P and a strong negative relationship to plasma lactate and

pyruvate (Fig. 1B and C) were found. Blood flow was unrelated to muscle L/P, lactate, or pyruvate (Fig, 1D. In contrast, blood flow in contralateral resting muscle was indifferent to plasma and muscle LP, lactate, or pyruvate (Fig,. 1B, C, and D). Also, plasma L/P did not correlate with LP in resting muscle or contracting muscle (r^2=0.04, P =0.4 for both muscles; Fig. 2). Specifically, muscle L/P was not increased by injection of lactate that increased blood flow. (This finding was confirmed in an independent experiment.)

Variation in lactate-evoked blood-flow changes in resting muscles with different protocols was related to differences in the total amount of lactate injected, the rate and duration of injection, and the time at which tissues were sampled during or following injection. Thus, blood flow in resting epitrochlearis was increased 73% at 1 min vs. 30% at 5 min after bolus injection of 1 mmol lactate/kg, and 3x after a 5-h infusion of lactate (1.35 mmol/kg/h). Resting soleus blood flow was increased 2x at 1 min vs. 86% at 5 min after bolus injection, but was unchanged by infusion,of I mmol lactate/kg over 15 min. Gastrocnernius flow was increased 2.5x at 1 min vs. 30% at 5 min after bolus injection. Plasma L/P ratios after bolus lactate injection (1 mmol/kg) were increased 3.5x at 20 s, 1.8x at 1 min, 1.4x at 2 min, 1.2x at 3 min, and back to normal at 5 min.

After 2 min of stimulation, contracting muscle L/P increased 11x, which reflected a 50% decrease in pyruvate and a 5x ,increase in lactate consistent with marked glycogenolysis. In muscle contracting for 15 min, L/P increased 16x as pyruvate levels fell by more than 85%, lactate levels did not change, and glucose levels tripled (Table 1). (Pyruvate production could be reduced by partial glycogen depletion and increased consumption by activation of pyruvate dehydrogenase (30), the rate-limiting enzyme for use of pyruvate for OP.) Because lactate levels correlate with pH_i (3 1), this finding indicates that pH_i did not differ in resting and contracting muscle. Thus, increased UP ratios in contracting muscle correspond to a ~16x increase in NADH/NAD$^+$ (262×10^{-4} vs. 17×10^{-4} in resting muscle) and therefore NADH. Plasma levels of glucose, pyruvate, lactate, and L/P all rose after 15 min of muscle stimulation (Table I). (The increase in plasma lactate levels was identical to the Micrease after a 5-h lactate infusion in resting tissues.) Diffusion of lactate and pyruvate (at a high L/P ratio) from

contracting muscle increases L/P in plasma from which lactate is taken up by the liver and other tissues and oxidized to pyruvate (8, 24); some pyruvate is used for gluconeogenesis by the Cori cycle and some diffuses back into plasma.

Worldng brain

Whisker stimulation increased blood flow 10.5% in contralateral vs. ipsilateral sornatosensory whisker barrel cortex (Fig. 3A). Whisker stimulation did not change flow to visual and olfactory cortices. A lactate bolus injected I min before stimulation doubled the increase in blood flow evoked by whisker stimulation but had no effect on flow in resting barrel, visual, and olfactory cortices. Injection of pyruvate completely prevented whisker-stimulated flow increases without affecting flow to the unstimulated side (Fig.3A). Lactate augmentation of blood flow in stimulated, but not in resting cortex, may be explained by a lactate-dependent increase in lactate transport between blood and brain (32). As in working muscle, effects of lactate or pyruvate alone on brain blood flow were abrogated when they were coinjected. Likewise, blood flows to stimulated cortex paralleled increased plasma L/P ratios but not plasma lactate and pyruvate levels (Fig. 3B), whereas flows in resting cortex were indifferent to plasma L/P, lactate, or pyruvate. In other experiments, visual stimulation increased blood flows in retina and visual cortex; these increased flows also were augmented by lactate, prevented by pyruvate, and strongly correlated with plasma L/Pratios (Ido et al., unpublished observations).

Hemodynamic, p1l, and electrolyte changes

Infusions for 15 min of lactate and pyruvate had no effect on blood $pC0_2$, pO_2, or MAP (Table 2). However, blood pH was increased from 7.44 ± 0.02 (saline controls) to 7.49 ± 0.02 - 7.51 ± 0.02 (P<0.02) with either 1 or 2 mmol pyruvate or 1 mmol lactate ± 2 mmol pyruvate. Lactate and/or pyruvate increased blood pH similarly yet evoked opposite changes in blood flow. These observations indicate that the opposite hemodynamic effects of lactate and pyruvate on peripheral resistance and blood flow are mediated by increases and decreases in $NADH/NAD^+$, (by lactate and pyruvate,

respectively) rather than by pH changes. This interpretation is consistent evidence that pH changes do not account for physiological work-induced increases in brain blood flow (9), lactate-induced relaxation of isolated rat mesenteric resistance arteries (33), or lactate-evoked reduction of tension development in working dog muscle (34).

Nitric oxide

Increases in Ca^{2+}_i activate constitutive (c)NOS to generate 'NO that relaxes smooth muscle. Preventing increases in Ca^{2+}_i by EGTA (a Ca^{2+} chelator) or by Dantrolene (which inhibits Ca^{2+} release from endoplasmic reticulum) blocked lactate-augmented flows in granulation tissue. L- NAME prevented lactate-induced flows in granulation tissue and other resting tissues and blocked increased flow to stimulated somatosensory cortex without affecting flow to resting cortex (Fig. 3A). L-NAME blocked increased blood flows to contracting muscle and reduced flow to contralateral. resting muscle by 50% (Fig. 1A) L-NAME increased MAP from 107 ± 4 to 131 ± 9 in whisker stimulation experiments and from 136 ± 10 to 182 ± 6 in muscle stimulation experiments.

Superoxide

The SOD_{mimic} prevented lactate-enhanced flow in granulation tissue and blocked increased flow in stimulated whisker cortex without affecting flow in resting cortex (Fig.3A) Catalase had no
effect on lactate-induced flow increases in granulation tissue. The SOD_{mimic} also reduced blood
flow in contracting muscle by 67% but increased flow in resting muscle by 32%(Fig.1A)

DISCUSSION
Observations in all experimental paradigms support the hypothesis that accumulation of electrons in NADH signals blood-flow need and regulates flow in resting and working tissues. The findings that blood flows in working muscle were strongly correlated with plasma, but not muscle L/P (Fig 1B,C and D), and that plasma and muscle L/P were not correlated

suggest that blood-flow need can be sensed by accumulation of electrons in NADH within vascular cells per se. Thus, high ratios of L/P diffusing from contracting muscle cells (which are ~16x higher than in contralateral resting muscle and ~20x higher than in plasma (Table I) increase interstitial L/P_{ec} to increase L/P_{ic} and NADH in vascular smooth muscle and endothelial cells (Equation 3 Fig. 4A). Similarly, elevation of plasma L/P_{ec} (by lactate injection) and interstitial L/P_{ec} (by addition of lactate to skin chambers) increases L/P_{ic} and NADH in vascular cells. Studies of isolated vessels support this interpretation (33, 35-37).

The strong correlation between blood flows and plasma L/P in contracting muscle, and the finding that both blood flow and plasma L/P_{ec} were unrelated to muscle L/P, may be explained by the gradients of L/P_{ec} and concentrations of lactate and pyruvate between $plasma_{ec}$ and L/P_{ic}. in skeletal muscle cells. When lactate and pyravate are injected, L/P_{ec} in plasma will be most affected. Interstitial L/P_{ec} will be less affected as lactate and pyruvate diffuse across the vessel wall (between and through vascular cells) and mix with lactate and pyravate diffusing from skeletal muscle cells. L/P_{ic} in vascular cells will be modulated by plasma L/P_{ec} on one side and interstitial L/P_{ec} on the other. Also, L/P_{ic} in resting and contracting skeletal muscle cells will be least affected, if at all. This condition is because the concentration of $lactate_{ic}$ in contracting and resting muscle cells (and of pyruvate in resting muscle cells) is much higher than in plasma (Table 1) and will "buffer" effects of lactate and pyrtivate diffusing from plasma on L/P_{ic}-$NADH/NAD^+$. Thus, injection of lactate and pyruvate will impact more on L/P_{ic}-$NADH/NAD^+$ in vascular endothelial cells and smooth muscle cells than in skeletal muscle cells.

Several studies in our laboratory indicate that increased blood flows induced by work and by lactate injection (Fig. 4A and B) are mediated by essentially the same redox signaling cascade that increases blood flows in resting tissues in response to elevated glucose levels in diabetes (14-17). The major difference is the source of electrons that activate the signaling cascade: glucose-derived sorbitol with diabetes, glucose-derived glyceraldehyde 3-phosphate (GA3P) with work, and lactate with lactate injection. In each case, electrons and protons are transferred to NAD^+ faster than they can be used by mitochondria for ATP synthesis. Electrons

accumulating in NADH fuel redox signaling pathways that augment blood flow coupled to reoxidation of NADH to NAD^+. Transfer of excess electrons from NADH to O2 by NADHoxidase generates O_{2-} (35), which elevates Ca^{2+}_i (14, 17, 38, 39) to activate 'NO production by cNOS. Excess electrons in NADH also favor de novo synthesis of diacylglycerol (14, 17) to activate PKC (protein kinase C)-mediated increased glucose transport in working muscle independent of insulin and hypoxia (40, 41).

This signaling cascade is supported by: 1) observations that reactive oxygen species (H_2O_2, O_{2-})
applied to the surface of the brain and to granulation tissue cause vasodilation and blood flow
increases, which, in granulation tissue, are prevented by inhibitors of NOS (16, 42); and 2) most,
but not all, reports that 'NO mediates increased blood flow in working skeletal muscle and somatosensory cortex (1, 2, 43, 44). Increased blood flow in stimulated somatosensory cortex appears to be mediated largely by neuronal (n)NOS, because flow increases with whisker
stimulation are blocked by NOS inhibitors in mice lacking endothelial (e)NOS (43). In skeletal
muscle, the cascade may be activated in contracting muscle cells that express nNOS and eNOS
and in which both reactive oxygen species and 'NO increase during work (45, 46). 'NO and O_2
could -then diffuse into vascular smooth muscle cells to cause vasodilation (Fig. 4A and B).
However, the finding that lactate injection increased blood flows in numerous resting tissues and in activated brain and muscle (without increasing muscle L/P) supports the likelihood that the signaling cascade can be initiated in vascular smooth muscle and/or endothelial cells by high ratios of L/P diffusing from plasma or skeletal muscle cells (Fig.4A) .

Lactate injection increased blood flows in resting tissues with widely differing metabolic profiles and functions. However, we found substantial differences in lactate-induced flow increases in different tissues at rest and between resting vs. stimulated skeletal muscle (Fig. I A) and whisker cortex (Fig. 3A). Tissue differences in metabolism, MCT activity, capacity to

reoxidize NADH to NAD$^+$, and content of SOD, NOS and other factors all influence the signaling cascade for increasing blood flow. Collectively, these factors contribute to a tissue- specific "threshold!' below which elevated L/P$_{ec}$ ratios have little impact on L/P$_{ic}$-NADH/NAD$^+$ and blood flow. This threshold is relatively high in heart (which is always working) and in resting brain in which blood flows were unaffected by lactate injection, intermediate in the diaphragm, and lower in a wide range of other tissues. Addition of lactate to the perfusate of isolated hearts increased myocardial NADH/NAD$^+$ ~5x without affecting coronary flow (47, 48). Also, infusion of lactate to increase plasma L/P 2.6x did not increase coronary sinus blood flow in vivo (49). Lactate infusion' does not increase cerebral blood flow in healthy subjects but augments regional flow elevations in individuals suffering from panic attacks (50).

Cytosolic free NADH is positioned strategically to sense blood flow need. NAD$^+$ is the initial acceptor of electrons from glucose metabolites during glycolysis and from oxidation of lactate (Fig. 4A) . Electrons accumulate in NADH when: 1) they are transferred to NAD$^+$ at an increased rate coupled to oxidation of metabolites in the cytosol, and 2) increased mitochondrial free NADH impairs transfer of electrons from cytosolic NADH into mitochondria. Electrons accumulating in NADH then fuel redox signaling pathways that augment blood flow and reoxidize NADH to NAD$^+$. Regardless of the cause of an increase in NADH, increased blood flow augments removal of lactate (accumulation of which limits glycolysis and ATP synthesis by SP) and delivery of oxygen to ensure maximal ATP synthesis- by OP. Also, increased delivery of blood with a low L/P ratio (in plasma and erythrocytes) promotes transfer of electrons and protons from NADH in vascular cells to pyruvate and facilitates their removal as lactate.

Sensing metabolic blood flow need by NADH complements the vital function of NAD as the principal and most efficient carrier of electrons from fuels for energy metabolism. ATP synthesis from glucose by substrate phosphorylation (SP) and OP is absolutely dependent on continuous redox cycling of NAD$^+$↔NADH (8), In hearts perfused with glucose, maximal glycolysis is the same under conditions of anoxia and aerobic work and is limited by reoxidation of NADH (5 1).

The basis for increased lactate in activated brain and muscle is hotly debated. Elevated lactate levels reflect a marked increase in glycolysis, which generates pyruvate faster than it can be used for mitochondrial ATP synthesis by OP; the excess pyruvate is reduced to lactate by lactate-DH under aerobic as well as hypoxic conditions. Why, then, is glycolysis increased to produce pyruvate faster than it can be used for OP? Production of pyruvate is the last of three reactions critical for ATP synthesis in the oxidoreduction-phosphorylation phase of glycolysis. The first reaction is the transfer of electrons and protons from GA311 to NAD^+; the second is synthesis of 2 ATP by SP coupled to dephosphorylation of oxidized metabolites of GA3P. Production of pyruvate is coupled to synthesis of the second ATP by pyruvate kinase. These reactions are vital for coordinating increased energy metabolism and blood flow evoked by work and for optimal ATP synthesis under aerobic and anaerobic conditions in general. However, they are limited by availability of NAD^+ for the first reaction (8, 5 1).

An important advantage of ATP synthesis by SP is that it can be produced ~2x as fast as by OP (8, 52) to support high rates of ATP use (as with vigorous muscle activity during a sprint to escape danger or to catch prey). In addition, ATP generated by SP is proximate to, and may be preferentially utilized by, plasma membrane-associated ATPases activated during electrical activity and work (53-55). When lactate (rather than glucose) is the source of pyruvate for ATP synthesis by OP, no ATP is synthesized by SP (Fig.4A)

The net yield of ATP per mole of glucose during aerobic glycolysis, is 2 ATP by SP and 28-30 ATP by OP (8). The ATP yield from SP is increased 50% with glycogen-derived glucose. Rapid ATP synthesis via SP with work is limited by reoxidation of NADH to NAD^+ (51, 52), inhibition, of glycolysis by lactate, and possibly by partial glycogen depletion and glucose uptake. In the longer Am, production of excess pyruvate ensures that pyruvate levels are not rate-limiting for OP, which, although slower, is far more efficient than SP.

The potential importance of ATP synthesis from SP during work is evident from data of Fox et al. (4). If all of the ~5% increase in oxygen consumption

in human visual cortex with photostimulation. were used for ATP synthesis from glucose by OP, and if all of the ~ 50% increase in glucose uptake not used for OP were metabolized to lactate, then ATP synthesis by SP would equal that by OP. With glucose derived from glycogen, the relative contribution of SP to ATP synthesis necessarily would be greater.

Chronic or frequent elevations of NADH may stimulate new vessel growth as in muscle by endurance training, in brain by neural activity and by hypoxia, and in retina by repeated lactate injections and by diabetes (14, 17, 56-59). Vascular endothelial growth factor (VEGF) mRNA and/or protein is increased in skeletal muscle by exercise (60, 61) and in cultured cells by elevated levels of glucose (via PKC), O_2-, and H_2O_2 (62--64). The impact of increased NADH on other signaling pathways and redox-sensitive transcription factors (e.g., NF-KB; nuclear transcription factor-KB), although likely, has not yet been shown.

In humans who suffer from "panic" attacks, lactate injection precipitates an attack coupled to increased regional cerebral blood flows (50). This finding suggests that accumulation of electrons in NADH mediates panic disorder as well as increased blood flow. Carr and Sheehan (65) hypothesized that the disorder may be caused by a defect within the redox-regulating apparatus of the brain. They proposed that infusion of pyruvate together with lactate might be expected to prevent the typical lactate-induced response. This prediction has not been tested.

Accumulation of electrons in NADH may contribute to other conditions associated with abnormal neural activity (e.g., galactosemia (14, 66), epileptic seizures, and defects in energy metabolism). These conditions could all be potential candidates for therapies designed to transfer electrons from NADH to substrates (e.g., pyruvate) that would not perturb signaling or other metabolic pathways. The observation that lactate injection doubled the increased blood flow in stimulated somatosensory cortex (without affecting flow in contralateral resting cortex) has implications for enhancing sensitivity of functional brain imaging. For example, injection of lactate could substantially amplify blood flows for mapping physiological neural activity in subtle tasks (67).

Accumulation of electrons in cytosolic; free NADH is an elegantly simple multifunctional sensor of local blood-flow need. It accounts for blood-flow increases by a remarkable range of stimuli from activation of excitable membranes of muscle and brain to hyperglycemia in diabetes.

REFERENCES:
1. Ladecola, C. (1993) Regulation of the cerebral microcirculation during neural activity: is nitric oxide the missing link? TINS **16**, 206-214
2. Lash, J.M. (1996) Regulation of skeletal muscle blood flow during contractions. Proc. Soc. Exp. Biol. Med **211**,218-235
3. Fox, P.T., and Raichle, M.E. (1986) Focal physiological uncoupling of cerebral blood flow and oxidative metabolism during somatosensory stimulation in human subjects. Proc. Nad. Acad Sci. USA **83**,1140-1144
4. Fox, P.T., Raichle, M.E., Mintun, M.A., and Dence, C. (1988) Nonoxidative glucose consumption during focal physiologic neural activity. Science **241**, 462-464
5. Lindauer, U., Megow, D., Schultze, I., Weber, J.R., and Dirnagl, U. (1996) Nitric oxide synthase inhibition does not affect somatosensory evoked potentials in the rat. Neurosci. Lett. **216,** 207-210
6. Powers, W.J., Hirsch, I.B., and Cryer, P.E. (1996) Effect of stepped hypoglycemia on regional cerebral blood flow response to physiological brain activation. Am. J Physiol. **270,** H554-H559
7. Cholet, N., Seylaz, J., Lacombe, P., and Bonvento, G. (1997) Local uncoupling of the cerebrovascular and metabolic responses to

somatosensory stimulation after neuronal nitric oxide synthase inhibition. J. Cereb. Blood Flow Metab. **17**, 1191-1201

8. Stryer, L. (1995) In Biochemistry, 4th ed. W. H. Freeman: New York; pp. 548-552, 577

9. Ueki, M., Linn, F., and Hossmann, K. -A. (1988) Functional activation of cerebral blood flow and metabolism before and after global ischemia of rat brain. J Cereb. Blood, Flow Metab. **8**,486-494

10. Katz, A., and Sahlin, K (1990) Role of oxygen in regulation of glycolysis and lactate production in human skeletal muscle. Exerc. Sport Sci. Rev. **18**, 1-28

11. Stainsby, W.N., and Brooks, G.A. (1990) Control of lactic acid metabolism in contracting muscles and during exercise. Exerc. Sport Sci. Rev. **18,** 29-63

12. Hyder, F., Chase, J.R., Behar, K.L., Mason, G.F., Siddeek, M., Rothman, D.L., and Shulman, R.G. (1996) Increased tricarboxylic acid cycle flux in rat brain during forepaw stimulation detected with 1H [13C] NMR_ proC. Nad. Acad Sci. USA **93**,7612-7617

13. Dernestre, M., Boutelle, M., and Fillenz, M. (1997) Stimulated release of lactate in freely rhoving rats is dependent on the uptake of glutamate. J. Physiol. **499**, 825-832

14. Williamson, J.R., Chang, K., Frangos, M., Hasan, K.S., Ido, Y., Kawamura, T., Nyengaard, J.R., Van den Enden, M., Kilo, C. and Tilton, R.G., (1993) Hyperglycemic pseudohypoxia and diabetic complications. Diabetes **42,** 801-813

15. Van den Enden, M., Nyengaard, J.R., Ostrow, E., Burgan, J.H., and Williamson, J.R. (1995) Elevated glucose levels increase retinal glycolysis and sorbitol pathway metabolism. Invest OphthalmoL Vis. Sci. **36**,1675-1685

16. Tilton, R.G., Kawamura, T., Chang, K.C., Ido, Y., Bjercke, R.J., Stephan, C.C., Brock, T.A., and Williamson, J.R. (1997) Vascular, dysfunction induced by elevated glucose levels in rats is mediated by vascular endothelial growth factor. J Clin. Invest. **99**, 21922202

17. Williamson, J.R., and Ido, Y. (1999) The vascular cellular consequences of hyperglycaemia. In Diabetic Angiopathy, , Chap. 10, ed., Tooke, J.E. Arnold. London and Oxford University Press Inc.: New York), pp. 161-185

18. Duffy, T.E., Nelson, S.R-, and Lowry, O.H. (1972) Cerebral carbohydrate metabolism during acute hypoxia and recovery. J Neurochem. **19,** 959-977

19. Siesjo, B.K., Berntman, L., and Rehncrona, S. (1979) Effect of hypoxia on blood flow and metabolic flux in the brain. Adv. **Neurol. 26,** 267-283

20. Rowell, L.R., Saltin, B., Kiens, B., and Christensen, N.J. (1986) Is peak quadriceps blood flow in humans even higher during exercise with hypoxemia? Am. J. Physiol. **251**, H1038-HI044

21. Williamson, D.H., Lund, P., and Krebs, H.A. (1967) The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. Biochem. J. **103,** 514-527

22. Veech, R.L. (1986) The toxic impact of parenteral solutions on the metabolism of cells: a hypothesis for physiological parenteral therapy, Am. J Clin. Nutr**. 44**, 519-551

23. Poole, R.C., and Halestrap, A.P. (1993) Transport of lactate and other monocarboxylates across mammalian plasma membranes. Am. J Physiol. **264,** C761-C782

24. Brooks, G.A. (1986) Lactate production under fully aerobic conditions: the lactate shuttle during rest and exercise. Federation Proc. **45,** 2924-2929

25. Hardy, M.M., Flickinger, A.G., Riley, D.P., Weiss, R.H., and Ryan, U.S. (1994) Superoxide dismutase mimetics inhibit neutrophil-mediated human aortic endothelial cell itijury in vitro. J. BioL Chem. **269**, 1853 5-18540

26. Kilgore, K.S., Friedrichs, G.S., Johnson, C.R-, Schasteen, C.S., Riley, D.P., Weiss, R.H., Ryan, U., and Lucchesi, B.R. (1994) Protective effects of the SOD-mimetic SC-52608 against ischemia/reperfusion damage in the rabbit isolated heart. J Mol. Cell. Cardiol. **26**,995-1006

27. Chang, K-, Ido, Y., LeJeune, W., Williamson, J.R-, and Tilton, R.G. (1997) Increased sciatic nerve blood flow in diabetic rats: assessment by "molecular" vs. particulate microspheres. Am. J Physiol. **273**, E164-E173

28. Dowling, J.L., Henegar, M.M, Liu, D., Rovainen, C.M., and Woolsey, T.A. (1996) Rapid optical imaging of whisker responses in the rat barrel cortex. J Neurosci. Meth. **66**, 113122

29. Strominger, R.N., and Woolsey, T.A. (1987) Templates for locating the whisker area in fresh, flattened mouse and rat cortex. J. Neurosci. Meth. **22**, 113-118

30. Parolin, M.L., Spriet, L.L., Hultman, E., Hollidge-Horvat, M.G., Jones, N.L., and Heigenhauser, G.J.F. (2000) Regulation of glycogen phosphorylase and PDH during exercise in human skeletal muscle during hypoxia. Am. J Physiol**. 278**, E522-E534

31. Sullivan, M.J., Saltin, B., Negro-Vilar, FL, Duscha, B.D., and Charles, H.C. (1994) Skeletal muscle pH assessed by biochemical and ^{31}P_MRS methods during exercise and recovery in men. J. Appl. Physiol. 77, 2194-2200

32. Lear, J.L., and Kasliwal, R.K. (1991) Autoradiographic measurement of cerebral lactate transport rate constants in normal and activated conditions. J Cereb. Blood Flow Metab. **11,**576-580

33. McKinnon, W., Aaronson, P.I., Knock, G., Graves, J., and Poston, L. (1996) Mechanism of lactate-induced relaxation of isolated rat mesenteric resistance arteries. J Physiol. **490,** 783-792

34. Hogan, MC, Gladden, L.B., Kurdak, S.S., and Poole, D.C. (1995) Increased [lactate] in working dog muscle reduces tension development independent of pH. Med Sci Sports Exere. **27,** 371-3 77

35. Wolin, M.S. (1996) Reactive oxygen species and vascular signal transduction mechanisms. Microcirculation 3, 1-17

36. Barron, J.T., Gu, L., and Parillo, J.E. (1997) Cytoplasmic redox potential affects energetics and contractile reactivity of vascular smooth muscle. J Mol. Cell Cardiol. **29,** 2225-2232

37. Barron, J.T., Gu, L., and Parillo, J.E. (1999) Relation of NADIUNAD to contraction in vascular smooth muscle. Mol. Cell Biochem. **194,** 283-290

38. Volk, T., Hensel, M., and Kox, W.J. (1997) Transient Ca'-'+ changes in endothelial cells induced by low doses of reactive oxygen species: role of hydrogen peroxide. MoL Cell Biochem. **171**, 11-21

39. Ikebuchi, Y., Masumoto, N., Tasaka, K., Koike, K., Kasahara, K., Miyake, A., and Tanizawa, 0. (1991) Superoxide anion increases intracellular pH, intracellular free calcium, and arachidonate release in human amnion cells. J. Biol Chem. **266,** 13233-13237

40. Hansen, P.A., Corbett, I.A., and Holloszy, J.O. (1997) Phorbol esters stimulate muscle glucose transport by a mechanism distinct from the insulin and hypoxia pathways. Am. J. Physiol **273,** E28-E36

41. Wojtaszewski, U., Laustsen, U., Derave, W., and Richter, E.A. (1998) Hypoxia and contractions do not utilize the same signaling mechanism in stimulating skeletal muscle glucose transport. Biochim. Biophys. Acta **1380**, 396-404

42. Wei, E.P., Christman, C.W., Kontos, H., and PovIishock, IT. (1985) Effects of oxygen radical on cerebral arterioles. Am. J. PhysioL **248**, H157-H162

43. Huang, P.L., and Lo, E.H. (1998) Genetic analysis of NOS isoforms using nNOS and eNOS knockout animals. Prog. Brain Res. **118**, 13-25

44. Hickner, R.C., Fisher, J.S., Ehsani, A.A., and Kohrt, W.M. (1997) Role of nitric oxide in skeletal muscle blood flow at rest and during dynamic exercise in humans. Am. I Physiol. **273,** H405-H410

45. Reid, M.B. (1996) Reactive oxygen and nitric oxide in skeletal muscle. News Physiol. Sci. 11, 114-119

46. Viguie, C.A., Frei, B., Shigenaga, M.K., Ames, B.N., and Brooks, G.A. (1993) Antioxidant status and indexes of oxidative stress during consecutive days of exercise. J AppL PhysioL **75**, 566-572.

47. Laughlin, M.R., and Heineman, F.W. (1994) The relationship between phosphorylation potential and redox state in the isolated working rabbit heart. J MoL Cell Cardiol **26**, 1525-1536

,48. Sholz, T.D., Laughlin, M.R., Balaban, R.S., Kupriyanov, V.V. and Heineman, F.W. (1995) Effect of substrate on mitochondrial NADH, cytosolic redox state, and phosphorylated compounds in isolated hearts. AM. J Physiol. **268**, H82-H91

49. Laughlin, M.R., Taylor, J., Chesnick, A.S., DeGroot, M., and Balaban, R.S. (1993) Pyruvate and lactate metabolism in the in vivo dog heart. Am. J. PhysioL **264,** H2068-H2079

50. Reiman, E.M., Raichle, M.E., Robins, E., Mintun, M.A., Fusselman, M.J., Fox, P.T., Price, J.L., and Hackman, X.A. (1989) Neuroanatomical correlates of a lactate-induced anxiety attack. Arch. Gen. Psychiatty **46,** 493-500

51. Kobayashi, K., and Neely, J.R. (1979) Control of maximum rates of glycolysis in rat cardiac muscle. Orc. Res. **44,** 166-175

52. McGilvery, R.W., and Goldstein, G.W. (1983) Glycolysis and gluconeogenesis. In Biochemistry:A Functional Approach, ed. 3. W. B. Saunders: Philadelphia; pp. 484-487

53. Weiss, J., and Hiltbrand, B. (1985) Functional compartmentation of glycolytic versus oxidative metabolism in isolated rabbit heart. J Clin. Invest. **75,** 436-447

Anderson, B.J., and Marniarou, A. (1992) Functional compartmentalization of energy production in neural tissue. Brain Research **585**, 190-195

55. Magistretti, P.J., and Pellerin, L. (1999) Cellular mechanisms of brain energy metabolism and their relevance to fimctional brain imaging. Phil. Trans. R. Soc. Lond (Biol.) **354,** 1155-1163

56. Imre, G. (1964) Studies on the mechanism of retinal neovascularization. Brit. J Ophthal. **48,**75-82

57. Coggan, AR, Spina, R.J., King, D.S., Rogers, M.A., Brown, M., Nemeth, P.M., and - Holloszy, J.0. (1992) Skeletal muscle adaptations to endurance training in 60- to 70-yrold men and women. J Appl. Physiol. **72,** 1780-1786

58. Isaacs, K.R., Anderson, B.J., Alcantara, A.A., Black~ J.E., and Greenough, W.T. (1992) Exercise and the brain: angiogenesis in the adult rat cerebellum after vigorous physical activity and motor s1cill learning. J. Cereb. Blood Flow Metab**. 12**, 110-119

59. Boero, LA., Ascher, J., Arregui, A., Rovainen, C., and Woolsey, T.A. (1999) Increased brain capillaries in chronic hypoxia. J. Appl. Physiol. **86,** 1211-1219

60, Roca, J., Gavin, T.P., Jordan, M. Siafakas, N., Wagner, H., Benoit, H., Breen, E., and Wagner, P.D. (1998) Angiogenic growth factor mRNA responses to passive and contraction-induced hyperperfusion in skeletal muscle. J. Appl. Physiol. **85**, 1142-1149

61. Gustafsson, T., Puntschart, A., Kaijser, L., Jansson, E., and Sundberg, C.J. (1999) Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. Am. J. Physiol. **276**, H679-685

62. Kurold, M., Voest, E.E., Amano, S., Berepoot, L.V., Takashima, S., Tolentino, M., Kim, R-Y., Roban, R.M., Colby, K.A., Yeo, K-T., and Adamis, A.P. (1996) Reactive oxygen intermediates increase vascular endothelial growth factor expression in vitro and in vivo, J Clin. Invest **98,** 1667-1675

63. Williams, B., Gallacher, B., Patel, H., and Orme, C. (1997) Glucose-induced protein kinase C activation regulates vascular permeability factor mRNA expression and peptide production by human vascular smooth muscle cells, in vitro. Diabetes **46,** 1497-1503

-64. Chua, C.C., Hamdy, R.C., and Chua, B.H. (1998) Upregulation of vascular endothelial growth factor by H_2O_2 in rat heart endothelial cells. Free Radic. Biol. Med. **15,** 891-897

65. Carr, D.B., and Sheehan, D.V. (1984) Panic anxiety: a new biological model. J Clin. Psych. **45,**323-330

66. Berry, G.T., Wehrli, S., Reynolds, R-, Palmieri, M., Frangos, M., Williamson, J.R. and Segal, S. (1998) Elevation of erythrocyte redox potential linked to galactonate biosynthesis: elimination by Tolrestat Metabolism **47,**1423-1428

67. Wagner, A.D., Schacter, D.L., Rotte, M., Koutstaal, W., Maril, A., Dale, A.M., Rosen, B.R., and Buckner, R.L. (1998) Building memonies: remembering and forgetting of verbal experiences as predicted by brain activity. Science **281**, 1188-1191