Prof. Birkmayer Health Products USA

Only Good Health Ensures Quality of Life!!!

Attention Deficit Hyperactivity Disorder (ADHD)

SPECT (Single Positron Emission

Computer Tomography)

before and 2 weeks after 20 mg

ENADAlert

You will note that the darker areas in the photographs below are where the brain is not in use (little activity) the brighter areas are activity. Therefore we see that after the 20mg NADH the brain is much more active.

DIAGNOSTICO DE MEDICINA NUCLEAR/DR.JUAN RIVERA
ROBERTO RAMIREZ ESPINOSA JULIO 20 2001-01 7/20/1 10:16 AM

IMAGEN CEREBRAL SPECT 3-D
FASE BASAL
VISTA FRONTAL

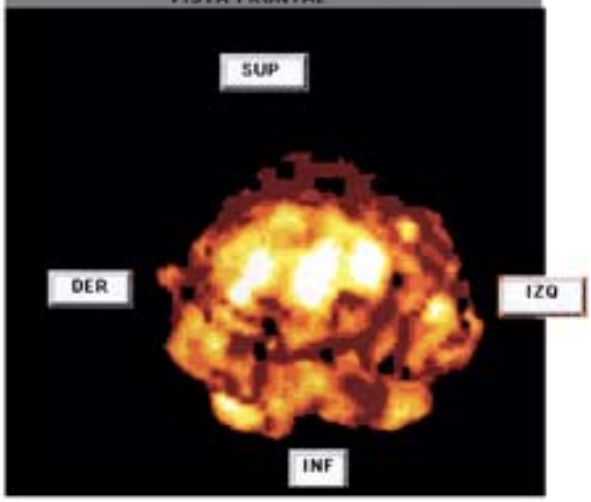

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IMAGEN 3 - D VISTA FRONTAL
FASE POST - ESTIMULACION

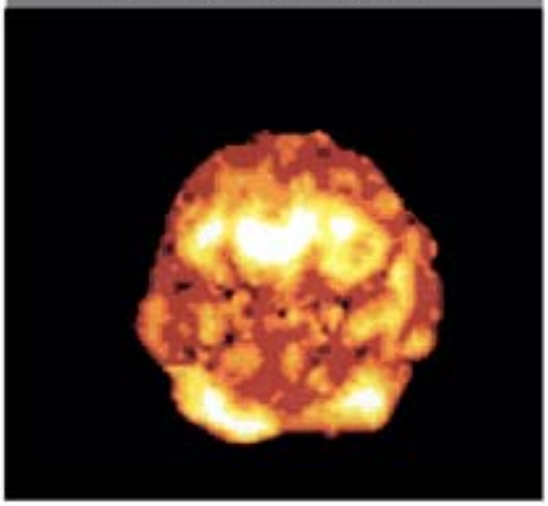

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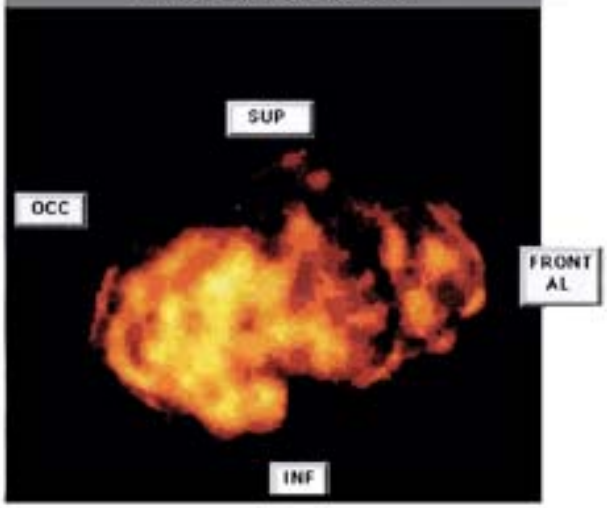
IMAGEN CEREBRAL SPECT 3-D
FASE BASAL
VISTAL LATERAL DERECHA



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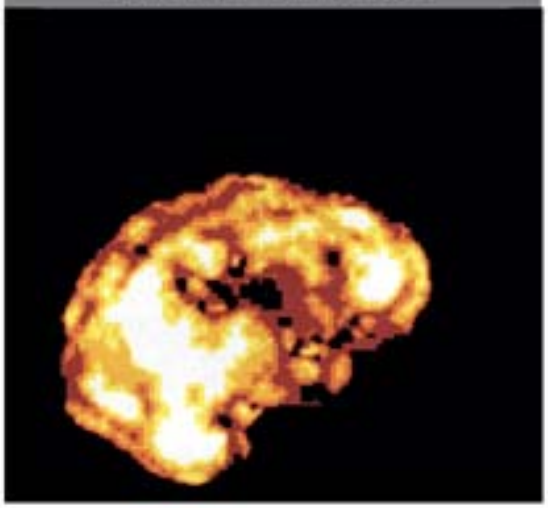

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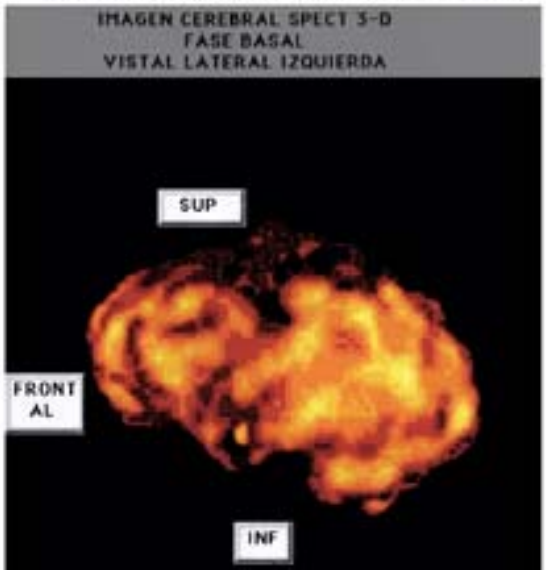
IMAGEN CEREBRAL SPECT 3-D
FASE BASAL
VISTAL LATERAL IZQUIERDA



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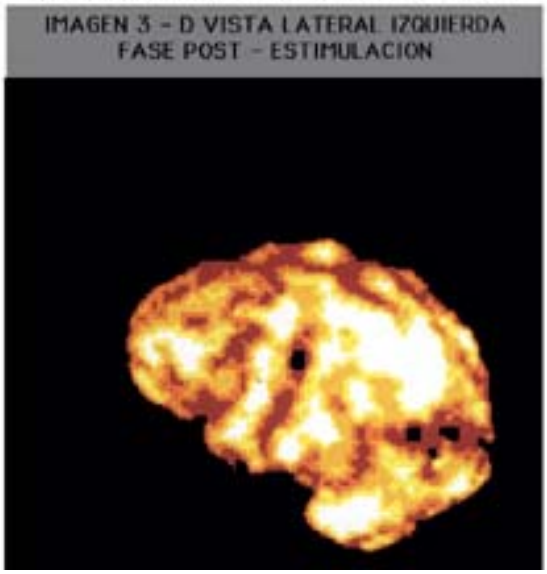

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IMAGEN CEREBRAL SPECT 3-D
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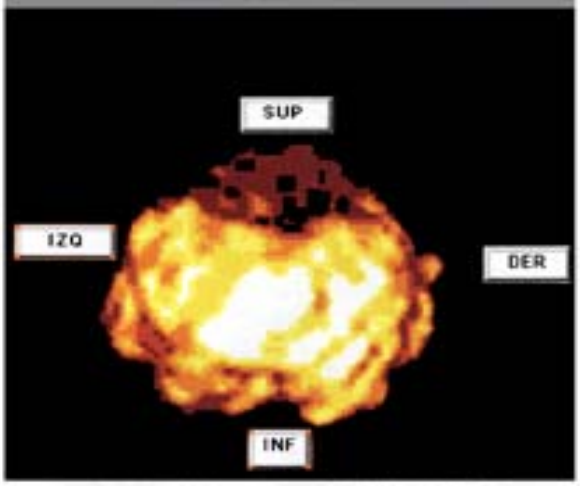
VISTA OCCIPITAL



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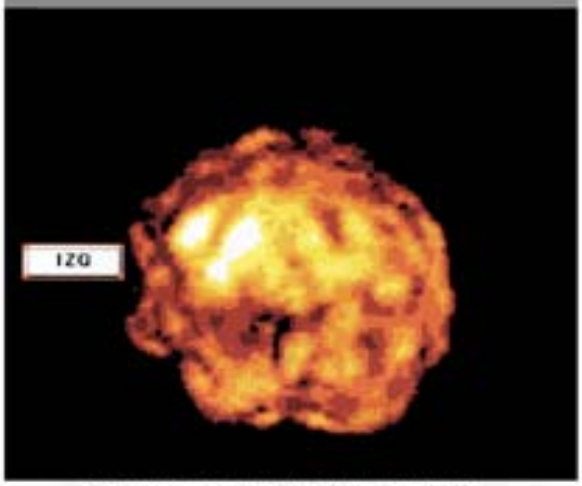

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IMAGEN CEREBRAL 3 - D
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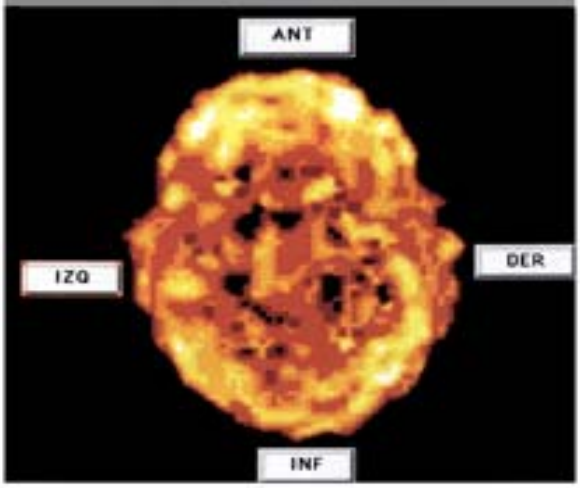
VISTA SUPERIOR



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VISTA SUPERIOR
FASE POST ESTIMULACION

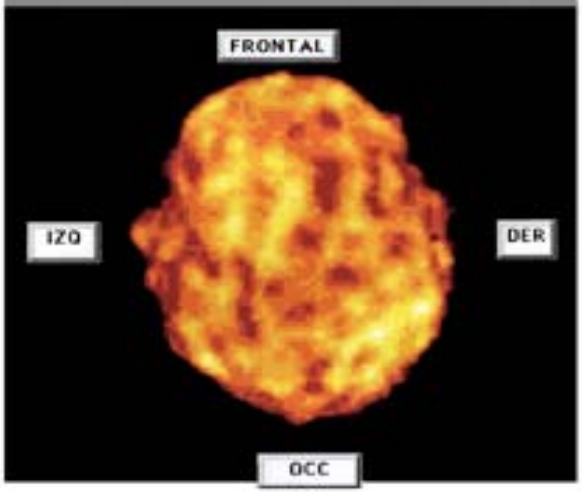

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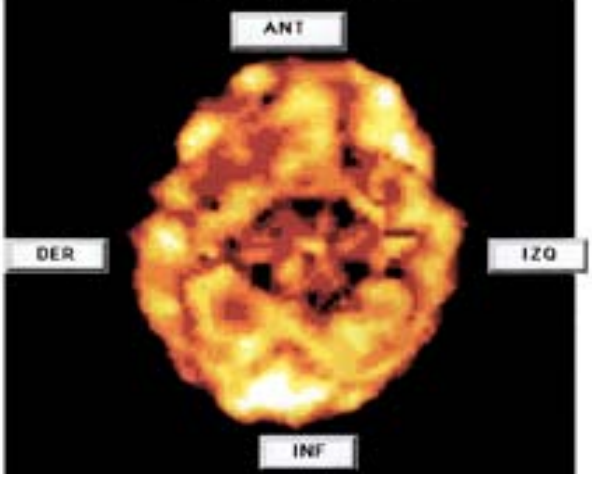
IMAGEN CEREBRAL 3 - D
FASE BASAL
VISTA INFERIOR



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VISTA INFERIOR
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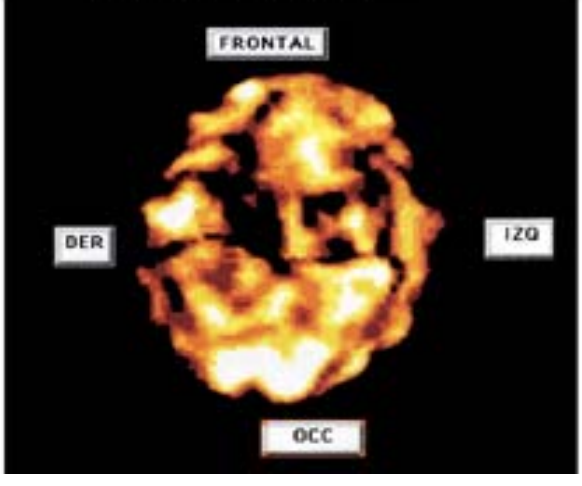

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NADH: sensor of blood flow need in brain, muscle, and other tissues

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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 vasodilation). 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 $K_{L\text{-}DH} = 1.11 \times 10^{-4}$ 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 ftee cytosofic 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-equilibria 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 extravascular 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 vasodilation 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 rnmol/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 ± SD), and blood pH increased (from 7.46 ± 0.05 to 7.54 ± 0.03). Coinfusion 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 coinfused 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 $NADH/NAD^+$ ($262x10^{-4}$ vs. $17x10^{-4}$ 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 O_2 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 $NAD^+ \leftrightarrow NADH$ (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-κB; nuclear transcription factor-κB), 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.

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Equation 1

$$\text{NADH} + \text{H}^+ + \text{pyruvate}_{ic} \overset{\text{L-DH}}{\rightleftharpoons} \text{NAD}^+ + \text{lactate}_{ic}$$

Equation 2

$$\text{NADH/NAD}^+ = [\text{lactate}]/[\text{pyruvate}]_{ic} \times K_{\text{L-DH}}$$

Equation 3

$$\therefore \text{NADH} \propto \text{NADH/NAD}^+ \propto \text{L/P}_{ic} \propto \text{L/P}_{ec}$$

Table 1

Effects of sciatic nerve stimulation at 10 Hz for 15 minutes on contracting and resting adductor magnus muscle and plasma metabolites.

Muscle	Glucose*	Pyruvate	Lactate	L/P ratio	Blood flow*
Resting	0.88 ± 0.11	0.536 ± 0.054	8.12 ± 2.41	15.2 ± 4.7	0.078 ± 0.009
Contracting	2.53 ± 0.63†	0.065 ± 0.033†	12.58 ± 5.27‡	237 ± 140†	0.630 ± 0.049†
Plasma					
Baseline	9.3 ± 0.9	0.140 ± 0.043	1.01 ± 0.28	7.3 ± 1.0	–
Stimulation	10.3 ± 1.6†	0.216 ± 0.046†	2.47 ± 0.88†	11.4 ± 2.8†	–

*Concentrations of glucose, pyruvate, and lactate are in μmol/g wet wt in muscle and mmol/liter for plasma. Blood flow is in ml/g wet wt per minute. All values are mean ± SD, N = 6.
† Different by paired t test from resting or baseline values at $P < 0.05$.
‡ Different by paired t test from resting value by $P = 0.095$.

Table 2

Effects of sciatic nerve stimulation at 10 Hz for 15 minutes on blood pH, blood gases and blood pressure (MAP)

Blood*	Baseline	15 min stim
pH	7.419 ± 0.10	7.438 ± 0.018†
pO_2	80.4 ± 3.9	80.7 ± 3.6
pCO_2	46.2 ± 2.6	44.4 ± 2.2
MAP	138 ± 8	138 ± 8

* Blood pO_2, pCO_2, and MAP are in mmHg. All values are mean ± SD, N = 6.
† Different by paired t test from resting or baseline values at $P < 0.05$.

Fig. 1

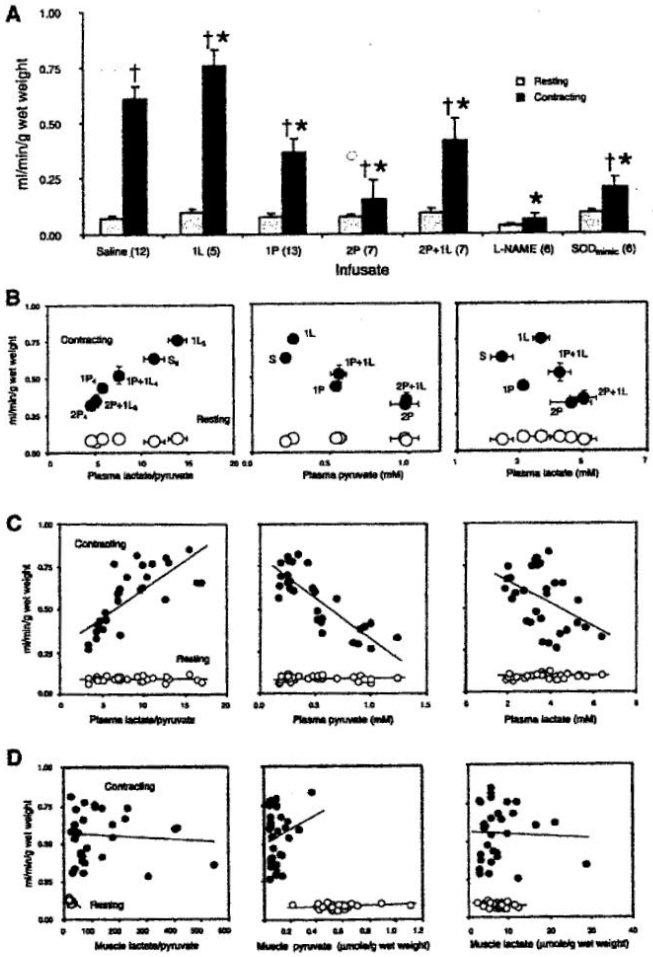

Figure 1. Effects of lactate and pyruvate vs. saline infusion during 15 min of muscle stimulation on blood flow in resting and contracting adductor magnus muscle. (A) In saline controls, blood flow increased 7× in contracting vs. resting muscle. Lactate injection further augmented the elevated blood flows in contracting muscle, whereas pyruvate attenuated them; these effects were blunted by coinjecting lactate and pyruvate. Lactate and pyruvate had no effect on blood flow in resting muscle. L-NAME and SOD_{mimic} both prevented increased flows in contracting muscle. Flows in the resting muscle were decreased by L-NAME and were increased by SOD_{mimic} ($P<0.05$ for both vs. saline controls). Infusions of saline (S), 1 or 2 mmol pyruvate (P), and/or 1 mmol lactate (L)/kg/15 min were initiated at the onset of stimulation. Infusions of SOD_{mimic} (1.45 μmol/kg/min) and L-NAME (0.5 μmol/kg/min) were initiated 5 min prior to muscle stimulation. **(B, C)** Relationships between blood flow in contracting and resting muscles and plasma L/P, pyruvate, and lactate levels after infusion of 1 or 2 mmol pyruvate and/or 1 mmol lactate or saline. Data from individual rats are shown in (C). Blood flows in contracting muscle correlated positively with plasma L/P ($r^2=0.57$, $P<0.0001$) and negatively with plasma pyruvate ($r^2=0.67$, $P<0.0001$) and lactate ($r^2=0.21$, $P<0.02$). Blood flows in resting muscle did not correlate with plasma L/P, pyruvate, or lactate ($r^2\leq0.01$, $P>0.6$ for all three). **(D)** Blood flows in contracting and resting muscle vs. muscle L/P, pyruvate, and lactate levels. Blood flow did not correlate with muscle L/P, pyruvate, or lactate in contracting muscle ($r^2<0.05$, $P>0.25$ for all three). In resting muscle, blood flow was weakly (negatively) correlated with muscle L/P ($r^2<0.14$, $P=0.042$) but not with pyruvate or lactate ($r^2<0.06$, $P>0.2$ for both). Mean SD in (A) and mean SE in (B); SE bars not visible are within the symbols. Numbers of animals ar e given at the bottom of bars in (A) and as subscripts in (B). † Different from resting muscle at $P < 0.05$. *Different from saline controls at $P < 0.05$.

Fig. 2

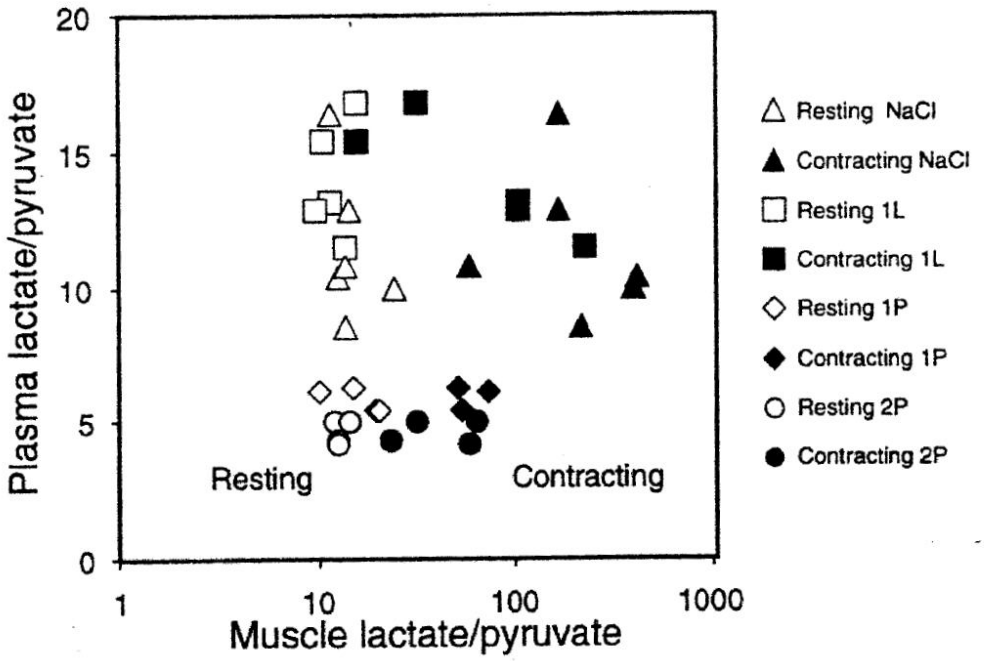

Figure 2. Correlation between plasma L/P and L/P in resting and contracting muscle during infusion of saline (n=6), 1 mmol lactate/kg (n=5), 1 mmol/pyruvate (n=4), and 2 mmol/pyruvate/kg (n=4) over 15 min. Data are from the same rats in **Figure 1B**, **C**, and **D**. Plasma L/P did not correlate with L/P in resting or contracting muscle (r^2=0.04, $P \geq 0.4$ for both). Muscle L/P values are plotted on a natural log scale.

Fig. 3

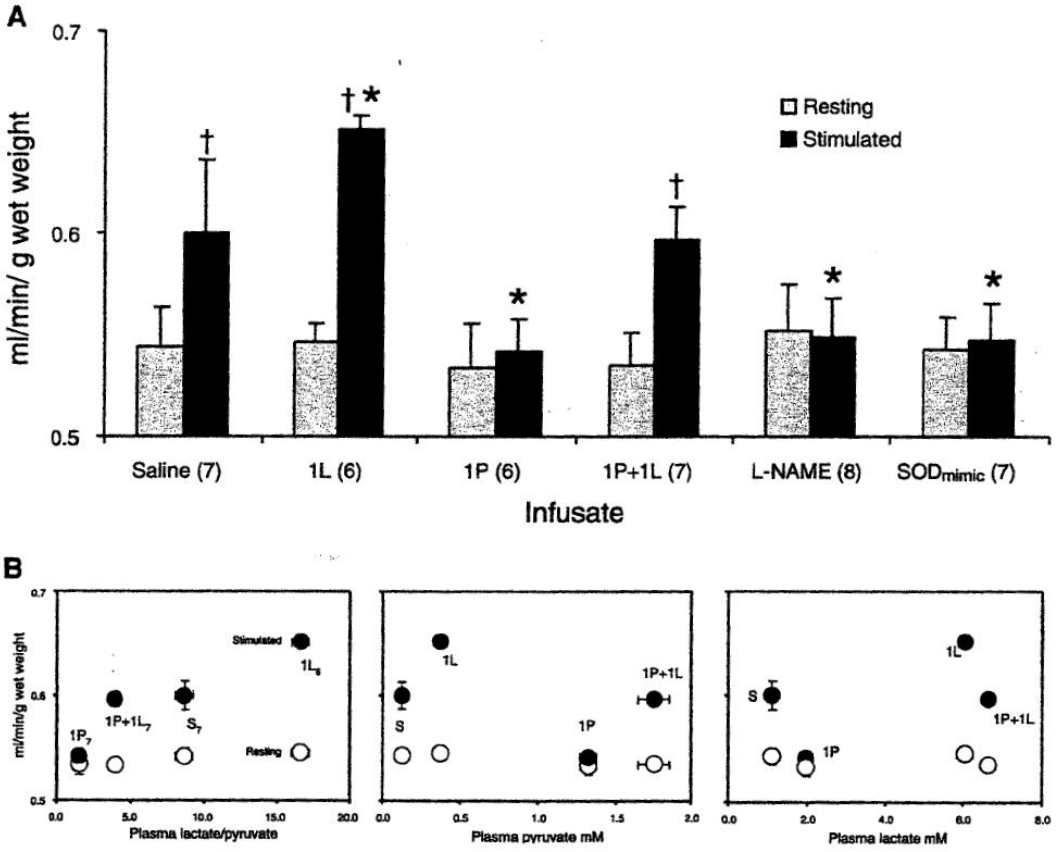

Figure 3. Blood flows in resting and contralateral somatosensory cortex after 5 s of whisker stimulation at 7.5 Hz. **(A)** Blood flow increased ~10% in stimulated vs. resting cortex. Bolus injection of L-lactate (1 mmol/kg in ~30 s) 1 min before stimulation further augmented the increased flows in stimulated cortex without affecting resting cortex. Stimulus-evoked increases in flow were prevented by a pyruvate bolus (1 mmol/kg) 1 min prior to stimulation, whereas c oinjection of lactate and pyruvate had no effect on blood flow in stimulated or resting cortex. Infusion of L-NAME (0.5 μmol/kg/min) or of SOD_{mimic} (0.6 μmol/kg/min) started 20 min before stimulation prevented increased flow in stimulated cortex without affecting flow to resting cortex. **(B)** Relationship of blood flows (from A) in stimulated and resting cortex to plasma L/P, pyruvate (P), and lactate (L) levels obtained in a separate experiment (S = saline controls). Flows in stimulated cortex parallel increasing plasma L/P independent of pyruvate or lactate levels. † Different from resting cortex at $P<0.05$. *Different from stimulated saline controls at $P<0.05$. Other conventions as in **Figure 1**.

Fig. 4

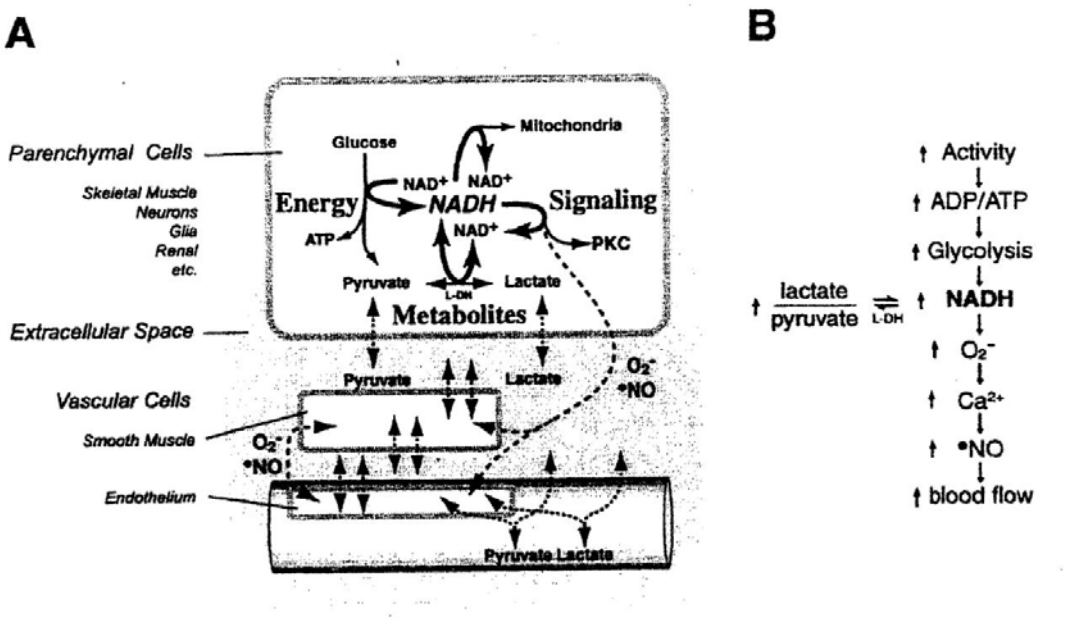

Figure 4. (A) Generic cell (i.e., skeletal muscle, neural, and glial cells, as well as vascular smooth muscle and endothelial cells) and nearby blood vessel showing central position of free cytosolic NAD in energy metabolism and blood flow signaling. Because intra- and extracellular L/P are in near-equilibrium with NADH/NAD^+ under steady-state conditions, changes in extracellular L/P and intracellular L/P in working cells cause corresponding changes in NADH in vascular smooth muscle and endothelial cells. Pyruvate and lactate diffusing from parenchymal cells and from plasma can pass through the vessel wall between and through smooth muscle and endothelial cells. **(B)** Proposed signaling pathway. Neural activity and muscle contraction are fueled by hydrolysis of ATP to ADP. Higher ADP/ATP accelerates glycolysis and transfer of electrons and protons to NAD^+, reducing it to NADH faster than electrons can be used for ATP synthesis. Excess electrons carried by NADH fuel redox signaling pathways to increase blood flow by augmenting O_2^- production and $^{\bullet}NO$ synthesis. (See text for details.)

NADH: sensor of blood flow need in brain, muscle, and other tissues[1]

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SPECIFIC AIM

The sensor of blood flow need in conditions as diverse as brain activation, exercise, high altitude, and diabetes mellitus is unknown. Our first aim was to test the hypothesis that accumulation of electrons in cytosolic free NADH, the reduced form of nicotinamide adenine dinucleotide (NAD), functions as this sensor. The rationale for testing this hypothesis was based on the near equilibria between extracellular and intracellular lactate/pyruvate (L/P) and free cytosolic NADH/NAD^+ ratios; NADH was increased or decreased by injecting lactate or pyruvate, respectively. Our second aim was to identify components of the signaling cascade that mediate increased flows.

PRINCIPAL FINDINGS

1. Increasing L/P increases blood flow in numerous tissues at rest

Bolus injection or brief infusion of lactate increased blood flows in all tissues examined except heart and brain: retina 50%, sciatic nerve 65%, epitrochlearis and gastrocnemius skeletal muscles 30%, soleus muscle 86%, and kidney 40%. Flow increases after lactate infusion for 5 h were retina 45%, sciatic nerve 2.4×, epitrochlearis muscle 3×, diaphragm 17%, and skin 77%. Lactate-augmented flows were prevented when pyruvate was coinjected; injecting pyruvate alone did not affect flow. Similar results were observed with direct application of lactate and/or pyruvate to granulation tissue in a wound-healing chamber model where systemic effects of intravascular injection of lactate and/or pyruvate are obviated.

2. Increasing L/P augments blood flow to contracting muscles

Hind limb skeletal muscle contraction evoked by stimulation of one sciatic nerve (10 Hz for 15 min) increased blood flow 7 to 10× in contracting vs. contralateral resting muscle. Stimulus-evoked flows to muscle were augmented by concurrent infusion of lactate. In contrast, they were prevented by pyruvate infusion, which had no effect on blood flow in contralateral resting muscle. These effects of lactate and pyruvate were abrogated when they were injected together (**Fig. 1**).

3. Increasing L/P augments stimulus-evoked blood flow to whisker barrel cortex

Unilateral whisker stimulation at 7.5 Hz for 5 s increased blood flow 10.5% in contralateral somatosensory whisker barrel cortex vs. ipsilateral unstimulated cortex. Whisker-evoked blood flow increases were doubled by concurrent infusion of lactate and were prevented by pyruvate infusion (which had no effect on blood flow in contralateral resting cortex). As in muscle, these effects of lactate and pyruvate were abrogated when they were coinjected (**Fig. 2**).

4. Blood flow changes correlate with plasma but not tissue L/P

Blood flow in stimulated cerebral cortex paralleled plasma L/P but not lactate and pyruvate levels. Blood flow in contracting skeletal muscle during infusion of saline or lactate and/or pyruvate was strongly and positively correlated with plasma L/P and negatively correlated with plasma lactate and pyruvate levels, but did not correlate with contracting muscle L/P, lactate, or pyruvate. (L/P in extracts of contracting muscle increased 16× vs. resting muscle.) Infusion of lactate or pyruvate during 15 min of muscle stimulation had no effect on blood pCO_2, pO_2, or mean arterial blood pressure; lactate and pyruvate both elevated plasma pH and each increased plasma lactate and pyruvate levels, whereas lactate increased and pyruvate decreased blood flow (Figs. 1 and 2).

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[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.00-0652fje; to cite this article, use *FASEB J.* (April 6, 2001) 10.1096/fj.00-0652fje

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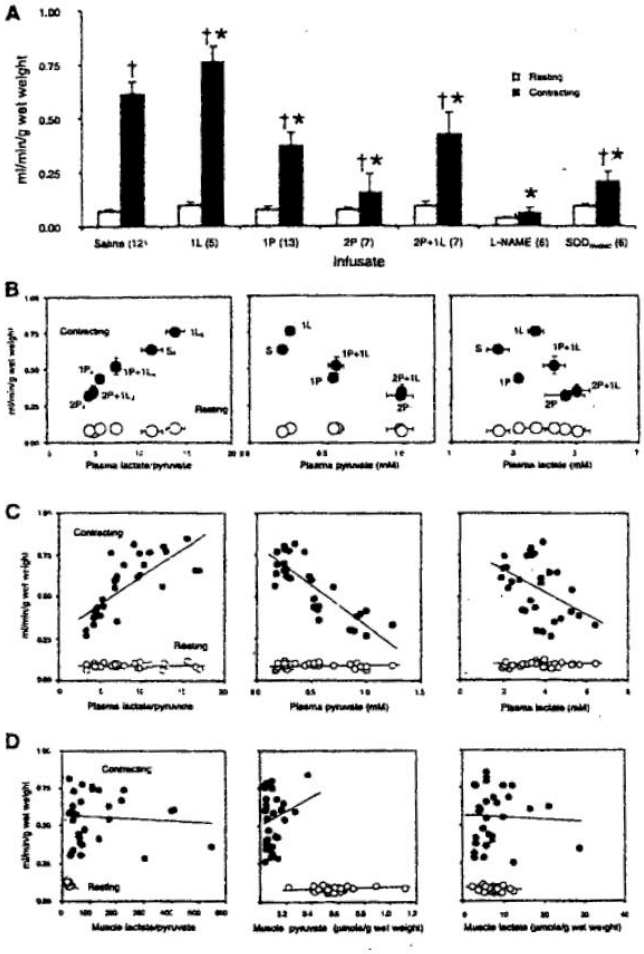

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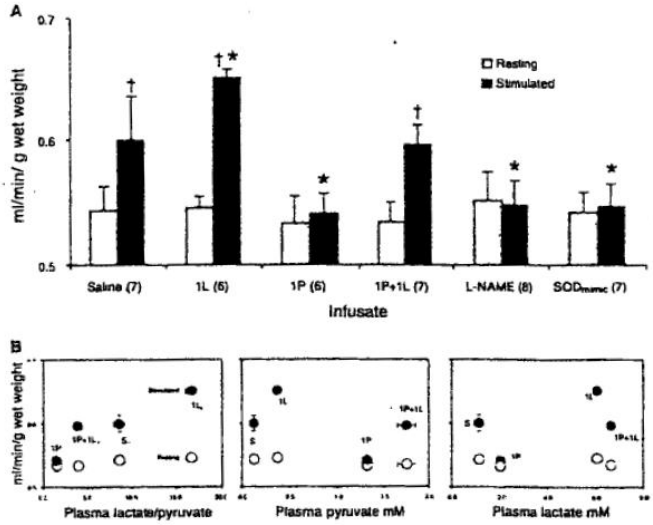

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5. Signaling pathways mediating blood flow augmentation by L/P

Injection of a highly selective superoxide dismutase $(SOD)_{mimic}$ (to block vascular effects of O_2^-) or of L-NAME [a nonselective inhibitor of nitric oxide synthase (NOS) to block $^{\bullet}NO$ vasodilation] prevented increased blood flows in working cortex and muscle. They also blocked L/P-augmented flow in skin chambers.

CONCLUSIONS AND SIGNIFICANCE

For more than a century it has been known that increased blood flow with muscle and neural work is coupled to increased energy metabolism and O_2 consumption. Here we show for a wide range of resting and

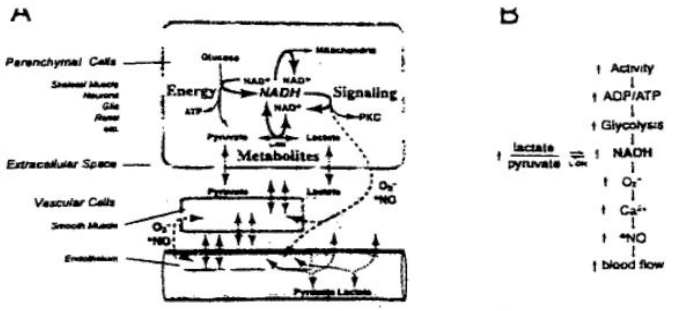
Numbers of animals are given at the bottom of bars (*A*) and as subscripts (*B*). †Different from resting muscle at $P < 0.05$; *different from saline controls at $P < 0.05$.

working tissues that blood flow need is sensed by a common mechanism that is not necessarily coupled to increased energy need or metabolism, O_2 consumption, or glycolysis. The observation that blood flow is augmented when L/P ratios are raised and is attenuated when they are lowered (Figs. 1 and 2) supports the conclusion that cytosolic free NADH senses blood flow need (**Fig. 3**). We have understood for more than half a century that the cofactor NAD is the major carrier of electrons (and protons) from fuels for energy metabolism. It is remarkable to discover only now that free cytosolic NAD also functions as the sensor to signal increased blood flow (Fig. 3).

Increased glycolysis during work and hypoxia accelerates production of pyruvate and transfer of electrons and protons from glucose to NAD^+ faster than they can be used for ATP synthesis by oxidative phosphorylation (OP) in mitochondria. The advantage of this high rate of glycolysis is that ATP can be synthesized up to 2× faster by substrate phosphorylation (SP) in the cytoplasm than by OP, albeit the yield of ATP (per mol of glucose) from SP is much less than from OP. The high rate of ATP synthesis by SP with increased glycolysis is short lived, however, since glycolysis is inhibited by increases in NADH and by accumulation of lactate (formed by reduction of excess pyruvate to lactate coupled to oxidation of NADH to NAD^+ by L-DH) under aerobic and hypoxic conditions. Increasing blood flow removes lactate and other products of energy metabolism and augments delivery of fuels and O_2 for energy metabolism and of pyruvate (which serves as a sink to remove more electrons and protons from NADH as well as fuel for energy metabolism).

Excess electrons accumulating in free cytosolic NADH from whatever cause (increased glycolysis with work, elevated extracellular L/P, increased oxidation of sorbitol in diabetes, and hypoxia) fuel redox signaling pathways (coupled to reoxidation NADH to NAD^+). The observations that pyruvate, an SOD_{mimic}, and an inhibitor of nitric oxide synthase each prevent lactate and work-induced increased blood flows supports the conclusion that excess electrons in NADH augment production of O_2^- and $^{\bullet}NO$, which mediate the increase in blood flow. Accumulation of electrons in NADH also augments de novo synthesis of diacylglycerol to activate PKC-mediated signaling pathways including PKC-mediated increased glucose transport.

The signaling cascade that mediates work-evoked increases in tissue blood flow is normally initiated by accumulation of electrons in NADH in working parenchymal cells, e.g., contracting muscle cells. The present experiments demonstrate that injection of lactate to increase extracellular (plasma) L/P also increased blood flows in both resting and working cells. These observations, together with the finding that increased



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blood flows evoked in contracting muscle by work and by lactate injection correlate with plasma (but not contracting skeletal muscle) L/P, indicate that the redox signaling cascade can be initiated in vascular smooth muscle and endothelial cells. Several lines of evidence suggest that chronic activation of these redox-mediated signaling pathways may stimulate new vessel growth to meet increased blood flow need over the longer term, such as with exercise, hypoxia, and diabetic retinopathy.

We observed a wide and seemingly appropriate range of tissue-specific responses or 'thresholds' to increases in plasma L/P. In the brain, elevation of plasma L/P increased flow only during work; in skeletal muscle elevation of plasma L/P increased flow in resting and working muscle, but the threshold was markedly decreased by work. These tissue differences likely relate to a number of tissue specific characteristics such as basal metabolism, activity of monocarboxylate transporters, capacity to reoxidize NADH, and content of SOD and NOS.

Our observations identify a novel central role for NAD in sensing blood flow need and augmenting flow that is elegant in its simplicity. FJ