

Insulin, ketone bodies, and mitochondrial energy transduction

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ABSTRACT Addition of insulin or a physiological ratio of ketone bodies to buffer with 10 mM glucose increased efficiency (hydraulic work/energy from O₂ consumed) of working rat heart by 25%, and the two in combination increased efficiency by 36%. These additions increased the content of acetyl CoA by 9- to 18-fold, increased the contents of metabolites of the first third of the tricarboxylic acid (TCA) cycle 2- to 5-fold, and decreased succinate, oxaloacetate, and aspartate 2- to 3-fold. Succinyl CoA, fumarate, and malate were essentially unchanged. The changes in content of TCA metabolites resulted from a reduction of the free mitochondrial NAD couple by 2- to 10-fold and oxidation of the mitochondrial coenzyme Q couple by 2- to 4-fold. Cytosolic pH, measured using ³¹P-NMR spectra, was invariant at about 7.0. The total intracellular bicarbonate indicated an increase in mitochondrial pH from 7.1 with glucose to 7.2, 7.5, and 7.4 with insulin, ketones, and the combination, respectively. The decrease in *Eh*⁷ of the mitochondrial NAD couple, *Eh*_{NAD⁺/NADH}, from -280 to -300 mV and the increase in *Eh*⁷ of the coenzyme Q couple, *Eh*_{Q/QH₂}, from -4 to +12 mV was equivalent to an increase from -53 kJ to -60 kJ/2 mol e in the reaction catalyzed by the mitochondrial NADH dehydrogenase multienzyme complex (EC 1.6.5.3). The increase in the redox energy of the mitochondrial cofactor couples paralleled the increase in the free energy of cytosolic ATP hydrolysis, ΔG_{ATP} . The potential of the mitochondrial relative to the cytosolic phases, *E*_{mito/cyto}, calculated from ΔG_{ATP} and ΔpH on the assumption of a 4 H⁺ transfer for each ATP synthesized, was -143 mV during perfusion with glucose or glucose plus insulin, and decreased to -120 mV on addition of ketones. Viewed in this light, the moderate ketosis characteristic of prolonged fasting or type II diabetes appears to be an elegant compensation for the defects in mitochondrial energy transduction associated with acute insulin deficiency or mitochondrial senescence. — Sato, K., Kashiwaya, Y., Keon, C. A., Tsuchiya, N., King, M. T., Radda, G. K., Chance, B., Clarke, K., Veech, R. L. Insulin, ketone bodies, and mitochondrial energy transduction. *FASEB J.* 9, 651-658 (1995)

porter (1) from endoplasmic reticulum to the plasma membrane where it catalyzes the movement of glucose across plasma membrane. However, recently it has become clear that in working heart perfused with 10 mM glucose without insulin, intracellular [glucose] is 1.9 mM, above the apparent *K_m* of about 0.07 mM of hexokinase (EC 2.7.1.1). Even under these conditions where insulin was absent, glucose transport is not the sole rate-determining step for glucose utilization (2). More recently, interest in insulin action has been directed to unraveling the complex signaling role of protein kinases (3-6) in the growth responses of tissue to insulin. However, these advances in our understanding of insulin's role as a growth factor so far have left unexplained the acute effects of insulin on mitochondrial (7, 8) energy transduction.

We have therefore studied the acute effects of insulin on mitochondrial energy transduction by determining the physiological performance of the working heart perfused with 10 mM glucose and comparing that to performance during perfusion in the presence of maximal insulin stimulation, a ratio of 4 mM D- β -hydroxybutyrate to 1 mM acetoacetate or the combination. In addition, we determined the changes in the tissue content of all components of the tricarboxylic acid (TCA)² cycle and their transaminase cosubstrates, the gradient of proton between cytosol and mitochondria, ΔpH , the free energy of cytosolic ATP hydrolysis, ΔG_{ATP} , and the potential between cytosolic and mitochondrial phases, *E*_{mito/cyto}, in the same tissue under identical experimental conditions using a combination of techniques. This allowed us for the first time to obtain in one tissue an integrated picture of the acute effects of insulin on mitochondrial energy transduction and physiological performance.

MATERIALS AND METHODS

Hearts from 400 to 450 g fed male rats were perfused in the working mode (9) with modified Krebs-Henseleit buffer containing 10 mM glucose, to which was added 100 nM insulin or 4 mM D- β -hydroxybutyrate and 1 mM acetoacetate, or both, in a manner previously described (2). The hearts were freeze-clamped, excess perfusate was removed under liquid N₂, and the frozen tissue was deproteinized with cold HClO₄ (2). The tissue contents

Key Words: tricarboxylic acid cycle intermediates • mitochondrial redox states • ΔpH • *E*_{mito/cyto} • ΔG_{ATP}

ON THE PRESUMPTION THAT THE intracellular glucose concentration is vanishingly small during insulin deficiency in muscle and adipose tissue, explanations of insulin's acute effects have focused on its ability to move the Glut 4 glucose trans-

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²Abbreviations: *Eh*, redox potential of a half-reaction; *E*_{mito/cyto}, electric potential of the mitochondrial relative to the cytosolic phase; ΔpH , pH gradient of mitochondrial relative to cytosolic phase; Q, ubiquinone; QH₂, ubiquinol; ΔG_{ATP} , free energy of ATP hydrolysis; TCA, tricarboxylic acid.

of glucose, pyruvate, L-lactate, citrate, L-isocitrate, α -ketoglutarate, L-glutamate, fumarate, L-malate, and L-aspartate were then measured using standard techniques of enzymatic analysis (10) with enzymes purchased from Boehringer Mannheim Co. (Indianapolis, Ind.). Total tissue bicarbonate plus CO_2 content was estimated using phosphoenolpyruvate carboxylase (EC 4.1.1.31) (11). Total tissue NH_4^+ plus NH_3 after charcoal treatment of the perchloric acid samples using glutamate dehydrogenase (EC 1.4.1.3) was measured enzymatically (12) and total P_i was measured using the method of Cornell et al. (13). Succinate was measured with succinate-CoA ligase (EC 6.2.1.4), purchased from Sigma Chemical Co. (St. Louis Mo.), pyruvate kinase (EC 2.7.1.40), and L-lactate dehydrogenase (EC 1.1.1.27) as listed above (14). Acetyl CoA, malonyl CoA, succinyl CoA, and CoA were measured by HPLC (15). Cytosolic ATP, PCr, and P_i and pH were determined by ^{31}P -NMR spectroscopic methods (16) whereas total tissue contents creatine, ATP, ADP, AMP, PCr, and P_i were measured enzymatically as previously described (17). The $[\text{P}_i]$ observed by ^{31}P -NMR was extramitochondrial because phosphorus nuclei present within mitochondrial are not NMR-visible (18). The monocarboxylic anions lactate $^-$, pyruvate $^-$, acetoacetate $^-$, and D- β -hydroxybutyrate $^-$ were all taken to distribute equally between mitochondrial and cytosolic phases in conformity with the $[\text{H}^+]$ gradient as dictated by the monocarboxylate-proton cotransporter (19). The concentration of malate $^{2-}$ was taken to be equal in both cytosolic and mitochondrial phases due to rapid malate $^{2-}$ /malate $^{2-}$ exchange. Because of active malate $^{2-}$ /dicarboxylate $^{2-}$ exchangers (20), the concentrations of the other dicarboxylates within mitochondrial phase—H-citrate $^{2-}$, H $^+$ -citrate $^{2-}$, α -ketoglutarate $^{2-}$, succinate $^{2-}$, and fumarate $^{2-}$ —were taken to be equal to measured total tissue contents.

Physiological measurements

The pO_2 , pCO_2 , and pH were determined in the arterial perfusate and the coronary venous effluent was measured potentiometrically (Corning Gas Analyzer, Corning, N.Y.). The afterload was set at 80 mmHg. Aortic and coronary outflow were measured volumetrically. Systolic, diastolic, mean aortic pressure (Spectramed P23X1, Oxnard, Calif.), heart rate, and dP/dt (Gould G4615-71, Valley View, Ohio) were measured as previously described (17).

Hydraulic work was calculated from:

$$\text{Cardiac hydraulic work (J} \cdot \text{min}^{-1} \cdot \text{g}^{-1} = \frac{(\text{aortic} + \text{coronary flow (ml/min)} \times 10^{-6} \text{ (m}^3/\text{ml)} \times \text{average systolic pressure (mmHg)})}{\text{heart wet weight (g)}} \times \frac{101,325 \text{ (Nm}^{-2}\text{)}}{760 \text{ (mmHg)}} \quad (\text{Eq. 1})$$

where 1 atm = 760, mmHg = 101,325 newton/m 2 (Nm $^{-2}$).

O_2 consumption was calculated from the measured decrease in pO_2 in the inflow and coronary venous outflow according to:

$$\text{O}_2 \text{ consumption } \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1} = \frac{(\text{Pa}_{\text{O}_2} - \text{Pv}_{\text{O}_2}) \times \text{coronary flow (ml/min)} \times \alpha_{\text{O}_2}}{\text{heart weight (g)} \times (\text{P}_{\text{atm}} - \text{P}_{\text{H}_2\text{O}}) \times \text{V}_{\text{O}_2}} \times 10^3 \quad (\text{Eq. 2})$$

where Pa_{O_2} and Pv_{O_2} are the partial pressure of oxygen in the aorta and coronary vein, respectively (mmHg); $\text{P}_{\text{H}_2\text{O}}$, the partial pressure of water, is 47.1 mmHg at 38°C; P_{atm} , the atmospheric pressure, is 760 mmHg. α_{O_2} is the solubility of O_2 , taken to be 0.0212 ml O_2 /ml human plasma at 38°C (21–24). V_{O_2} is the mole volume of oxygen gas, taken to be 25.5 l O_2 /mol corrected to 38°C.

Cardiac efficiency was calculated, using a slight modification of the method of Neely et al. (25), as the ratio of hydraulic work/redox energy available from mitochondrial $\text{NADH} + \text{H}^+$ combining with $1/2 \text{ O}_2$, expressed as percent:

$$\text{Cardiac efficiency (\%)} = \frac{\text{hydraulic work (J} \cdot \text{min}^{-1} \cdot \text{g}^{-1}\text{)}}{\text{O}_2 \text{ consumption } (\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}) \times \text{respiratory chain energy (J}/\mu\text{mol)}} \times 100 \quad (\text{Eq. 3})$$

Respiratory chain energy is that energy derived from the transfer of 2 electrons from mitochondrial NADH to O_2 as described below (Eq. 21).

Tissue water distribution

Total H_2O , determined by drying to a constant weight, was 0.853 ml/g wet weight. Intracellular and extracellular H_2O spaces were measured using $^3\text{H}_2\text{O}$ and ^{14}C mannitol as previously described (17). Extracellular H_2O comprised 0.593 ml/ml total H_2O and intracellular H_2O comprised 0.407 ml/ml total H_2O .

Intracellular H_2O distribution was estimated in hearts (perfused under the four conditions tested) that were rapidly frozen, cryosectioned, left unstained, and dehydrated at -80°C (26). In livers treated in this manner, 22.0% of the morphometric volume was mitochondria that contained only 11.8% of intracellular potassium, where the nonmitochondrial morphometric volume of 78.0% contained 88.2% of intracellular potassium as determined by X-ray microanalysis (Veech et al., unpublished results). Morphometric volume of mitochondria in the perfused hearts did not differ significantly between the four groups studied, from about 34% of the intracellular area compared with an area of 35% in hearts frozen in situ. Taking the ratio of the morphometric volume of mitochondria to the mitochondrial free H_2O volume to be the same as that for liver, then the 34% of the total intracellular morphometric volume composing mitochondria contained $(11.8\%/22.0\%) \times 34\%$ or 18.2% of intracellular H_2O , whereas the nonmitochondrial phase contained 81.8% of water. This finding is consistent with many earlier reports suggesting that a much greater percentage of mitochondrial volume is occupied by protein and inner membrane than is occupied by protein and membranes in the cytosol, endoplasmic reticulum, and nucleus (27). Using this water distribution between mitochondrial and nonmitochondrial phases, and naming all nonmitochondrial water cytosolic, the measured total metabolite ($\mu\text{mol}/\text{ml}$ intracellular H_2O) was distributed between compartments by:

$$[\Sigma\text{P}_i]_{\text{total}} = V_{\text{cyto}} \times [\Sigma\text{P}_i]_{\text{cyto}} + V_{\text{mito}} \times [\Sigma\text{P}_i]_{\text{mito}} \quad (\text{Eq. 4})$$

where V_{cyto} is the fractional H_2O volume of cytosol and V_{mito} is that of mitochondria. Mitochondrial $[\Sigma\text{P}_i]$ can be calculated from Eq. 4 using total $[\Sigma\text{P}_i]$ measured enzymatically, and cytosolic $[\Sigma\text{P}_i]$ taken to be equal to the ^{31}P -NMR visible P_i and reported as $\mu\text{mol}/\text{ml}$ cytosolic H_2O .

Compartment pH and HCO_3^- distribution

Cytosolic pH was measured by the chemical shift of P_i relative to PCr in ^{31}P -NMR spectra. It was assumed that the $[\text{CO}_2]$ was the same in both the mitochondrial and cytosolic phases and equivalent to the coronary venous $[\text{CO}_2]$. Knowing cytosolic pH from NMR measurements and $[\text{CO}_2]$ from measurements of coronary effluent, cytosolic $[\text{HCO}_3^-]$ was calculated using the Henderson-Hasselbalch equation:

$$\text{pH} = 6.1 + \log \frac{[\text{HCO}_3^-]}{[\text{CO}_2]} \quad (\text{Eq. 5})$$

The total $[\text{HCO}_3^-]$ measured enzymatically was apportioned between the mitochondrial and nonmitochondrial phases according to:

$$[\text{HCO}_3^-]_{\text{mito}} = ([\text{HCO}_3^-]_{\text{total}} - V_{\text{cyto}} \times [\text{HCO}_3^-]_{\text{cyto}}) / V_{\text{mito}} \quad (\text{Eq. 6})$$

Mitochondrial pH was then estimated by inserting the calculated $[\text{HCO}_3^-]_{\text{mito}}$ into Eq. 5.

Free energy of cytosolic ATP hydrolysis, ΔG_{ATP} , and potential between cytosolic and mitochondrial phases, $E_{\text{mito/cyto}}$

The ΔG_{ATP} was calculated according to the statements:

$$\frac{[\Sigma\text{ATP}]}{[\Sigma\text{ADP}]} = \frac{[\Sigma\text{PCr}]}{[\Sigma\text{creatine}]} \times K'_{\text{CK}} \quad (\text{Eq. 7})$$

$$\Delta G_{\text{ATP}} = \Delta G_{\text{ATP}}^0 + RT \ln \frac{[\Sigma\text{creatine}][\Sigma\text{P}_i]_{\text{cyto}}}{K'_{\text{CK}}[\Sigma\text{PCr}]_{\text{cyto}}} \quad (\text{Eq. 8})$$

where R , the gas constant, is $8.3145 \times 10^{-3} \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$; T , the absolute temperature, is 311.15 K. Taking $[\Sigma\text{ATP}]$ from the ^{31}P visible NMR spectra, the free cytosolic $[\Sigma\text{ADP}]$ was calculated from the creatine kinase reaction (EC 2.7.3.2) (17). K'_{CK} and ΔG_{ATP}^0 were corrected (28) for the cytosolic pH determined by ^{31}P -NMR and free $[\text{Mg}^{2+}]$ estimated from the measured $[\Sigma\text{citrate}]/[\Sigma\text{isocitrate}]$ (29). Free $[\Sigma\text{P}_i]_{\text{cyto}}$ and $[\Sigma\text{PCr}]_{\text{cyto}}$ were taken to be equal to the ^{31}P -NMR visible P_i and PCr. $[\Sigma\text{Creatine}]$ was determined enzymatically and cytosolic $[\text{H}^+]$ was determined using ^{31}P -NMR as described above.

Estimation of the $E_{\text{mito/cyto}}$ was made using the stoichiometry of 4 H^+ (30–32) moved from mitochondrial to cytosolic phases by NADH dehydrogenase and QH_2 cytochrome c oxidoreductase (EC 1.10.2.2). Because 4 H^+ reenter the mitochondria (33) through the ATP synthase (34) and H^+/P_i cotransporter (35) for each ATP molecule synthesized in the mitochondrion and translocated to the cytosol (36), then the concentration and electric work of the proton may be represented as:

$$\frac{\Delta G_{\text{ATP}}}{4} = RT \ln \frac{[\text{H}^+]_{\text{m}}}{[\text{H}^+]_{\text{c}}} + FE_{\text{m/c}} \quad (\text{Eq. 9})$$

where F , the Faraday constant, is $96,485 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{V}^{-1}$.

Cytosolic redox state

Cytosolic $[NAD^+]/[NADH]$ was estimated from the measured components of the lactate dehydrogenase reaction (EC 1.1.1.27) (37):

$$\frac{[NAD^+]_{cyto}}{[NADH]_{cyto}} = \frac{[\text{pyruvate}^-]_{total} [H^+]_{cyto}}{[L\text{-lactate}^-]_{total}} \times \frac{1}{1.11 \times 10^{-11} \text{ M}} \quad (\text{Eq. 10})$$

Mitochondrial redox state and estimation of oxaloacetate

The distribution of the measured tissue NH_4^+ between the cytosolic and mitochondrial phases was calculated, assuming that NH_3 is permeant across the mitochondrial membrane, solving the following equations simultaneously:

$$[NH_4^+]_{total} = V_{cyto} \times [NH_4^+]_{cyto} + V_{mito} \times [NH_4^+]_{mito} \quad (\text{Eq. 11})$$

$$\frac{[NH_4^+]_{cyto} [H^+]_{mito}}{[NH_4^+]_{mito} [H^+]_{cyto}} = 1 \quad (\text{Eq. 12})$$

The free mitochondrial $[NAD^+]/[NADH]$ was estimated from the measured components of the glutamate dehydrogenase reaction (EC 1.4.1.2) taking K' at pH 0 from Engel and Dalziel (38) by:

$$\frac{[NAD^+]_{mito}}{[NADH]_{mito}} = \frac{[\alpha - \text{ketoglutarate}^{2-}]_{total} [NH_4^+]_{mito} [H^+]_{mito}}{[L\text{-glutamate}^-]_{total}} \times \frac{1}{3.87 \times 10^{-13} \text{ M}^2} \quad (\text{Eq. 13})$$

When ketones were added, mitochondrial $[NAD^+]/[NADH]$ was also calculated from the D- β -hydroxybutyrate dehydrogenase reaction (EC 1.1.1.30) taking K' from Williamson et al. (37) according to:

$$\frac{[NAD^+]_{mito}}{[NADH]_{mito}} = \frac{[\text{acetoacetate}^-]_{total} [H^+]_{mito}}{[D\text{-}\beta\text{-hydroxybutyrate}^-]_{total}} \times \frac{1}{4.93 \times 10^{-9} \text{ M}} \quad (\text{Eq. 14})$$

Mitochondrial [oxaloacetate] was calculated from malate dehydrogenase reaction (EC 1.1.1.37), taking K' from Guynn et al. (39), and mitochondrial $[NAD^+]/[NADH]$, assuming equal distribution of malate $^{2-}$ between mitochondrial and cytosolic phases:

$$[\text{oxaloacetate}^{2-}]_{mito} = \frac{[L\text{-malate}^{2-}]_{total} [NAD^+]_{mito}}{[NADH]_{mito} [H^+]_{mito}} \times 2.86 \times 10^{-12} \quad (\text{Eq. 15})$$

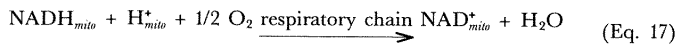
The potential of the half-reaction of mitochondrial NAD $^+$ couple at pH 7, $Eh_{NAD^+/NADH}$ was calculated from the mitochondrial $[NAD^+]/[NADH]$:

$$Eh_{NAD^+/NADH} = E_{NAD^+/NADH}^0 + \frac{RT}{nF} \ln \frac{[NAD^+]_{mito}}{[NADH]_{mito}} \times \frac{10^{-7}}{[H^+]_{mito}} \quad (\text{Eq. 16})$$

where $n = 2$ and $E_{NAD^+/NADH}$ at pH 7.0 is -0.32 V (40).

Free energy of respiratory chain, ΔG_{CHAIN}

The reaction transferring 2 electrons from NADH to oxygen is expressed as:



The free energy of the respiratory chain can be estimated by combining the half-reactions at pH 7, Eh_{O_2/H_2O} (41) and $Eh_{NAD^+/NADH}$:

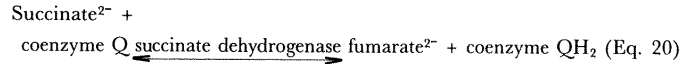
$$Eh_{O_2/H_2O} = +0.814 \text{ V} \quad (\text{Eq. 18})$$

$$\Delta G_{CHAIN} = -nF(Eh_{O_2/H_2O} - Eh_{NAD^+/NADH}) \quad (\text{Eq. 19})$$

where $n = 2$.

Relationship between free mitochondrial $[Q]/[QH_2]$ and $[NAD^+]/[NADH]$ couples

The Eh^0 of the mitochondrial coenzyme Q couple, Eh_{Q/QH_2}^0 , was estimated from the measured [fumarate $^{2-}$]/[succinate $^{2-}$] ratio, taking the reversible (42) succinate dehydrogenase reaction (EC 1.3.5.1):



to be in near-equilibrium with its diffusible cofactor, coenzyme Q. At equilibrium:

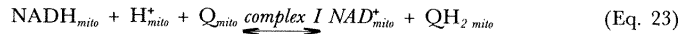
$$Eh_{Q/QH_2}^0 = Eh_{fum/succ}^0 \quad (\text{Eq. 21})$$

then:

$$Eh_{Q/QH_2}^0 = E_{fum-succ}^0 + \frac{RT}{nF} \ln \frac{[\text{fumarate}^{2-}]}{[\text{succinate}^{2-}]} \quad (\text{Eq. 22})$$

where $E_{fum-succ}^0$ is $+0.031 \text{ V}$ (43) and $n = 2$.

The free energy of the reaction catalyzed by the mitochondrial NADH dehydrogenase multienzyme complex was calculated from the relationship:



$$\Delta G_{QH_2/NAD^+} = -nF(Eh_{Q/QH_2} - Eh_{NAD^+/NADH}) \quad (\text{Eq. 24})$$

where $n = 2$.

RESULTS

The heart rate was between 290 and 300 min^{-1} in all groups tested. The peak systolic pressure was 90.5 mmHg in hearts perfused with glucose alone and increased to 96, 98, and 94 mmHg on addition of insulin, ketones, and the combination, respectively. Mean systolic pressure was 81 mmHg, which increased to 83 mmHg with insulin. Cardiac output (Table 1) was 25 $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}$ wet weight $^{-1}$ and increased to 28 after addition of insulin. Hydraulic work (Eq. 1) was 0.30 $\text{J} \cdot \text{min}^{-1} \cdot \text{g}$ wet weight heart $^{-1}$ and increased to 0.34 and 0.37 $\text{J} \cdot \text{min}^{-1} \cdot \text{g}$ wet weight $^{-1}$ on insulin and ketones, respectively. O_2 consumption (Eq. 2 and refs 17–19) was 6.5 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}$ wet weight $^{-1}$ and decreased to 5.6, 5.9, and 5.4 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}$ wet weight $^{-1}$ after addition of insulin, ketones, and the combination, respectively. The efficiency of cardiac hydraulic work (Eq. 3) was 10.5% in hearts perfused with glucose alone, and increased to 28% with insulin, to 24% with ketones, and to 36% on addition of the combination.

Addition of insulin to the perfusion buffer containing 10 mM glucose increased the intracellular glucose concentrations from 1.9 to about 10 mM (2), but increased [pyruvate $^-$] only from 0.05 to 0.08 $\mu\text{mol}/\text{ml}$ intracellular H_2O . Addition of insulin, ketones, or the combination al-

TABLE 1. Effect of insulin and/or ketones on physiological parameters and efficiency of the working rat heart^a

	Control, $n = 8$	Insulin, ^b $n = 5$	Ketones, ^c $n = 5$	Ketones and insulin, $n = 5$
Cardiac output, $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}$ wet wt $^{-1}$	24.9 \pm 0.6	26.7 \pm 0.9	28.3 \pm 0.9 ^d	25.8 \pm 0.7
Hydraulic work, $\text{J} \cdot \text{min}^{-1} \cdot \text{g}$ wet wt $^{-1}$	0.30 \pm 0.01	0.34 \pm 0.01 ^d	0.37 \pm 0.01 ^d	0.32 \pm 0.01
O_2 consumption, $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}$ wet wt $^{-1}$	6.5 \pm 0.1	5.6 \pm 0.1 ^d	6.0 \pm 0.2 ^d	5.4 \pm 0.4 ^d
Cardiac efficiency, %	10.5 \pm 0.3	13.4 \pm 0.6 ^d	13.0 \pm 0.2 ^d	14.3 \pm 1.3 ^d

^aData are means \pm SE. Control = 10 mM glucose perfusion. ^bInsulin: 100 nM. ^cKetone bodies: 4 mM D- β -hydroxybutyrate + 1 mM acetoacetate. ^d $P < 0.05$ compared to control, determined using the Mann-Whitney U test.

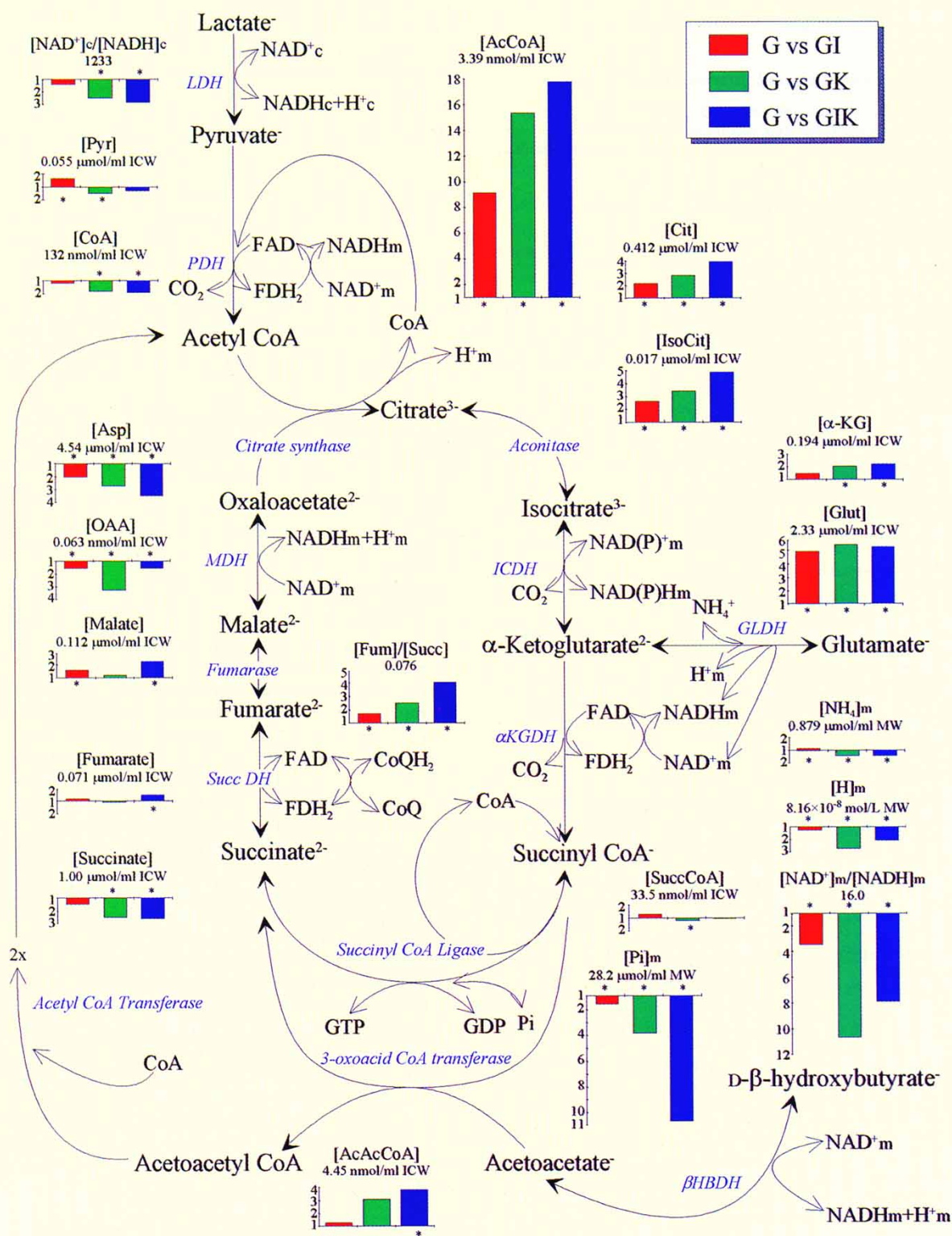


Figure 1. The mitochondrial TCA cycle in the working rat heart showing the proportional changes (bar graphs), relative to the glucose-perfused rat heart, that occur with the addition of insulin and/or ketone bodies. (Experimental value/control values)ⁿ: where $n = 1$ when the experimental value > control, and $n = -1$ when experimental value < control. Component concentrations and ratios in the glucose-perfused hearts are shown below the headings. Symbols are: ICW, intracellular H₂O; G, glucose alone in the perfusate; GI, glucose plus 100 nM insulin; GK, glucose plus 4 mM D-β-hydroxybutyrate 1 mM acetoacetate; GIK, glucose plus ketone bodies and insulin.

tered the levels of TCA cycle components in the working rat heart. Addition of insulin, ketones, and the combination increased acetyl CoA 9-fold, 15-fold, and 18-fold, respectively, with corresponding decreases in CoA (Fig. 1). Malonyl CoA was increased from 0.14 nmol/ml intracellular H₂O in the groups perfused with glucose alone to 5 on addition of insulin, 3 on addition of ketones, and 6 nmol/ml intracellular H₂O with the combination. In the first third of the TCA cycle, citrate increased 2- to 4-fold, isocitrate from 3- to 5-fold, and α -ketoglutarate 1.5- to 2-fold.

The cytosolic pH was not changed from 7.05 by any of the perfusion conditions (Table 2, Fig. 2). The cytosolic [HCO₃⁻] (Eq. 5) was about 14 μ mol/ml intracellular H₂O in hearts perfused with or without insulin and decreased to about 12 on addition of ketones. The difference between the total measured intracellular [HCO₃⁻] measured enzymatically and the cytosolic [HCO₃⁻] showed that the mitochondrial [HCO₃⁻] (Eq. 6), and with it mitochondrial pH, increased from 7.09 during perfusion with glucose alone to 7.21, 7.52, and 7.42 on addition of insulin, ketones, and the combination.

The cytosolic [P_i] determined from the ³¹P-NMR visible spectra was 6.9 μ mol/ml intracellular H₂O or 8.4 μ mol/ml cytosolic H₂O in glucose perfused hearts, and was not significantly changed by any treatment (Table 2). In contrast, the enzymatically measured [P_i] was about 12 μ mol/ml intracellular H₂O in control hearts, but decreased to 8-9 μ mol/ml intracellular H₂O after addition of insulin or ketones and to 7 μ mol/ml intracellular H₂O in hearts perfused with the combination. The mitochondrial [P_i] (Eq. 4), deduced from the difference between the ³¹P-NMR visible [P_i] and the enzymatic measurements, was estimated to be 28 μ mol/ml mitochondrial H₂O in glucose perfused hearts; it decreased to 17 on addition of insulin, to 6 on addition of ketones, and to 3 on addition of the combination (Table 2). Mitochondrial [P_i] varied inversely with the mitochondrial [HCO₃⁻], conforming to the requirement for electroneutrality in a system in which a process of net H⁺-P_i⁻ cotransport or OH⁻/P_i⁻ exchange was operating.

There was a 5-fold increase in tissue glutamate content with all additions that, with the decreased [NH₄⁺] (Eq. 11 and Eq. 12) resulting from the alkalization of the mitochondria, reflected a 3- to 11-fold decrease in the free mitochondrial [NAD⁺]/[NADH] (Fig. 1) from the control value of 16. This ratio was decreased to 1.51 and 2.04 on addition of ketones or ketones plus insulin, respectively. Because in these conditions the hearts were being perfused with 4 mM D- β -hydroxybutyrate⁻ and 1 mM acetoacetate⁻, the free mitochondrial [NAD⁺]/[NADH] calculated from the β -

hydroxybutyrate dehydrogenase reaction (Eq. 14) was 1.54 and 1.93, respectively, which agrees remarkably well with the ratio calculated from the reactants of the glutamate dehydrogenase reaction. As glutamate dehydrogenase (Eq. 13) is located within mitochondrial matrix and D- β -hydroxybutyrate dehydrogenase (Eq. 14) is located in mitochondrial inner membrane, this agreement demonstrates that both matrix and mitochondrial membrane enzymes share a common pool of pyridine nucleotides in heart, as has been previously demonstrated in liver (37). The Eh' of the mitochondrial NAD couple, $Eh'_{NAD^+/NADH}$ (Eq. 16), was -280 mV and decreased to about -300 mV on addition of insulin, ketones, and the combination.

In contrast to the increased tissue contents of the initial components of the TCA cycle, the [succinyl CoA⁻] was 33.5 nmol/ml intracellular H₂O and was essentially invariant, showing only a 10% drop on addition of ketones (Fig. 1). Unexpectedly, [succinate²⁻] decreased 1.5- to 3-fold, whereas [fumarate²⁻] and [L-malate²⁻] increased slightly on addition of the combination of ketones and insulin, and [L-aspartate⁻] and calculated [oxaloacetate²⁻] decreased 2- to 4-fold on the addition of insulin and/or ketones (Fig. 1). The ratio [fumarate²⁻]/[succinate²⁻] increased about 1.5-fold on addition of insulin, about 2.5-fold on addition of ketones, and about 4-fold on addition of the combination (Fig. 1). Taking this change to indicate the redox state of the [Q]/[QH₂] couple, which is the cofactor for the succinate dehydrogenase (EC 1.3.5.1) reaction, the observed increase in [fumarate²⁻]/[succinate²⁻] indicates a 1.5- to 4-fold oxidation of mitochondrial [Q]/[QH₂] on addition of insulin, ketones, or the combination. The Eh'_{Q/QH_2} , calculated from the succinate dehydrogenase reaction (Eqs. 20-22, Fig. 2), increased progressively from -4 mV during perfusion with glucose alone to +15 mV on the addition of insulin and ketones. When the oxidation of the mitochondrial Q couple was combined with the reduction of the mitochondrial NAD couple resulting from these additions to the glucose perfused hearts, the estimated energy available in the transfer of 2 e from the mitochondrial NAD to the Q couple (Eq. 23 and Eq. 24) catalyzed by the NADH dehydrogenase multienzyme complex, $\Delta G_{QH_2/NAD^+}$, increased from -53 kJ/2 mol e during perfusion with glucose alone to -60 kJ/2 mol e with addition of insulin and ketones. This increase was paralleled by an increase in the cytosolic free energy of ATP hydrolysis, ΔG_{ATP} (Eq. 7 and Eq. 8), determined independently using NMR spectroscopy, which increased from -56 kJ/mol in control hearts to about -59 kJ/mol on addition of insulin or insulin plus ketones (Table 3, Fig. 2). The potential between mitochondrial and cytosolic phases, $E_{mito/cyto}$ (Eq. 9, Fig. 2,

TABLE 2. Effect of insulin and/or ketones on biochemical parameters of the working rat heart^a

	Control, n = 8	Insulin, ^b n = 5	Ketones, ^c n = 5	Ketones and insulin, n = 5
Cytosolic pH	7.06 ± 0.01	7.05 ± 0.01	7.04 ± 0.01	7.02 ± 0.00
Mitochondrial pH	7.09 ± 0.02	7.21 ± 0.02 ^d	7.52 ± 0.02 ^d	7.42 ± 0.00
Coronary venous CO ₂ , μ mol/ml intracellular H ₂ O	1.58 ± 0.02	1.60 ± 0.06	1.37 ± 0.05 ^d	1.39 ± 0.02 ^d
Total [HCO ₃ ⁻], μ mol/ml intracellular H ₂ O	14.6 ± 0.9	15.7 ± 1.2	16.3 ± 1.3	14.9 ± 1.8
Cytosolic [HCO ₃ ⁻], μ mol/ml cytosolic H ₂ O	14.3 ± 0.2	14.4 ± 0.3	12.1 ± 0.3 ^d	11.5 ± 0.1 ^d
Mitochondrial [HCO ₃ ⁻], μ mol/ml mitochondrial H ₂ O	15.6 ± 0.7	20.9 ± 1.1 ^d	36.1 ± 1.5 ^d	29.9 ± 0.3 ^d
Total [P _i], μ mol/ml intracellular H ₂ O	11.8 ± 0.9	8.25 ± 0.88 ^d	8.83 ± 0.70 ^d	6.93 ± 0.64 ^d
Cytosolic [P _i], μ mol/ml cytosolic H ₂ O	8.38 ± 0.68	6.08 ± 0.48	9.50 ± 0.61	7.99 ± 1.60
Mitochondrial [P _i], μ mol/ml mitochondrial H ₂ O	28.2 ± 3.3	17.1 ± 1.9 ^d	5.71 ± 2.81 ^d	2.62 ± 6.51 ^d

^aData are means ± SE. Control = 10 mM glucose perfusion.

^bInsulin: 100 nM.

^cKetone bodies: 4 mM D- β -hydroxybutyrate + 1 mM

acetoacetate. ^dP < 0.05 compared to control, determined using the Mann-Whitney U test.

Table 3), was -143 mV in hearts perfused with glucose alone and unchanged on the addition of insulin but decreased to -120 mV on addition of ketones or -130 mV on addition of the combination.

DISCUSSION

Addition of insulin, with or without the addition of ketones, decreased O_2 consumption by about 14% (Table 1). The free cytosolic ADP, $[\Sigma ADP]_{cyto}$, was calculated from the measured components of the creatine kinase reaction (Eq. 7), and the free cytosolic inorganic phosphate, $[\Sigma P_i]_{cyto}$, was taken directly from the ^{31}P -NMR spectra. The K_{mADP} for the ADP/ATP translocator is known to be about $20 \mu M$ (44), and the K_{mP_i} of the H^+ - P_i cotransporter about 1 mM (45). We determined the V_{max} of this perfused heart system for O_2 consumption to be $24 \mu mol \cdot min^{-1} \cdot ml^{-1}$ intracellular H_2O . Using a simple initial Michaelis-Menten rate equation (46),

$$V_{O_2} = \frac{V_{max}}{1 + \frac{K_{mADP}}{[\Sigma ADP]_{cyto}} + \frac{K_{mP_i}}{[\Sigma P_i]_{cyto}}} \quad (\text{Eq. 25})$$

one may then calculate a predicted rate of O_2 consumption. The predicted values (see line on bar graph, Fig. 2) correlated extremely well with the measured values (see bar graph, Fig. 2). However, the $[\Sigma ADP]_{cyto}$ was $0.14 \mu mol/ml$ intracellular H_2O in hearts perfused with glucose alone, which decreased to 0.08 with insulin and about to 0.06 with ketones or the combination; $[\Sigma P_i]_{cyto}$ was $6 \mu mol/ml$ intracellular H_2O in all groups (Table 2). Because both $[\Sigma ADP]_{cyto}$ and $[\Sigma P_i]_{cyto}$ were so much greater than their respective K_m s for the adenine nucleotide translocator and the H^+ - P_i cotransporter, the availability of these substrates is unlikely to determine the rate of O_2 consumption in the working rat heart.

Addition of insulin increased the efficiency of hydraulic work per mole of O_2 consumed in heart 28% by decreasing O_2 consumption by 14% and increasing cardiac work 13% (Table 1). Addition of ketones, on the other hand, increased the efficiency mainly by increasing hydraulic work, at the same time decreasing O_2 consumption by only a small percentage. From the energy of ATP hydrolysis measured independently with ^{31}P -NMR spectroscopy and the ΔpH (Eq. 9), the calculated potential of the mitochondrial phase relative to the cytosolic phase was -143 mV in hearts per-

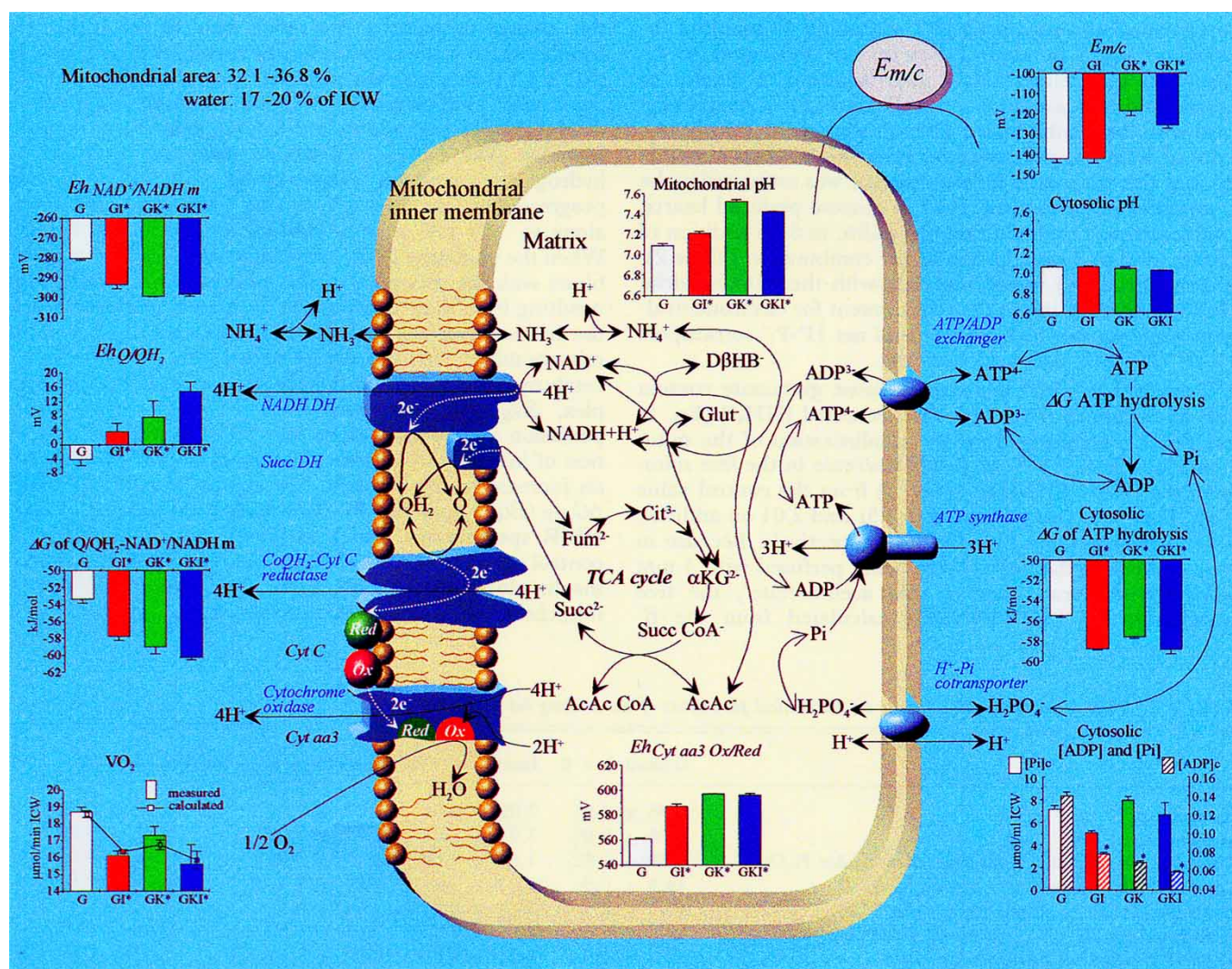


Figure 2. A representation of the mitochondrion showing the TCA cycle, oxidative phosphorylation, and various transport processes in the working rat heart. Changes in calculated mitochondrial and cytosolic parameters under different perfusion conditions are indicated on the bar graphs.

TABLE 3. Effect of insulin and/or ketones on mitochondrial energy parameters^a

	Control, <i>n</i> = 8	Insulin, ^b <i>n</i> = 5	Ketones, ^c <i>n</i> = 5	Ketones and insulin, <i>n</i> = 5
<i>E</i> _h ⁷ of mitochondrial [NAD ⁺]/[NADH], mV	-280 ± 1	-293 ± 2 ^d	-299 ± 0 ^d	-298 ± 1 ^d
<i>E</i> _h ⁷ of mitochondrial [Q]/[QH ₂], mV	-4.1 ± 1.6	3.6 ± 2.3 ^d	7.8 ± 4.3 ^d	14.8 ± 2.6
Δ <i>G</i> of mitochondrial NAD to Q couple, kJ/2 mol e	-53.4 ± 0.5	-57.8 ± 0.5 ^d	-59.1 ± 0.8 ^d	-60.3 ± 0.3 ^d
Δ <i>G</i> of cytosolic ATP hydrolysis, kJ/mol	-56.6 ± 0.2	-58.9 ± 0.1 ^d	-57.6 ± 0.2 ^d	-58.9 ± 0.4 ^d
<i>E</i> _{mito/cyto} mV	-143 ± 2	-143 ± 2	-120 ± 2 ^d	-128 ± 1 ^d

^aData are means ± SE. Control = 10 mM glucose perfusion. ^bInsulin: 100 nM. ^cKetone bodies: 4 mM D β-hydroxybutyrate + 1 mM acetoacetate. ^d*P* < 0.05 compared to control, determined using the Mann-Whitney U test.

fused with or without insulin, but decreased to -120 mV on addition of ketones and -128 mV with addition of the combination (Fig. 2, Table 3). The increase in efficiency caused by ketones therefore was compatible with a decrease in proton leakage across mitochondrial membrane due simply to a decrease in potential (47), as has been previously suggested. The increase in efficiency associated with insulin administration is not readily explained by such a straightforward mechanism; other factors, such as reduction of the mitochondrial NAD couple or specific effects like covalent modification of mitochondrial membrane protein, will have to be considered as possible factors altering efficiency of ATP synthesis.

Clearly, the administration of insulin increased the cardiac content of acetyl CoA almost 10-fold whereas [pyruvate] increased less than twofold, confirming previous inferences that insulin activates the pyruvate dehydrogenase multienzyme complex. This stimulation was independent of changes in total calcium and altered the activity of the α-ketoglutarate dehydrogenase multienzyme complex (8), as indicated by the observation that succinyl CoA content was unaltered by addition of insulin (Fig. 1). Also, insulin was able to alter the pattern of TCA cycle metabolites by a simultaneous reduction of the mitochondrial NAD couple and an oxidation of the mitochondrial coenzyme Q couple. A physiological ratio of ketone bodies at a total concentration of only 5 mM thus was able to duplicate most of the acute effects of insulin by providing increased amounts of acetyl CoA by a mechanism not involving activation of pyruvate dehydrogenase multienzyme complex (Fig. 1), and at the same time induce similar changes in the contents of TCA cycle metabolites and mitochondrial redox couples. This suggests that the major acute effect of insulin results simply by increasing the supply of mitochondrial acetyl CoA in a manner not dependent on insulin's ability to activate the pyruvate dehydrogenase multienzyme complex. Provision of acetyl moieties within mitochondria has been suggested to reverse many age-related defects in mitochondrial ATP synthesis (48). Use of ketones may therefore provide unexpected benefits in the treatment of elderly patients or others suffering from oxidative damage to mitochondria.

Because the estimation of redox potential of the Q couple was done by using only the fumarate²⁻/succinate²⁻ couple, and because the equilibrium constant of the succinate dehydrogenase reaction has not been measured under appropriate physiological condition (43), some uncertainty as to the precise potential of Q/QH₂ must remain. However, taking the values available from this study, there appeared to be an inverse correlation between O₂ consumption and oxidized/reduced aa₃ (Fig. 2). The aa₃ redox couple was calculated assuming an equal free energy for each proton exporting site (49), because all sites are related to each other through the NAD, Q, and cytochrome c redox cofactors present in mitochondria in excess relative to the other com-

ponents of the respiratory chain (50). The ratio of oxidized/reduced cytochrome c was on the order of 10:1, yet that of oxidized/reduced cytochrome aa₃ was 1–4 × 10⁵:1, indicating that dominant control is exerted at cytochrome aa₃. Thus, it would appear that changes in O₂ consumption respond to changes in mitochondrial redox couples. The significant increase in the energy available in redox span between mitochondrial complexes I and II and the similar significant increase in the energy of ATP hydrolysis suggest that the redox energy in the NADH dehydrogenase reaction is the major determinant of the free energy of ATP hydrolysis in cytosol in this relatively intact preparation.

The close similarities in the functional and energetic effects of insulin and ketone bodies may have important clinical consequences. Elevation of blood ketones to levels that are observed after a 48 h fast (51) almost completely reverses the mitochondrial abnormalities associated with insulin deficiency. As such, mild ketosis should be viewed as a beneficial compensation for insulin deficiency and perhaps also for geriatric patients or others with peroxidative damage to the processes of mitochondrial energy transduction. [F]

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