

Evidence that stimulation of gluconeogenesis by fatty acid is mediated through thermodynamic mechanisms

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We have studied the stimulatory effects of palmitate on the rate of glucose synthesis from lactate in isolated hepatocytes. Control of the metabolic flow was achieved by modulating the activity of enolase using graded concentrations of fluoride. Unexpectedly, palmitate stimulated gluconeogenesis even when enolase was rate-limiting. This stimulation was also observed when the activities of phosphoenolpyruvate carboxykinase and aspartate aminotransferase were modulated using graded concentrations of quinolinate and aminooxyacetate, respectively. Linear force-flow relationships were found between the rate of gluconeogenesis and indicators of cellular energy status (i.e. mitochondrial membrane and redox potentials and cellular phosphorylation potential). These findings suggest that the fatty acid stimulation of glucose synthesis is in part mediated through thermodynamic mechanisms.

Gluconeogenesis; Metabolic regulation; Linear force-flow relationship; Irreversible thermodynamics; Electrochemical potential; Isolated hepatocyte

1. INTRODUCTION

In a recent study [1] we demonstrated that the pathway of gluconeogenesis from lactate can be under thermodynamic control. We here report work that suggests that the stimulatory effects of fatty acid on gluconeogenesis may operate through thermodynamic-dependent mechanisms. The ability of fatty acids to stimulate glucose synthesis has been known for more than 20 years but their mode of action still remains uncertain [2-8]. A widely held idea is that the stimulation is a consequence of activation of pyruvate carboxylase by acetyl CoA arising from fatty acid oxidation [4-6]. In another view it is the reducing equivalents derived from this oxidation that promote glucose synthesis by in-

creasing the activity of glyceraldehyde-3-P dehydrogenase [5,6]. Other suggested modes of action of fatty acid include the depression of pyruvate oxidation and consequent promotion of pyruvate carboxylation through inhibition of pyruvate dehydrogenase [3,4], the stimulation of phosphoenolpyruvate carboxykinase and phosphoglycerate kinase activities due to enhancement of ATP synthesis [8], and the suppression of a futile cycle between fructose 6-P and fructose 1,6-P by a citrate-dependent inhibition of phosphofructokinase [5,7]. All these suggested mechanisms can be categorized as kinetic. That is to say they entail an increase in the catalytic activity of putative regulatory enzymes of the gluconeogenic pathway, either by increasing the availability of substrate or alternatively the level of allosteric effectors. Because evidence for the operation of these processes in vivo is meagre we have explored the possibility that the fatty acid stimulation of

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glucose synthesis may be mediated through thermodynamic rather than kinetic mechanisms.

2. MATERIALS AND METHODS

Collagenase and enzymes for metabolite determination were from Boehringer-Mannheim (FRG) as was bovine serum albumin (fraction V); defatted according to [9]. Palmitate, rotenone and aminooxyacetic acid were obtained from Sigma (USA). Quinolinic acid was from Matheson Coleman and Bell Inc. (USA) and sodium fluoride from Ajax Chemicals Limited. [^{14}C]Methyltriphenylphosphonium iodide was purchased from Amersham (England). Palmitate (8 mM) was prepared in isotonic saline containing 9% (w/v) bovine serum albumin. Water-insoluble compounds were dissolved in dimethylsulphoxide.

Isolated liver cells from male hooded Wistar rats (250–280 g body wt), starved for 24 h to deplete liver glycogen, were prepared and incubated at 37°C for 35 min in a bicarbonate-buffered medium as described [10,11]. Unless stated otherwise, the added substrates were a mixture of lactate (10 mM) and pyruvate (1 mM).

Metabolites were measured by standard enzymic techniques as in [12] on neutralized perchloric acid extracts of the incubated cells and medium [11]. The level of 2-phosphoglycerate was derived from measurement of 3-phosphoglycerate on the basis of studies which consistently showed a constant relationship between the concentrations of these metabolites. The metabolite assays were performed in a Cobas FARA centrifugal analyser (Roche Diagnostics, Switzerland) and the data transferred to a PDP 11/73 computer (DEC, USA) for subsequent processing. All fitted, straight-line relationships were calculated by least-squares linear regression analysis of untransformed data. Each plot is a representative example from at least three related experiments.

Mitochondrial membrane, redox and cellular phosphorylation potentials were determined as previously described [1,13]. Values for cytoplasmic redox potential (E_{hc}) defined as the half-cell reaction of free $[\text{NAD}^+]/\text{free}[\text{NADH}]$ ($E_h = E_o' + (RT/nF)\ln[\text{pyruvate}]/[\text{lactate}]$) were obtained by measurement of the concentrations of pyruvate and lactate on the assumption of an E_o' at 37°C of -215 mV [14].

3. RESULTS AND DISCUSSION

In this study of the effects of palmitate on gluconeogenesis the rate of the metabolic flow from lactate to glucose has been modulated using graded concentrations of fluoride, an inhibitor of enolase [15] which is not regarded as a rate-limiting enzyme of the pathway under physiological conditions [16]. Enolase was chosen for this study because it is believed to catalyse a 'near-equilibrium' reaction [16,17], and is not known to be affected by fatty acid at concentrations which stimulate gluconeogenesis.

Fig.1 illustrates the inhibitory effect of fluoride on glucose formation. The degree of depression of gluconeogenesis and accumulation of phosphoenolpyruvate, reflecting inhibition of enolase activity, increased as a function of fluoride concentration. Fluoride also depressed palmitate-stimulated gluconeogenesis, but for any given concentration of the inhibitor the rate of glucose synthesis was always greater in the presence of palmitate than in its absence. Nevertheless, palmitate had no significant effect on the steady-state concentrations of the components of the enolase-catalysed reaction (fig.1), tending to discount any direct action of the fatty acid on that step. We also observed that when the rate of glucose synthesis in the presence of each concentration of fluoride was expressed as a percentage of the rate observed in the absence of inhibitor, rather than in absolute units, the degree of inhibition of gluconeogenesis for any given fluoride concentration was the same whether or not palmitate was present (fig.2). This observation implies that the amount of enolase-inhibitor complex formed by exposure of the hepatocytes to a particular concentration of fluoride was unaffected by the presence of palmitate.

These findings are not readily explicable in terms of current concepts that account for metabolic regulation entirely on the basis of kinetic mechanisms [16,18,19]. For many years a cardinal element of these concepts has been the importance of a 'rate-limiting step' for control of flux through a metabolic pathway. It has been argued that, for a given pathway, the enzyme with the lowest catalytic activity will in large measure determine the overall rate of flux [16]. In the experiments described here, however, an artifactual rate-limiting step was created by exposing hepatocytes to fluoride, thereby reducing the activity of enolase (which is normally not rate-limiting) below that of the physiologically limiting regulatory enzyme activity. Evidence for the rate-limiting nature of the enolase reaction was provided by the incremental accumulation of phosphoenolpyruvate in response to increasing fluoride concentration. Paradoxically, palmitate though stimulating glucose synthesis, had no effect on this accumulation. We concluded from this that palmitate was not exerting a specific stimulatory effect on the enolase reaction, a conclusion in keeping with current views concerning the mechanisms whereby gluconeogenesis is stimu-

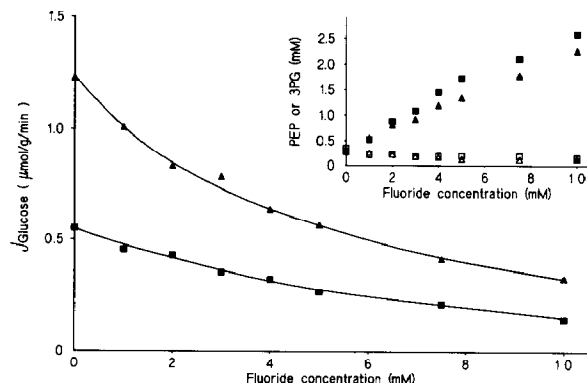


Fig.1. Inhibition of gluconeogenesis from lactate by fluoride. Hepatocytes were incubated for 35 min at 37°C with 10 mM lactate and 1 mM pyruvate in the absence (■) or presence (▲) of 2 mM palmitate. Fluoride concentrations in the range 1–10 mM were included to inhibit gluconeogenesis. The inset depicts changes in the cellular concentrations of phosphoenolpyruvate (PEP) (solid symbols) or 3-phosphoglycerate (3PG) (open symbols) in the absence (■, □) or presence (▲, △) of palmitate. The data are presented from a single representative experiment.

lated by palmitate [2–8]. It was therefore necessary to seek an alternative mechanism to explain how palmitate could stimulate synthesis under circumstances where enolase was rate-limiting.

To examine this question further we employed two other gluconeogenic inhibitors capable of depressing the rate of glucose synthesis from lactate in the absence or presence of palmitate, name-

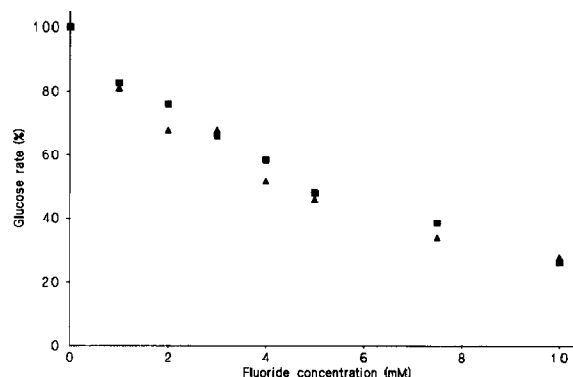


Fig.2. Percentage inhibition of gluconeogenesis from lactate by fluoride. The rates of glucose synthesis shown in fig.1 are plotted as percentages with the uninhibited rate of glucose formation being taken as 100%. Conditions and symbols are as for fig.1.

ly quinolinate [20] and aminooxyacetate [21]. Quinolinate inhibits phosphoenolpyruvate carboxykinase, whereas aminooxyacetate is an inhibitor of aspartate aminotransferase and hence impairs the flow of reducing equivalents between cytoplasm and mitochondria [22]. In each case the absolute rate of gluconeogenesis in the presence of a given concentration of inhibitor was greater in the presence of fatty acid (fig.3). Again, it is not immediately obvious on the basis of current concepts [16] why palmitate, which has no known

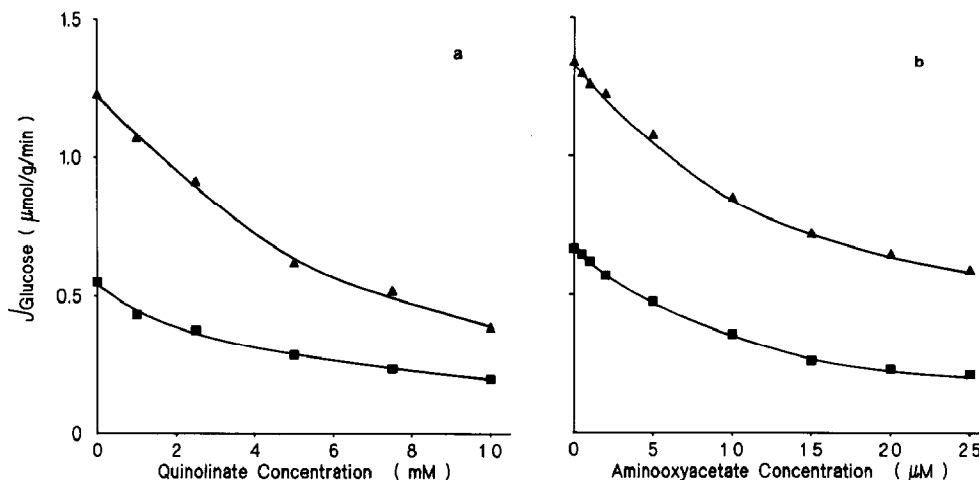


Fig.3. Effect of quinolinate and aminooxyacetate. Hepatocytes were incubated as described for fig.1, with (a) quinolinate concentrations in the range of 0–10 mM; and (b) aminooxyacetate in the range of 0–25 μM. Gluconeogenesis from lactate in the presence and absence of palmitate is shown as a function of inhibitor concentration for both inhibitors. Each set of data is from a single representative experiment. Symbols are as for fig.1.

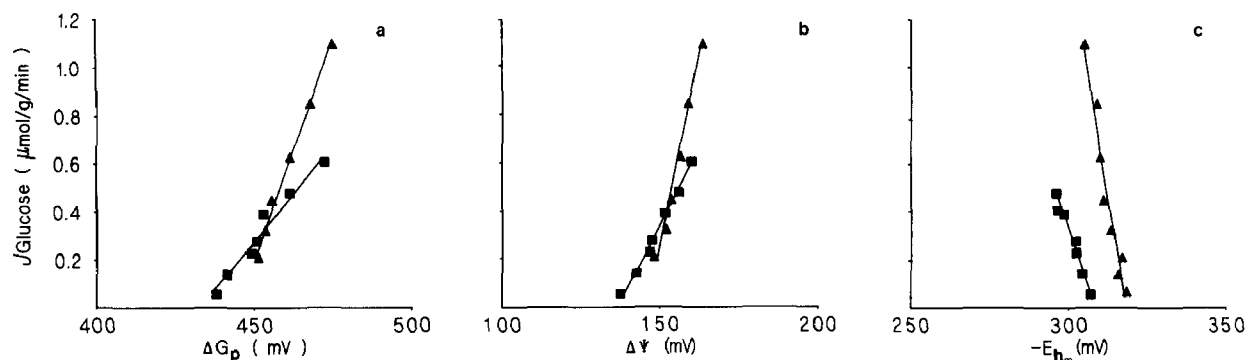


Fig.4. Relationships between gluconeogenesis, mitochondrial membrane and redox potentials and cellular phosphorylation potential. Hepatocytes were incubated as described in fig.1 and gluconeogenesis titrated with graded concentrations of rotenone (0–1.0 μ M). The rate of glucose synthesis from lactate in the presence and absence of palmitate is shown as a function of (a) cellular phosphorylation potential (ΔG_p); (b) mitochondrial membrane potential ($\Delta \Psi$); and (c) mitochondrial redox potential ($-E_{hm}$). Each set of data is from the same representative experiment. Symbols are as for fig.1.

specific effects on phosphoenolpyruvate carboxykinase or aspartate aminotransferase, should be able to enhance gluconeogenesis when either of these reactions has been made rate-limiting with a specific inhibitor.

In view of recent studies [1] in which we obtained evidence that gluconeogenesis under some circumstances may be under thermodynamic control, we examined the effects of alteration of cellular energy state on the stimulation of glucose synthesis by palmitate. Fig.4 demonstrates that for cells exposed to rotenone plots of gluconeogenic rate against various measures of energy state (cellular phosphorylation potential, mitochondrial membrane potential and mitochondrial redox potential), yielded straight lines. Except in the presence of high concentrations of inhibitor the rate of glucose synthesis at a given potential was substantially greater when the cells were incubated in the presence of palmitate. Similar results were obtained with other inhibitors of energy metabolism, e.g. oligomycin and carbonylcyanide-*p*-trifluoromethoxy-phenylhydrazone (not shown).

Elsewhere we have demonstrated other examples of linear relationships between mitochondrial 'forces' and cytoplasmic 'flows' in isolated hepatocytes [1,13] and have interpreted these findings in terms of irreversible thermodynamics [23], arguing that the observed flow-force linearity reflects some kind of balanced interplay between energy-coupled nonequilibrium forces [23–25], resulting in a thermodynamic-dependent control of metabolism.

We suggest that, under our experimental conditions, the gluconeogenic pathway is subject to such control, with the enzyme-catalyzed steps being poised (apparently) near equilibrium by the application of an external far-from-equilibrium force. This would entail modulation of the kinetic constants of the relevant enzymes (or transport proteins) [1]. Such modulation could be achieved only if the enzymes of the pathway exist in an organized array, thereby avoiding the equilibrium constraints of the Haldane relation [26]. If, indeed, the gluconeogenic pathway functions as an organized enzyme system under thermodynamic control, the flux through the pathway will be sensitive to changes in the far-from-equilibrium force which drives the system. Accordingly, it can be inferred that palmitate leads to an increase in this driving force (as do other fatty acids) and hence stimulates flux through the gluconeogenic pathway.

Although the driving force induced by palmitate oxidation is obviously a consequence of mitochondrial electron transport, the energy transfer to the cytoplasm does not appear to be mediated by ATP, since palmitate stimulates glucose synthesis without affecting the phosphorylation potential. Nor can the effects of palmitate be attributed to changes in mitochondrial membrane or redox potentials although such changes are taken to reflect the dynamics of the fundamental mitochondrial driving force. The manner in which intramitochondrial events are sensed beyond the

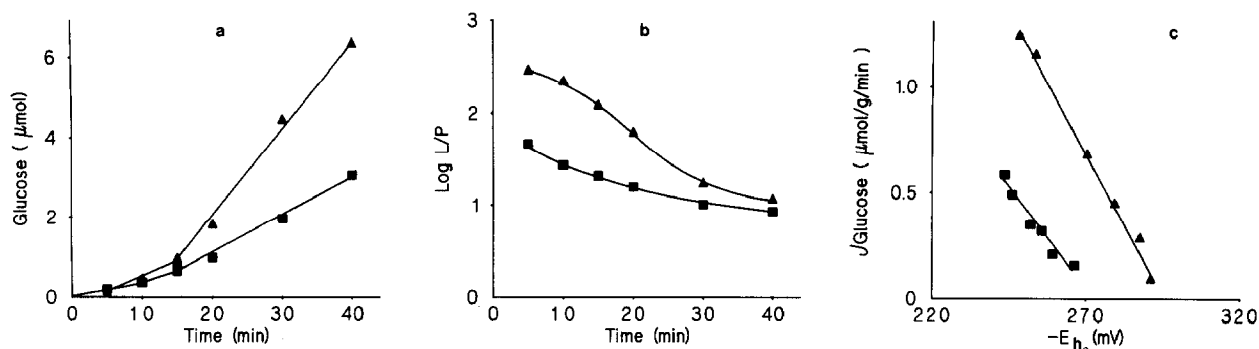


Fig.5. Gluconeogenesis from lactate and cytoplasmic redox state as a function of time. Glucose formation was measured as a function of time when hepatocytes were incubated with 10 mM lactate, 1 mM pyruvate, with or without 2 mM palmitate (a). (b) The variation with time of the redox state of the cytoplasm as reflected by $[\log_{10}(\text{lactate/pyruvate})]$. (c) The data of a and b are combined to show the relationship between the rate of glucose formation and the cytoplasmic redox potential ($-E_{nc}$). Each set of data is from the same representative experiment. Symbols are as for fig.1.

immediate locality of the inner mitochondrial membrane, as well as the molecular mechanisms by which the kinetic properties of enzymes (and transport proteins) are altered, must remain a matter for speculation. However, it is reasonable to assume that enzymes bound to the outer mitochondrial membrane (or, for that matter, various cytoplasmic membranes) could come within the domain of electric fields generated as a consequence of the protonmotive force originating within the mitochondrion [27]. Under these circumstances, ΔG for reactions catalysed, for example, by dehydrogenases and kinases (involving uptake or release of protons) would be expected to be considerably different from ΔG for the same reactions taking place in the bulk aqueous phase. A rudimentary argument along these lines has been advanced previously [28]. Stucki has suggested that modulation of enzyme-kinetic parameters by local electric fields may lie at the heart of the force-flow linearity of some mitochondrial processes [24].

It should be emphasized that linear force-flow relationships are not an artefact seen only in cells exposed to inhibitory agents, but can readily be demonstrated in hepatocytes without resorting to the use of inhibitors. It is well recognized that when hepatocytes are incubated with lactate there is a substantial lag phase before the rate of gluconeogenesis becomes maximal, whether or not palmitate is present [29] (fig.5a). If the cytoplasmic

redox potential, determined from measurement of lactate and pyruvate concentrations (fig.5b) at each time point is plotted against gluconeogenic rate, a linear relationship is achieved both in the absence and presence of fatty acid (fig.5c). This implies that the unknown mechanism responsible for the lag phase is dependent on thermodynamic rather than kinetic processes; that is to say it reflects rate control through variation in the degree of energy transfer rather than by means of alteration of enzyme activity.

These proposals are not intended to discount the importance of kinetic control mechanisms in metabolic regulation. Certainly, the amount of enzyme present, the action of allosteric effectors, etc., do influence metabolic flux. Notwithstanding, the results presented here, in addition to others reported previously [1], suggest that thermodynamic control is superimposed on kinetic control processes. Validation of this proposition will probably require elucidation of the mechanism whereby a far-from-equilibrium mitochondrial force (of presently unknown nature) can influence the kinetic parameters of extra-mitochondrial enzymes. Our results should encourage the search for such a mechanism.

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