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Philip John Randle
Minkowski Award, 1966, Aarhus

Sir Philip John Randle M.A. PhD. MD, FRCP, FRS. Emeritus Professor of Clinical Biochemistry, University of Oxford. Born 1926 in Nuneaton Warwickshire, UK. Educated King Edward VI Grammar School Nuneaton (1936–1944), Sidney Sussex College, University of Cambridge (1944–1947) and University College Hospital, London (1947–1950). Read Natural Sciences Tripos at Cambridge (Biochemistry in Part II) with First Class Honours; given an interest in metabolic regulation and insulin by Professor T.R.Mann, FRS and Dr. G.D.Greville. Medical education at University College Hospital; given a lifelong perspective and interest in diabetes by Professors (later Sir) Harold Himsworth and Frank Young. Appointed Lecturer in Biochemistry in Cambridge (1955–1964), Professor of Biochemistry, University of Bristol (1964–1975), Professor of Clinical Biochemistry, University of Oxford (1975–1993). With a group of outstanding graduate students (two Minkowski prizewinners) and fellows formulated in 1963 the Glucose Fatty Acid Cycle and the proposition that the metabolism of glucose stimulates insulin release from pancreatic beta cells and that glucokinase is the glucoreceptor for this process. Have spent most of my years since then converting these propositions into solid reality. Elected FRS, 1983 and knighted, 1985.

Mechanisms modifying glucose oxidation in diabetes mellitus

P.J.Randle, D.A.Priestman, S.Mistry, A.Halsall

Nuffield Department of Clinical Biochemistry, University of Oxford, John Radcliffe Hospital, Oxford, UK

Summary The Glucose Fatty Acid Cycle as formulated 30 years ago and reviewed in the Minkowski lecture in 1966 described short term effects of fatty acids (minutes) to decrease uptake, glycolysis and oxidation of glucose in heart and skeletal muscles. Such short term effects have since been extended to include inhibition of glucose uptake and glycolysis and stimulation of gluconeogenesis in liver and these effects have also been convincingly demonstrated in man in vivo. More recently a longer term effect of fatty acid metabolism to decrease glucose oxidation (hours) has been shown in heart and skeletal muscle and liver. This effect increases the specific activity of pyruvate dehydrogenase kinase, which in turn results in enhanced phosphorylation and inactivation of the pyruvate dehydrogenase complex. Activity of the pyruvate dehydrogenase complex is the major determinant of glucose oxidation rate. It seems likely that longer term effects of fatty acids on this and other aspects of glucose metabolism could be important in the development of insulin resistance in diabetes mellitus in man. [Diabetologia (1994) 37 [Suppl 2]: S155–S161]

Key words Glucose fatty acid cycle, glucose oxidation, diabetes mellitus, starvation, pyruvate dehydrogenase complex, pyruvate dehydrogenase kinase, pyruvate dehydrogenase phosphatase, fatty acids, cyclic AMP, cultured hepatocytes, cultured cardiac myocytes, soleus muscle, insulin action, insulin resistance.

Corresponding author: Professor P.Randle, Nuffield Department of Clinical Biochemistry, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, UK

Abbreviations: PDH, Pyruvate dehydrogenase; KAP, kinase activator protein.

Historical and general information

The concept of a Glucose Fatty Acid Cycle which was first described some 30 years ago in 1963 [1] provided the basis for the Minkowski lecture by one of us (P.J.R.) in 1966 [2]. The essential components of this regulatory cycle were: (a) the relationship between glucose and NEFA metabolism is reciprocal and not dependent; (b) in vivo, oxidation of NEFA and ketone bodies released into the circulation in diabetes and starvation may inhibit uptake and oxidation of glucose in muscle; (c) in vitro, the oxidation of NEFA released from muscle triacylglycerol may have similar effects; (d) these effects of NEFA and ketone body oxidation are mediated by inhibition of the pyruvate dehydrogenase (PDH) complex, phosphofructo 1-kinase and hexokinase; (e) the central mechanism is an increase in the mitochondrial ratio of [acetyl CoA]/[CoA] which inhibits the PDH complex directly, and which indirectly leads to inhibition of phosphofructo-1-kinase by citrate and of hexokinase by glucose 6-phosphate; (f) the effect of physiological concentrations of insulin to activate glucose transport in heart muscle is inhibited by NEFA and ketone bodies. The mechanism of citrate accumulation (unspanning of the citrate cycle) was detailed subsequently [3].

This concept became controversial in the early 1970s because of doubts about its applicability to skeletal muscle, but these doubts have since been dispelled by more extensive studies [reviewed in 4]. Other studies have shown that fatty acid oxidation stimulates gluconeogenesis, and inhibits glucose uptake and glycolysis, in liver [5–7]. Acute inhibitory effects of NEFA oxidation on glucose disposal and oxidation in man have been shown unequivocally and repeatedly by (a) indirect calorimetry in conjunction with glucose and insulin/glucose clamp [8–12] (b) Positron Emission Tomography in heart, and in hind limb and fore limb skeletal muscles [13] and c) forearm and hind limb perfusion studies [14–16].

Since these concepts were formulated in the 1960s two further major discoveries have been made with respect to mechanism. These are regulation of the PDH complex by reversible phosphorylation [17]; and the discovery of fructose 2,6 bisphosphate and the bifunctional phosphofructo-2-kinase/ fructose 2,6 bisphosphatase [18]. At the first meeting of Minkowski prize winners in Capri 1976, the subject had advanced to the point that it was possible to talk on "Diabetes and the Metabolism of Pyruvate" with particular reference to the potential for new drugs for the treatment of diabetic patients and based upon the ability of these drugs to interfere with lipolysis, or fatty acid oxidation or inactivation of the PDH complex by phosphorylation [19].

The original concept of the Glucose Fatty Acid Cycle and its later extensions to incorporate effects of fatty acid oxidation on gluconeogenesis, hepatic glucose utilization, and reversible phosphorylation in the PDH complex were based upon short term mechanisms regulating glucose uptake and oxidation and glucose production. There is now firm evidence for longer term regulation of reversible phosphorylation in the PDH complex and hence of glucose oxidation, by fatty acid oxidation, and by cyclic AMP. This provides the major new theme in this paper.

Regulation of the PDH complex by reversible phosphorylation

The PDH complex: is a mitochondrial multienzyme complex catalysing the reaction: pyruvate + CoA + NAD $\rightarrow$ acetylCoA + $NADH_2$ + CO_2. It utilises intramitochondrial substrates and coenzymes and it provides acetyl CoA for the citrate cycle and for fatty acid synthesis. The complex contains multiple copies of three component enzymes; E_1 which catalyses the non-reversible removal of CO_2 from pyruvate; E_2 which forms acetylCoA and E_3 which reduces NAD to $NADH_2$ (reviewed in [20, 21])

Reversible phosphorylation; component enzymes. The PDH complex of animal tissues including man contains an intrinsic PDH kinase which with ATPMg phosphorylates and inactivates the complex. PDH phosphatase, which separates from the complex during its purification catalyses dephosphorylation and reactivation. There are no known allosteric activators of the phosphorylated form of the complex. The two regulatory enzymes, PDH kinase and PDH phosphatase, are mitochondrial and utilise intramitochondrial substrates and effectors. Phosphorylation is on up to three seryl residues in the alpha chain of the E1 component of the PDH complex the relative rates being site 1 >> site 2 > site 3. Inactivation is due largely (> 90 %) to phosphorylation of one serine residue (site 1); phosphorylation of the other two sites slows reactivation by PDH phosphatase (reviewed in [20, 21]. Regulation of the PDH complex by reversible phosphorylation is widespread in animal tissues containing the complex including those of man [22]. PDH kinase has been purified and contains two subunits alpha (M_r = 46 kDa) and beta (M_r = 43 kDa). The alpha-subunit is the catalytic subunit. The kinase is much more active towards its substrate (E1 alpha) when E2 is present. PDH phosphatase has two subunits, alpha (M_r = 97 kDa) and beta (M_r = 50 kDa; catalytic subunit). Relative rates of dephosphorylation of the three sites are site 2 > site 1 = site 3. A second phosphatase has been described in bovine kidney mitochondria but its function is uncertain. Reversible phosphorylation is now accepted as the major mechanism regulating the PDH complex in animal tissues and the major deter-

minant of the rate of glucose oxidation in animal tissues including those of man.

PDH complex; regulation by reversible phosphorylation in vivo and in vitro. The active (dephosphorylated) and inactive (phosphorylated) forms of PDH complex are referred to as PDHa and PDHb. Except in adipose tissue of rats fed a high fat diet for a prolonged period, the total amount of PDH complex in rat tissues (PDHa + PDHb) is not altered by the physiological and pathophysiological variations to be detailed. Except where individual references are given, full bibliographies may be found in [4, 21].

In the rat tissues examined (heart, skeletal muscles, liver, kidney, adipose tissue and intestine) per cent of PDHa is decreased by starvation, high fat/low carbohydrate diet and insulin-deficient diabetes. The maximum changes require 24–48 h and reversal by carbohydrate refeeding or insulin treatment also requires 24–48 h. Per cent PDHa is also decreased in heart muscle of goldthioglucose obese hyperinsulinaemic rats in which glucose oxidation is impaired in muscles and adipocytes. Effects of starvation, diabetes, high fat diet or obesity to decrease per cent PDHa persist into isolated tissues incubated in vitro and effects of contraction in skeletal muscles and of increased work in heart to increase per cent PDHa are also demonstrable with in vitro preparations. The effect or exercise to increase per cent PDHa is decreased in hearts of starved or diabetic rats as compared with controls.

Short term (rapid) in vitro effects of hormones are as follows. Insulin increases percentage of PDHa in rat adipocytes by 1.8- to 2-fold in 5–8 min and a smaller effect (approximately 1.2-fold) is seen in rat hepatocytes. Consistent in vitro effects of insulin in heart or skeletal muscle have yet to be demonstrated. Rapid effects of other hormones comprise mainly increases in percentage of PDHa induced in liver by alpha-adrenergic agonists, glucagon and vasopressin and in heart muscle by beta-adrenergic agonists. The role of hormones in longer term regulation is discussed in a later section.

In vitro effects of short chain and long chain fatty acids (NEFA) or of ketone bodies to decrease per cent PDHa have been shown in perfused rat heart, perfused rat liver (NEFA only) and skeletal muscles (ketone bodies only). Effects of elevating plasma NEFA to decrease per cent PDHa in skeletal muscles have been shown in vivo [23]. The effects of long chain NEFA (but not of ketone bodies) are blocked by inhibitors of beta-oxidation such as sodium 2-tetradecylglycidate.

Short term regulation of PDH kinase and PDH phosphatases (reviewed in [4, 21]). PDH kinase activity is enhanced by increasing mitochondrial concentration ratios of acetylCoA/CoA, $NADH_2$/NAD and ATP/ADP. Pyruvate is an inhibitor of PDH kinase and the degree of inhibition is enhanced by increasing ADP concentration. These regulatory interactions have been shown with purified PDH complexes and in studies with isolated rat heart and skeletal muscle mitochondria. These short term regulators of PDH kinase modulate phosphorylation of all three sites in PDH complex. PDH phosphatase requires Mg^{2+} for activity and in the presence of Mg^{2+} the enzyme is activated by Ca^{2+} in the physiological range (0.1 to 10 mmol/l; Km 0.5 mmol/l). The effects of Ca^{2+}, at concentrations in the physiological range, to effect conversion of PDHb to PDHa have been readily demonstrable in isolated mitochondria. PDH phosphatase may also be inhibited by $NADH_2$ (reversed by NAD).

Mechanism of effects of exercise and short term actions of hormones. The increase in per cent PDHa in muscle during contraction is largely effected by Ca^{2+} activation of PDH phosphatase. The increase in cytosolic [Ca^{2+}] which initiates contraction increases mitochondrial [Ca^{2+}]. The increases in per cent PDHa induced in liver by alpha-adrenergic agonists, glucagon and vasopressin, and in heart by beta-adrenergic agonists are likewise mediated by Ca^{2+}. The action of insulin to effect conversion of PDHb into PDHa in rat adipocytes is effected through activation of PDH phosphatase by a mechanism which is not mediated by known effectors such as Ca^{2+} [24].

Longer term regulation of PDH kinase

Longer term regulation of reversible phosphorylation in the PDH complex was discovered through the observation that the effect of starvation or diabetes to lower per cent PDHa in heart muscle persists into mitochondria prepared from the tissue and incubated in vitro with 2-oxoglutarate/malate or succinate. It was shown later by direct analysis that this was not mediated by known mitochondrial effectors of PDH kinase [25]. This led to the demonstration that 48 h starvation or alloxan-diabetes increase the activity of PDH kinase 2- to 3-fold in extracts of heart, skeletal muscle or liver mitochondria [26–28]. This is a stable form of activation persisting through isolation, purification, and incubation of mitochondria at 30 °C with uncoupler to effect conversion of PDHb to PDHa, and extraction. This effect of starvation or diabetes required 24 to 48 h, and reversal by refeeding of starved rats also took 24–48 h to complete. More detailed time courses have shown that the major decreases in per cent PDHa in response to starvation occur between 12–18 h in skeletal muscles and between 4–8 h in heart and liver [29, 30]. After refeeding (48 h starvation) restoration of per cent PDHa in heart began between 1 and 2 h but most of the restoration

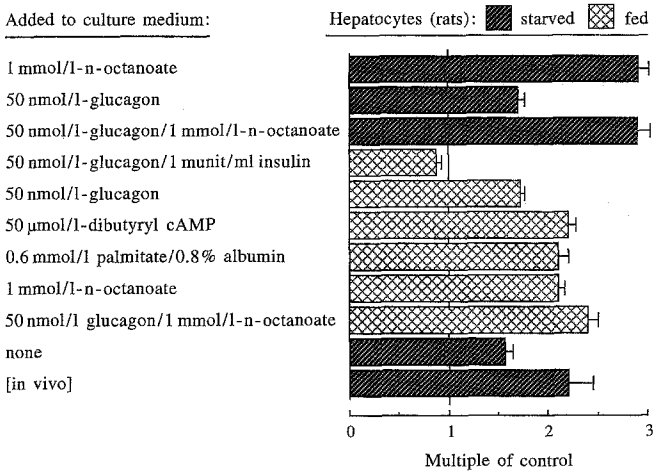

Fig. 1. The PDH kinase activity of extracts of mitochondria from livers of fed or 48 h starved rats, together with the effects in tissue culture (25 h) of 50 nmol/l-glucagon ± insulin (1 mU/ml), 1 mmol/l-n-octanoate, or 0.6 mmol/l-palmitate on 0.6 % (w/v) bovine plasma albumin. Results are mean ± SEM for multiple of control (hepatocytes from fed rats, no additions to medium 199). For further experimental details see [32]

(70 %) occurred after 4 h. In liver restoration was complete by 4 h [30].

Factors that may mediate longer term effects of starvation and diabetes on PDH kinase. The question as to which hormones or metabolites may mediate longer term effects of starvation on the activity of PDH kinase has been investigated with rat hepatocytes, cardiac myocytes and soleus muscle strips in tissue culture [31–34]. These studies have shown uniformly that culture of cells from fed rats with agents which increase cAMP (glucagon or dibutyryl-cAMP), or with NEFA (n-octanoate or albumin bound palmitate) increases PDH kinase activity 2- to 3-fold. The effect of glucagon was detectable within 1 h of culture, took 21 h to reach 2-fold and was blocked by insulin. Culture of hepatocytes from fed rats had no effect on PDH kinase in the absence of these agonists. Culture of hepatocytes from starved rats reversed the effect of starvation on PDH kinase activity by about 60 % in 21 h; this reversal was blocked by n-octanoate, dibutyryl cAMP, glucagon or a combination. Representative data for hepatocytes are in Figure 1.

PDH kinase activator protein (KAP). It was possible to separate from PDH complex in mitochondrial extracts a fraction which enhanced the PDH kinase activity of highly purified pig heart PDH complex. This separation was achieved by ultracentrifugation or, more effectively, by gel filtration on Sephacryl S300. The activity thus separated was thermolabile, non-dialysable and inactivated by trypsin and was termed kinase activator protein [35, 36] because it increased the PDH kinase activity of purified pig heart PDH complex which contains intrinsic PDH kinase. It could therefore be either free PDH kinase or a protein activator of the intrinsic PDH kinase.

There are now five lines of evidence that rat liver KAP is a free PDH kinase. KAP phosphorylates and inactivates pig heart PDHE1 that is devoid of PDH kinase activity. It also phosphorylates and inactivates E1 in *S cerevisiae* PDH complex which, though devoid of PDH kinase, is nevertheless a substrate for mammalian PDH kinases. The fed/starved difference is retained in assays with these substrates. KAP, like PDH kinase, is inactivated by thiol reactive reagents such as N-ethylmaleimide and p-chloromercuribenzoate (the latter is reversed by dithiothreitol). KAP was also shown to undergo pseudo first order inactivation by fluorosulphonylbenzoyladenosine which is known to block the ATP binding sites of protein kinases [37]. More recent and as yet unpublished studies of the authors have shown that antibodies prepared against highly purified KAP cross react with the alpha-chain of bovine kidney PDH kinase (shown by Western blots with E2-X-kinase subcomplex kindly provided by Dr. T. E. Roche).

Longer term regulation of enzyme activity usually involves a change in enzyme concentration, but this does not appear to be the case with the increase in PDH kinase activity effected by starvation. Initial evidence was provided by the effect of varying concentrations of KAP, prepared from liver mitochondria of fed and starved rats, on PDH kinase activity of purified pig heart PDH complex. The effect of starvation was to increase the V_{max} of PDH kinase at saturating KAP without changing the KAP concentration required for 0.5 V_{max} [28]. This conclusion was confirmed by purifying KAP from fed and starved rats to apparent homogeneity as established by SDS-PAGE and N-terminal sequence analysis. The specific activity of purified KAP from starved rats was 4.5-fold greater than that from fed rats [38]. SDS-PAGE gave a single band of M_r = 45 kDa suggesting that KAP is the free alpha-subunit of PDH kinase (the M_r of KAP on SDS-PAGE was lower by about 1 kDa than the 46 kDa given by the alpha-subunit of PDH kinase in the bovine kidney $PDHE_1$-X-kinase subcomplex provided by Dr. T. E. Roche).

Further support for this conclusion has been obtained in more recent unpublished studies employing polyclonal antibodies in an ELISA assay to measure the concentration of KAP protein (alpha subunit of PDH kinase) in mitochondrial extracts. This showed a possible small increase in the concentration of this protein in extracts of liver mitochondria of starved rats that could account for at most 15 % of the increase in activity [39].

Two alternative hypotheses consistent with these findings are under consideration viz. that starvation increases the specific activity of KAP by covalent modification; or that there are two isoforms of PDH

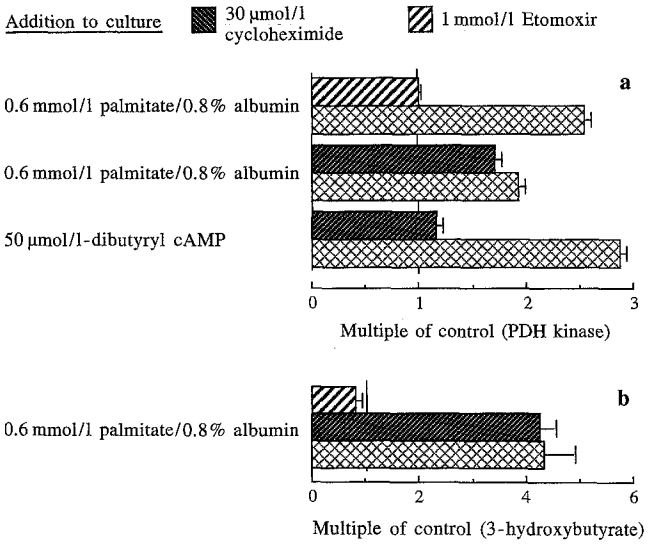

Fig. 2 a, b. The effect of (**a**) the protein synthesis inhibitor cycloheximide on the increase in PDH kinase activity effected in cultured hepatocytes by dibutyryl cAMP or palmitate; and of the fatty acid oxidation inhibitor Etomoxir on the increase effected by palmitate: and (**b**) the effects of Etomoxir and cycloheximide on 3-hydroxybutyrate production in the presence of palmitate (as an index of fatty acid oxidation). Hepatocytes were prepared from the livers of fed rats and cultured for 25 h as described in [32]. Results are mean ± SEM

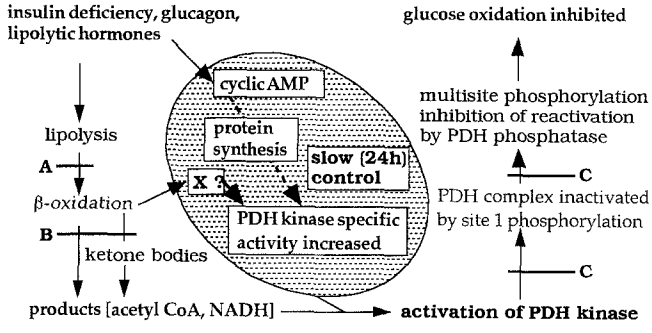

Fig. 3. Mechanisms leading to phosphorylation and inactivation of PDH complex in rat tissues by 48 h starvation or insulin-deficient diabetes. A, B and C are inhibitors of lipolysis (e.g. Acipimox, 5-methylpyrazole 3-carboxylate) beta oxidation of fatty acids (e.g. 2-tetradecylglycidate, Etomoxir) and of PDH kinase (e.g. dichloroacetate) respectively. X is an unidentified metabolite of the beta oxidation pathway

kinase which differ in specific activity and that starvation increases the relative concentration of the more active isozyme. It has not been possible to ascertain in vivo whether protein synthesis is required for the effect of starvation on PDH kinase activity because rats undergoing starvation are intolerant of protein synthesis inhibitors such as cycloheximide. However in studies with hepatocytes in tissue culture it has been shown that 30 μmol/l-cycloheximide, which inhibited [^{14}C]leucine incorporation into protein, blocked completely the effect of dibutyryl cAMP to increase PDH kinase activity, but that it did not block the effect of palmitate. Etomoxir (1 mmol/l), which inhibits carnitine acyltransferase I, blocked the effect of palmitate (Fig. 3) i.e. acylCoA or a product of beta-oxidation is likely to mediate the effect. Representative data are shown in Figure 2. With the ELISA assay it has been found that dibutyryl cAMP increased the specific activity of PDH kinase in cultured hepatocytes and had no effect on the concentration of the alpha subunit of PDH kinase. This, in conjunction with the results with cycloheximide, might indicate that cAMP either directly or indirectly switches on the synthesis of a more active isoform of PDH kinase or an enzyme effecting conversion of PDH kinase into a more active form or both.

Mechanisms by which starvation and diabetes decrease activity of the PDH complex and glucose oxidation

Figure 3 summarises the current state of knowledge of the mechanisms by which starvation or diabetes lead to phosphorylation and inactivation of PDH complex in rat heart, skeletal muscle and liver (the only tissues studied in all aspects). The short term mechanisms occupy the periphery of the figure and the longer term mechanisms the central shaded area.

That shorter term mechanisms based on oxidation of lipid fuels are of continuing importance in starvation and diabetes can be deduced from rapid (minutes) restoration of per cent PDHa to normal in rat heart muscle following in vivo and in vitro administration of the beta-oxidation inhibitor sodium tetradecylglycidate [40]. Sodium tetradecylglycidate was not effective in skeletal muscle [30, 40] but the lipolysis inhibitor 5-methylpyrazole 3-carboxylic acid inhibited the decrease in per cent PDHa in skeletal muscles effected by starvation whereas elevation of plasma NEFA with corn oil/heparin accelerated it [30]. There is some evidence to suggest that effects of NEFA to decrease per cent PDHa and glucose oxidation can be inhibited by hyperinsulinaemia (reviewed in [23]). The likely role of cyclic AMP in mediating longer term effects of starvation and diabetes on PDH kinase is assumed from the known increases in tissue cAMP in starved and/or diabetic rats.

In man starvation, insulin-dependent and non-insulin-dependent diabetes, and elevation of plasma NEFA in normal people, decrease the utilization and oxidation of glucose and increase the oxidation of NEFA; and elevation of plasma NEFA increases muscle [acetyl CoA]/[CoA] [8, 11, 41–43]. It is not known whether per cent active PDHa in tissues is changed in man by starvation, diabetes or elevation of plasma NEFA. In man inhibitors of lipolysis (Acipimox), NEFA oxidation (Etomoxir, tetradecyl-glycidate) and PDH kinase (dichloroacetate) increase glucose oxidation [44–48].

Conclusion

The Glucose Fatty Acid cycle is 30 years old this year and during the past decade there has been a substantial resurgence of interest in NEFA glucose interaction and the regulation of glucose disposal. Part of this renewed interest is due to technical developments which have made it possible to study NEFA glucose interactions in vivo in man under controlled conditions of circulating glucose and insulin and with much greater precision.

The discovery of longer term regulation of PDH kinase by NEFA in liver and muscles is a novel departure which should pave the way for more general studies of longer term regulation of glucose metabolism by NEFA. Virtually all of the glucose utilized by animal tissues is either stored as glycogen or triacylglycerol or oxidised by a combination of glycolysis, the PDH complex reaction and the citrate cycle. Quantitatively, storage is the more important pathway of disposal. Oxidation is limited by O_2 consumption and physical activity is the major determinant of O_2 consumption. Glucose oxidation is enhanced by intake of the sugar but the maximum rate at rest is about 16 g/h for a 70 kg man whereas the maximum rate of non oxidative disposal (= glycogen synthesis) is greater than 118 g/h [49].

Although there is convincing evidence that NEFA, acting through short term mechanisms, inhibit glucose utilization in man, the general consensus is that this is mediated almost wholly through inhibition of glucose oxidation i.e. there may be no short term effect on the quantitatively more important pathway of glucose conversion to glycogen. There is already some metabolic evidence for longer term regulation of muscle glucose uptake and glycogen synthesis by NEFA in rat and man [8, 25, 50] including decreased percentage of the active (dephosphorylated) form of glycogen synthase [50]. Obvious targets for such study are regulatory enzymes in glycogen synthesis (and glucose production), and glucose transporters.

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