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The relationship between changes in lipid fuel availability and tissue fructose 2,6-bisphosphate concentrations and pyruvate dehydrogenase complex activities in the fed state

Timothy J. FRENCH,* Anthony W. GOODE,* Mark J. HOLNESS,† Peter A. MACLENNAN† and Mary C. SUGDEN†‡

†Department of Biochemistry, The London Hospital Medical College, Turner Street, London E1 2AD, U.K., and *Surgical Unit, The London Hospital, Whitechapel, London E1 1BB, U.K.

An elevated concentration of non-esterified fatty acids in the fed state elicited inhibition of cardiac, but not hepatic, pyruvate dehydrogenase complex (PDH). There was a modest decline in fructose 2,6-bisphosphate (Fru-2,6-P_2) concentration in heart, and, to a lesser extent, in liver. Surgical stress decreased PDH activities and Fru-2,6-P_2 concentrations in both heart and liver. Only the former response was abolished if post-operative lipolysis was inhibited. Surgery also decreased the [Fru-2,6-P_2] in gastrocnemius: this response was abolished if lipolysis was inhibited.

INTRODUCTION

The oxidation of fatty acids and ketone bodies has been demonstrated to inhibit glucose utilization in the heart, and in some circumstances (e.g. exercise), skeletal muscle (the glucose/fatty acid cycle) [1–3]. Inhibition of glucose utilization occurs at multiple sites, including 6-phosphofructo-1-kinase (PFK-1) and the pyruvate dehydrogenase complex (PDH) (reviewed in ref. [4]). The glucose-sparing effect of lipid-derived fuels has been demonstrated in working muscles in starvation [5,6], and can be reproduced in perfused hearts from fed rats by the provision of ketone bodies or acetate [1–7]. PDH is inactivated [1], and specific inhibition of PFK-1 has been demonstrated by measurements of the detritiation of [3-^{3}H]glucose [7]. Inhibition of PDH has been correlated with an increased [acetyl-CoA]/[CoA] ratio [8,9], whereas inhibition of glycolytic flux has been correlated with reciprocal dose-dependent changes in the concentration of fructose 2,6-bisphosphate (Fru-2,6-P_2) (decreased) [7] and citrate (increased) [1–3,7].

The hepatic response to starvation also involves inactivation of PDH (e.g. ref. [9]), and a decrease in the concentration of Fru-2,6-P_2 (see ref. [10] for details). *In vitro*, exogenous fatty acid decreases the percentage of PDH in the active form [11,12], decreases the concentration of Fru-2,6-P_2 [7], and inhibits flux through PFK-1 [7]. The results obtained with isolated tissue preparations therefore suggest that the activity of the PDH complex and the concentration of Fru-2,6-P_2 should respond immediately and in parallel after changes in the availability of lipid substrates *in vivo*. However, we have failed to observe a significant, immediate, effect of a decreased lipid fuel supply on tissue [Fru-2,6-P_2], even under conditions where PDH is activated. Thus inhibition of lipolysis in the fed state leads to significant activation of cardiac PDH, without an analogous increase in Fru-2,6-P_2 concentration [6]. Moreover, when lipid fuel concentrations are decreased in response to re-feeding after starvation, restoration of hepatic PDH activity precedes any change in the Fru-2,6-P_2 concentration [10]. For this reason, we considered it important to examine in more detail the effects of variation in fatty acid supply on cardiac and hepatic PDH activities and Fru-2,6-P_2 concentrations both in the normal fed state, and in a pathophysiological condition where it is known that lipolysis and fat utilization are accelerated, namely that of surgical stress.

MATERIALS AND METHODS

Materials

Sources of material were as in [6]. 5-Methylpyrazole-3-carboxylic acid (MPCA) was generously given by Upjohn Ltd., Crawley, West Sussex, U.K.

Rats

Female albino Wistar rats (180–220 g) were subjected to a 12 h-light/12 h-dark cycle, and were fed *ad libitum* on a standard rodent diet containing (by wt.) 52% digestible carbohydrate and 2% fat. Water was provided *ad libitum*. Experiments were started at 08:30 h, at which time the food was removed. Inhibition of lipolysis was achieved by the administration of MPCA [13] (0.66 mg/100 g body wt. in 0.2 ml of 0.9% NaCl, pH 7.4, intraperitoneally) at 2.5 h before the rats were killed. An elevation of fatty acid concentration was achieved by the administration of corn oil followed by heparin as detailed previously [6]. Rats were sampled at 1 or 2 h after heparin injection.

The model of surgical stress utilized comprised laparotomy and liver manipulation. Rats were lightly anaesthetized with diethyl ether, and an incision (3 cm) was made in the anterior abdominal wall. The large median lobe and the smaller left lateral lobe of the liver were

Abbreviations used: PDH, pyruvate dehydrogenase complex; PDH_a, its active form; PFK-1, 6-phosphofructo-1-kinase; Fru-2,6-P_2, fructose 2,6-bisphosphate; NEFA, non-esterified fatty acids; MPCA, 5-methylpyrazole-3-carboxylic acid.

‡ To whom correspondence should be addressed.

eventrated and then re-inventrated. The abdominal wall and skin were sutured and the rats removed from ether. The entire surgical procedure took 5–7 min. Zero time was taken as the time after removal of the rat from ether. Where indicated, MPCA was given at 0.5 h before the initiation of the surgical procedure.

Metabolite and enzyme assays

Rats were killed while under sodium pentobarbital anaesthesia (5 min; 6 mg/100 g body wt). Blood (0.5 ml) was sampled from the abdominal aorta. Tissues were rapidly freeze-clamped and stored in liquid N_2. Concentrations of glucose and the ketone bodies were measured in KOH-neutralized $HClO_4$ extracts of whole blood, concentrations of non-esterified fatty acids (NEFA) in plasma, and concentrations of free and acylated carnitine, citrate and Fru-2,6-P_2 in extracts of freeze-clamped tissues (see ref. [6] for details).

The active form of the pyruvate dehydrogenase complex (PDH_a) and citrate synthase activities were measured in freeze-clamped tissue extracts as described in ref. [9]. Further details are given in ref. [8]. PDH activities have been expressed relative to citrate synthase to correct for possible variation in the efficiency of mitochondrial extraction (see refs. [4,6,10]). There was no effect of the experimental procedures on citrate synthase activities or on the total activity of PDH (results not shown). One unit of enzyme activity is that which converts 1 μmol of substrate into product per min at 30 °C.

Expression of results

Statistical significance of difference was assessed by Student's unpaired t test. Results are given as means ± S.E.M. for the numbers of rats specified.

RESULTS AND DISCUSSION

Fatty acid availability and PDH activities in normal rats

Values of cardiac and hepatic PDH_a observed in tissues of control (fed) rats were similar to those observed in other studies (e.g. [9,14]), representing 13.2% (heart) and 19.1% (liver) of the complex in the active form. In a preliminary study [15], we observed that an acute elevation of NEFA concentration in the fed rat was accompanied by inactivation of cardiac, but not hepatic, PDH. The effect of a 10-fold variation in exogenous NEFA concentration on cardiac and hepatic PDH activities in the fed state is shown in Table 1. A more than 3-fold variation in cardiac PDH activity was observed in response to changes in fatty acid supply achieved by the administration of corn oil plus heparin (increased NEFA), where the percentage of the complex in the active form decreased to 6.2%, or inhibition of lipolysis (decreased NEFA), where the percentage of the complex in the active form increased to 21.1%. In contrast, hepatic PDH activity was unchanged over the entire range of NEFA concentrations, maintaining a value of 15.9–20.6% of the complex in the active form. Others have demonstrated that the culture of hepatocytes from fed rats with octanoate for periods $\leqslant 4$ h does not lead to a significant decrease in liver PDH activity, even when glucagon (a hormone known to promote hepatic fat oxidation) is included in the medium [14]. The failure of an acute elevation in NEFA to diminish hepatic PDH_a contrasts with the marked effect of prolonged starvation, where the percentage of the complex in the active form decreased to 2.3%, although, in this study, values of hepatic PDH in the starved state were rather lower than have been observed previously (4–7% of the complex in the active form; see, e.g. refs. [9,14]).

Fatty acid availability and Fru-2,6-P_2 concentrations in normal rats

The decline in cardiac Fru-2,6-P_2 concentration observed after prolonged exposure to elevated NEFA concentrations (Table 1) was modest compared with the change in activity of PDH, but was comparable with that observed in perfused hearts in response to 3-hydroxy-

Table 1. Effects of altered NEFA concentrations on cardiac and hepatic PDH_a and Fru-2,6-P_2 concentrations in normal fed rats

Fed rats were given either MPCA to decrease fatty acid concentrations, or corn oil and heparin to increase fatty acid concentrations, as described in the text, and were sampled at the times indicated. Total PDH activities were 83.4 ± 7.8 (heart) and 275.8 ± 21.2 (liver) munits/unit of citrate synthase. Mean activities of citrate synthase in heart and liver were 90.0 ± 3.9 and 9.5 ± 0.3 units/g wet wt. respectively. Results are means ± S.E.M. for three to 11 rats. Values of blood metabolite or tissue Fru-2,6-P_2 concentrations or of tissue PDH activities significantly different from the control (fed) values are indicated: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

	Fed	Decreased fatty acids	Increased fatty acids		48 h-starved
			1 h	2 h	
Blood concn. (mM) of:					
NEFA	0.21 ± 0.03	0.10 ± 0.02*	1.05 ± 0.08**	1.06 ± 0.11**	0.68 ± 0.05***
3-Hydroxybutyrate + acetoacetate	0.23 ± 0.05	0.25 ± 0.04	0.55 ± 0.14***	0.44 ± 0.09***	2.09 ± 0.25***
Glucose	7.83 ± 0.31	7.80 ± 0.43	7.56 ± 0.34	7.35 ± 0.12	3.70 ± 0.12***
PDH_a (munits/unit of citrate synthase)					
Heart	11.0 ± 2.5	17.6 ± 2.7*	5.4 ± 1.9**	5.2 ± 1.2***	0.2 ± 0.1***
Liver	52.8 ± 7.8	44.9 ± 4.7	43.8 ± 7.4	56.8 ± 13.2	6.4 ± 0.2***
Fru-2,6-P_2 (nmol/g wet wt.)					
Heart	1.45 ± 0.07	1.72 ± 0.06**	1.41 ± 0.20	1.13 ± 0.09*	0.56 ± 0.07***
Liver	9.53 ± 0.63	10.30 ± 0.66	8.80 ± 1.00	7.30 ± 0.80*	1.90 ± 0.36***

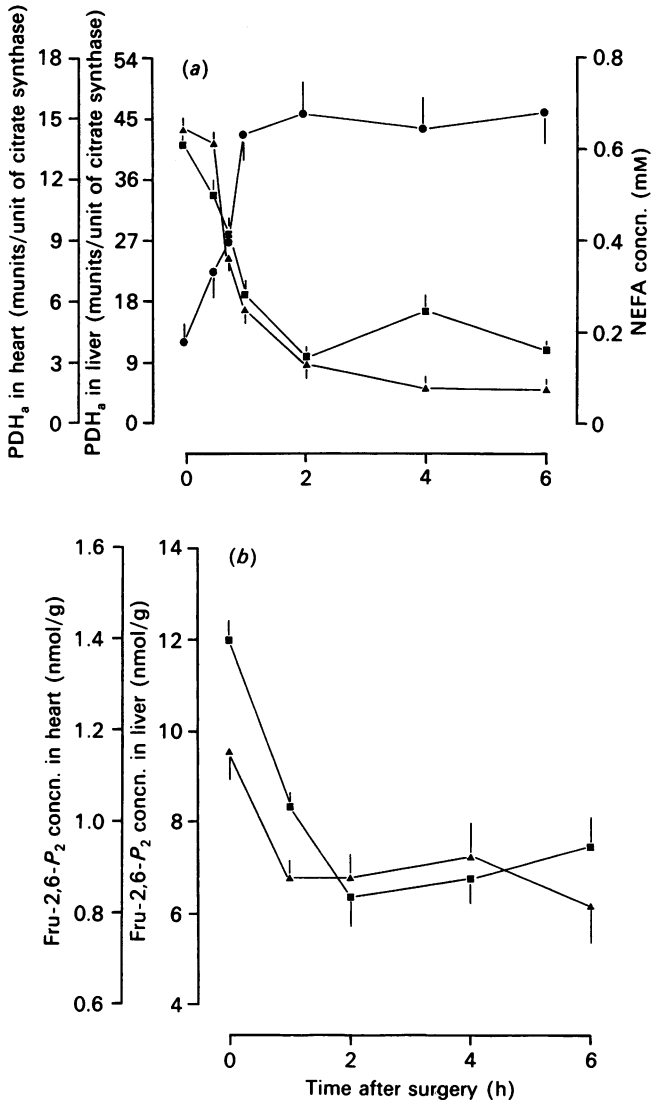

Fig. 1. Time courses of plasma NEFA concentrations, cardiac and hepatic PDH activities and cardiac and hepatic Fru-2,6-P_2 concentrations after surgery

Post-operative changes in plasma concentrations of NEFA (●), cardiac PDH$_a$ (■) and hepatic PDH$_a$ (▲) are shown in (*a*), and of cardiac (■) and hepatic (▲) Fru-2,6-P_2 concentrations in (*b*). Results are means ± S.E.M. for at least six values. The effects of surgery on all five variables were significant ($P < 0.001$) at 1, 2, 4 and 6 h after surgery. The significance of the effect of surgery at 0.5 h after surgery is $P < 0.01$.

butyrate (an approx. 50% decrease at 5 mM-3-hydroxybutyrate [7], compared with a 34% decrease in the present experiments). The results predict that any pathological or physiological situation leading to increased fatty acid availability in the fed state should be associated with a decrease in cardiac Fru-2,6-P_2, and, assuming that observations *in vitro* can be extrapolated to the situation *in vivo*, decreased glycolysis.

In the study of the effects of increasing fatty acid concentrations on hepatic Fru-2,6-P_2 *in vitro* [7], the initial concentration of Fru-2,6-P_2 (15 nmol/g) was obtained in the absence of exogenous fatty acid, and at a high glucose concentration. The major decline in Fru-2,6-P_2 concentration occurred when the fatty acid concentration was increased from 0 to 0.2 mM (where the Fru-2,6-P_2 concentration was approx. 9.5 nmol/g), and further increases in fatty acid concentrations from 0.2 to 1.0 mM were not associated with marked decreases in Fru-2,6-P_2. The mean Fru-2,6-P_2 concentration *in vitro* over the range of NEFA concentrations from 0.4 to 1 mM was approx. 6.75 nmol/g. In the present experiments, the hepatic Fru-2,6-P_2 concentration at 0.2 mM-NEFA was 9.53 nmol/g (Table 1), and over a range of NEFA concentrations from 0.4 to 1 mM (mean = 0.78 ± 0.08, $n = 8$) was 8.90 ± 0.52 nmol/g (not significant versus 0.2 mM). Because of the remarkable similarity in the values for hepatic Fru-2,6-P_2 found in the fed state *in vivo* and in isolated hepatocytes from fed rats at a comparable fatty acid concentration (0.2 mM), it might be tempting to conclude that, even in the fed state, the availability of exogenous NEFA may be sufficient to exert a restrictive influence on hepatic glycolytic flux. However, *in vivo*, inhibition of lipolysis did not increase hepatic Fru-2,6-P_2, and the response to an increased NEFA was attenuated. There is therefore no evidence from either PDH activities or from Fru-2,6-P_2 concentrations for suppression of hepatic carbohydrate utilization by fatty acids in normal fed rats. As ketonaemia is not increased to the extent that might be expected from the degree of elevation of NEFA concentrations (Table 1), it is suggested that available fatty acyl-CoA is preferentially esterified until further changes in hormone or substrate supply dictate that the liver enters the ketogenic mode, and only at this time will fat oxidation occur at rates adequate to lead to significant suppression of carbohydrate utilization.

Response of tissue PDH activity to surgery

Previous work has demonstrated increased NEFA concentrations, together with decreased cardiac and hepatic PDH activities, at 2 and 4 h after abdominal surgery in the rat [16,17]. A more detailed time course of the early post-operative changes in NEFA supply and PDH activities in the same animal model is shown in Fig. 1. An increase in NEFA concentration was observed within 0.5 h of surgery and was accompanied by decreased activity of cardiac PDH; significant decreases in hepatic PDH$_a$ were observed after 1 h. The maximum NEFA concentration was reached after 2 h, and concentrations remained high for the subsequent 4 h. The greatest percentage change in cardiac and hepatic PDH activity was observed within 2 h of surgery.

On the basis of the close temporal relationship between the increase in NEFA and the decline in cardiac PDH activity after surgery (Fig. 1), together with the observation that increased NEFA concentrations are associated with inactivation of cardiac PDH in fed rats (Table 1), it might be concluded that the post-operative decline in cardiac PDH activity is simply a consequence of the effects of an increased NEFA supply, with an associated increase in fatty acid oxidation. However, the activity of cardiac PDH in surgically stressed rats was significantly lower than that observed in normal rats given corn oil plus heparin over a comparable range of NEFA concentrations. Thus, in normal rats at a mean NEFA concentration of 0.64 ± 0.10 mM, cardiac PDH$_a$ was 11.3 ± 1.4 munits/unit of citrate synthase, compared with 3.3 ± 0.3 munits/unit of citrate synthase in surgically stressed rats (Fig. 1a). Nevertheless, under conditions where post-operative lipolysis was suppressed by the prior administration of MPCA (see Table 2), the activity

Table 2. Blood metabolite concentrations and tissue Fru-2,6-P_2 concentrations at 2 h after surgical stress, with or without prior treatment with MPCA

Results are means ± S.E.M. for four to ten observations. Statistically significant effects of inhibition of lipolysis with MPCA are indicated by: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Concentrations of Fru-2,6-P_2 in gastrocnemius and soleus of rats not subjected to surgery were 1.42 ± 0.11 and 1.22 ± 0.09 nmol/g respectively in the fed state, and 0.69 ± 0.06 and 0.46 ± 0.06 nmol/g respectively after 48 h starvation.

	Surgical stress	Surgical stress with inhibition of lipolysis
Blood concn. (mM) of:		
NEFA	0.63 ± 0.07	0.17 ± 0.02***
3-Hydroxybutyrate + acetoacetate	0.29 ± 0.03	0.21 ± 0.03
Glucose	8.67 ± 0.22	7.15 ± 0.31**
Fru-2,6-P_2 concn. (nmol/g) in:		
Heart	0.88 ± 0.62	0.94 ± 0.10
Liver	8.21 ± 0.62	8.00 ± 0.60
Gastrocnemius	1.08 ± 0.04	1.41 ± 0.15*
Soleus	1.05 ± 0.04	1.07 ± 0.09

of cardiac PDH_a was within the normal range for the prevailing fatty acid concentration (12.8 ± 0.3 munits/unit of citrate synthase; 15.3% active form). The results suggest that the response to surgical stress acts to augment the effects of an increased NEFA supply to decrease cardiac PDH activity by accelerating cardiac fat oxidation.

As an acute elevation of NEFA concentration failed to decrease hepatic PDH_a in normal fed rats, the decline in hepatic PDH observed after surgery cannot be directly attributed to increased lipolysis alone, although (as in the heart) post-operative decreases in activity were not observed if triacylglycerol mobilization was prevented [a value of 45.9 ± 6.7 munits/unit of citrate synthase (16.6% active) in MPCA-treated rats]. The results suggest that surgical stress may exert a permissive effect on hepatic fat oxidation, thereby facilitating PDH inactivation in response to an increase in plasma NEFA.

Tissue Fru-2,6-P_2 concentrations after surgery

Surgical stress led to a progressive decrease in the Fru-2,6-P_2 concentration in both the heart and the liver, with the major effect of surgery being observed within the first 2 h (Fig. 1*b*), as is the case for the post-operative elevation in NEFA concentration and decline in PDH activity (Fig. 1*a*). From a comparison of the range of Fru-2,6-P_2 concentrations seen in the heart after 2 h of exposure to increased NEFA (Table 1) and at 2 h after surgery (Table 2), it can be concluded that the response of cardiac Fru-2,6-P_2 to surgery exceeds that which might be attributable to the effects of an increased NEFA concentration alone. Furthermore, the post-operative decrease in cardiac Fru-2,6-P_2 was observed even when the lipolytic response was prevented (Table 2). Although it is not excluded that surgery may be accompanied by mobilization of endogenous cardiac triacylglycerol which may not be inhibited by MPCA [18], this appears unlikely, not only because of the low cardiac triacylglycerol concentrations found in the fed state, but also because of the effect of MPCA to prevent the inhibitory effect of surgery on cardiac PDH. The results suggest that factors in addition to the operation of the glucose/fatty acid cycle may assume regulatory significance for the diminution in cardiac Fru-2,6-P_2 concentration after surgery.

The decline in the hepatic concentration of Fru-2,6-P_2 observed after surgery was comparable in magnitude with that induced by prolonged exposure to an elevated NEFA concentration, but, as in the heart, inhibition of lipolysis failed to abolish the effect of surgery on Fru-2,6-P_2 (Table 2), while reversing that on PDH. It is therefore improbable that the post-operative increase in NEFA either causes or is permissive for the response of hepatic Fru-2,6-P_2 to surgery. The post-operative decreases in cardiac and hepatic PDH and Fru-2,6-P_2, although occurring in parallel, thus appear to be achieved by different mechanisms.

Fru-2,6-P_2 in skeletal muscle

Despite the marked effect of surgery to decrease the cardiac concentration of Fru-2,6-P_2 within 2 h, there was only a limited response of skeletal-muscle Fru-2,6-P_2 to surgery within the same post-operative period (Table 2). A 24% decline in Fru-2,6-P_2 concentration was observed in gastrocnemius muscle. However, significant changes were not observed in soleus muscle, which, like the heart, is a working muscle containing a high percentage of oxidative fibres. The finding that prior treatment with MPCA evokes a significant increase in the Fru-2,6-P_2 concentration in gastrocnemius muscle of surgically stressed rats may indicate that, in certain muscle types, fatty acids may inhibit glucose utilization after surgery. Relief of such inhibition may explain the hypoglycaemic effect of MPCA (Table 2).

Relationship between carbohydrate and fat utilization in the heart after surgery

The inactivation of cardiac PDH after surgery was accompanied by a decrease in the concentration of free (non-esterified) carnitine, and an increase in the concentration of esterified carnitine. These concentration changes are consistent with accelerated fat oxidation [19]. The concentration of long-chain acylcarnitine was doubled, to a value of 48 ± 4 nmol/g, which is similar to that observed after prolonged starvation (see [6]). The ratio of short-chain acylcarnitine to free carnitine was increased from 0.34 ± 0.04 (6) to 0.68 ± 0.07 (6) ($P < 0.01$). Since in the heart short-chain acylcarnitine is predominantly acetylcarnitine, and the activity of carnitine acetyltransferase is high, it may be inferred that the [acetyl-CoA]/[CoA] ratio is increased [19]. Caterson *et al.* [9] have demonstrated that such an increase is responsible for the inactivation of cardiac PDH, which can be attributed to increased utilization of NEFA in starvation. The changes in free and esterified carnitine concentrations observed after surgery were abolished by MPCA (results not shown).

It has been suggested that the decreases in Fru-2,6-P_2 observed in response to 3-hydroxybutyrate *in vitro* are secondary to increases in citrate concentrations [7]. In the present experiments, cardiac citrate concentrations after surgery were approximately similar to those seen after prolonged exposure to elevated NEFA concentrations [0.41 ± 0.03 (7) and 0.36 ± 0.06 (4) μmol/g respec-

tively], yet the decrease in Fru-2,6-P_2 was rather greater. The mechanisms by which the post-operative changes in Fru-2,6-P_2 are achieved thus remain to be clarified.

Relationship between carbohydrate and fat utilization in the liver after surgery

There was no evidence from measurements of ketonaemia (Table 2) or acylcarnitine concentrations (results not shown) that hepatic lipid oxidation was substantially increased after surgery, but the hepatic [3-hydroxybutyrate]/[acetoacetate] ratio was significantly increased [from 0.12 ± 0.03 (6) to 0.40 ± 0.05 (6), $P < 0.01$]. This indicates an increased mitochondrial [NADH]/[NAD^+] ratio, which would be expected to inhibit PDH. The change in [3-hydroxybutyrate]/[acetoacetate] ratio was not observed when lipolysis was inhibited [a value of 0.20 ± 0.05 (6) in MPCA-treated surgically stressed rats].

In view of the pronounced effect of surgery to inhibit hepatic PDH, it is perhaps surprising that the decline in Fru-2,6-P_2 concentration is not more marked. A significant ($P < 0.01$) post-operative increase in glycaemia (cf. Tables 1 and 2 for control and surgically stressed rats) may compensate for any decrease which may be occasioned by an increased availability of NEFA. Although it is appreciated that under some conditions (e.g. fructose re-feeding [20]) the hepatic Fru-2,6-P_2 concentration may not give a good indication of the relative rates of glycolysis and gluconeogenesis, experiments with isolated hepatocytes from fed rats [21] would suggest that the decrease in hepatic Fru-2,6-P_2 induced by surgery would be insufficient to restrict net glycolytic flux severely. This is compatible with our previous observations that, in the short term, net hepatic glucose output is unaffected by abdominal surgery [16].

Concluding remarks

Although plasma NEFA concentrations after the administration of corn oil plus heparin or surgical stress were comparable with those found after 48 h starvation (Table 1), the responses of cardiac and hepatic PDH activities or of tissue Fru-2,6-P_2 concentrations were generally less, and, in some cases, were not observed. These findings emphasize the importance of long-term adaptive changes in tissue enzyme complement for achieving the extent of glucose conservation observed after prolonged food withdrawal. The results do, however, indicate that an acute elevation in NEFA concentration may rapidly lead to a restriction of glucose utilization in cardiac (and possibly skeletal) muscle, even when carbohydrate is available. This is of potential importance both in stress and in other conditions leading to increased fatty acid availability in the fed state.

From the present experiments there is no indication for a controlling influence of fatty acids over hepatic carbohydrate utilization, at least in the well-fed normal rat. Indeed, from the literature there is more compelling evidence for a regulatory role of hepatic carbohydrate metabolism on fatty acid disposal. Thus, when presented with the same concentration of long-chain fatty acid in the medium, isolated liver preparations from fed rats exhibit lower rates of fat oxidation than do those from starved animals [22], increased fat oxidation in the starved livers being correlated with depletion of hepatic glycogen [23]. Such intrahepatic control of fat utilization will dampen the response of hepatic PFK-1 to sudden or transient changes in lipid supply, thereby preventing any activation of gluconeogenesis from preceding that of glycogenolysis and thereby minimizing inessential glucose re-cycling. At the same time, the maintenance of hepatic PDH activity permits the continued utilization of lactate generated via glycolysis in non-oxidative tissues in the absence of an alternative route of disposal.

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