Insulin and non-esterified fatty acids

Acute regulators of lipogenesis in perfused rat liver

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Livers from fed rats were perfused with whole rat blood and infused with oleate to maintain constant concentrations of serum non-esterified fatty acids over a wide physiological range. Infusion of insulin opposed the antilipogenic effects of increasing concentrations of serum non-esterified fatty acids. Secretion of very-low-density-lipoprotein triacylglycerols was directly proportional to the concentration of serum non-esterified fatty acids and was increased by insulin. The secretion of newly-synthesized fatty acids in very-low-density-lipoprotein triacylglycerols decreased with increasing concentrations of serum non-esterified fatty acid. Insulin opposed this change. Cholesterol biosynthesis was unaffected by alterations in concentration of serum non-esterified fatty acid but was increased by insulin. Equilibrium concentrations of perfusate lactate and glucose were increased by serum non-esterified fatty acids but steady-state rates of hepatic glucose output and lactate uptake were unchanged. Insulin decreased perfusate glucose concentrations and abolished the increase in its concentration that resulted from increases in non-esterified fatty acid concentrations.

An inverse relationship has been observed in the perfused rat liver between the rate of fatty acid synthesis *de novo* (lipogenesis) and the serum non-esterified fatty acid concentration (Mayes & Topping, 1974). These findings are consistent with the hypothesis that long-chain fatty acids play an important role as acute regulators of hepatic lipogenesis. *In vivo*, this is supported by the fact that in the starving, fat-fed or diabetic states, where circulating non-esterified fatty acids are increased in concentration, lipogenesis is depressed (Masoro, 1962). Conversely, in the carbohydrate-fed state lipogenesis is enhanced, and not only is the concentration of non-esterified fatty acids decreased, but, in addition, plasma insulin concentration is increased (Masoro, 1962).

Direct effects of insulin on hepatic fatty acid synthesis *in vivo* have been difficult to differentiate from indirect effects of the hormone arising from its antilipolytic action in adipose tissue and the consequent decrease in non-esterified fatty acid concentrations. Acute effects of insulin on hepatic metabolism *in vitro* have also been inconclusive. For

example, Haft (1967) reported that insulin stimulated lipogenesis (as measured by incorporation of ^{14}C -labelled acetate) in perfused liver but this occurred only at high concentrations of glucose. On the other hand we have shown that the hormone inhibited fatty acid oxidation and increased VLD-lipoprotein triacylglycerol secretion in rat livers perfused with whole blood (Topping & Mayes, 1972). However, in these experiments, there were no consistent effects of insulin on lipogenesis as measured by the incorporation of $[^3\text{H}]$ acetate.

The lack of effect of insulin may arise from the use of perfusion media other than blood of physiological haematocrit (Storer *et al.*, 1980; Topping *et al.*, 1981). However, the incorporation of isotopically-labelled acetate as an index of lipogenesis is also open to criticisms (Windmueller & Spaeth, 1966; Lowenstein, 1972) which may be largely overcome by measurement of the incorporation of ^3H from $^3\text{H}_2\text{O}$ into fatty acids.

We therefore decided to investigate the effects of insulin on the rate of fatty acid synthesis by rat liver perfused with homologous whole blood in the presence of $^3\text{H}_2\text{O}$. The study was carried out over a wide range of physiological serum non-esterified fatty acid concentrations to determine whether the hormone modified the antilipogenic effects of long-chain fatty acids, which we had previously observed (Mayes & Topping, 1974).

Abbreviation used: VLD lipoproteins, very-low-density lipoproteins ($d < 1.006$).

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A preliminary account of some of this work has been presented (Topping & Mayes, 1976a).

Materials and methods

Liver perfusions

Livers from fed male Wistar albino rats (340–360 g body wt.) were perfused *in situ* by a modification of the method of Mayes & Felts (1966). The perfusate was 120 ml of defibrinated rat blood, dialysed for 24 h against Krebs–Henseleit (1932) buffer to remove vasoconstrictive factors. The buffer contained glucose (10 mM), mixed amino acids (500 mg/litre) and half the specified concentration of Ca^{2+} . The $p\text{O}_2$ of the perfusate was monitored continuously and maintained at 90–100 mmHg (12–13 kPa). The rate of perfusion was 12 ml of blood/min (0.9–1.0 ml/min per g of liver).

At the start of the experiment (designated zero time), 5 mCi of $^3\text{H}_2\text{O}$ (The Radiochemical Centre, Amersham, Bucks., U.K.) in 1.0 ml of 0.15 M-NaCl was added to the perfusate. Infusions of oleate and insulin were started simultaneously. Oleic acid (Sigma Chemical Co., Poole, Dorset, U.K.) was added as a complex with bovine serum albumin (Armour Co., Eastbourne, Sussex, U.K.) to maintain a constant concentration of non-esterified fatty acid within the range 0.3–1.8 $\mu\text{mol/ml}$ of serum (Mayes & Topping, 1974; Topping & Mayes, 1972). Crystalline bovine insulin, as a solution in 0.15 M-NaCl, was infused to maintain a concentration of 80 ng/ml of serum. In control perfusions 0.15 M-NaCl was substituted for the insulin solution.

Samples of the perfusate were taken at zero time and at 45 and 90 min, after which the liver was removed, blotted, weighed and homogenized in 150 ml of chloroform/methanol (2:1, v/v).

In calculating rates of production or utilization of metabolites by the liver, corrections were applied for changes in volume due to additions to, and withdrawals from, the perfusate (Topping & Mayes, 1972, 1976b).

Analytical techniques

A commercial glucose oxidase method (Boehringer Corp., Lewes, E. Sussex, U.K.) was used to estimate perfusate glucose. L(+)-Lactate was determined by the method of Hohorst (1963).

Serum was obtained by centrifugation of the blood perfusate and 2 ml was layered under 0.15 M-NaCl in cellulose nitrate tubes. VLD lipoproteins were separated by ultra-centrifugation and the lipids were extracted with chloroform/methanol (2:1, v/v) as described previously (Topping & Mayes, 1972). Serum non-esterified fatty acids were measured titrimetrically by the modification by Trout *et al.* (1960) of the Dole procedure.

Portions of the chloroform extract of liver and lipoprotein lipids were dried down under N_2 at 40–60°C and saponified with ethanolic KOH by a modification of the method of Exton *et al.* (1972). After acidification, fatty acids and cholesterol were extracted with heptane and separated by t.l.c. (Topping & Mayes, 1976b). Lipids were extracted from silica gel using chloroform/methanol (2:1, v/v) and the radioactivity determined as described previously (Topping & Mayes, 1972, 1976b). Perfusate (0.2 ml) was diluted, deproteinized and counted for determination of $^3\text{H}_2\text{O}$ specific radioactivity and rates of fatty acid and sterol biosynthesis were calculated by using the factors and assumptions of Windmueller & Spaeth (1966, 1967).

Statistical methods

The statistical significance of effects of serum non-esterified fatty acid concentration was established by linear regression analysis (Brownlee, 1949). Differences between control and insulin-infused livers were established by multiple regression analysis and are expressed as either differences between the slopes of the regression lines or the distance between them. A value of $P < 0.05$ was taken as the criterion of statistical significance.

Results

Lipogenesis

In the absence of infused oleate, the non-esterified fatty acid concentration in the perfusate was 0.3 $\mu\text{mol/ml}$ of serum and the rate of long-chain fatty acid synthesis was maximal at approx. 7 $\mu\text{mol/h}$ per g of liver. With increasing concentrations of serum non-esterified fatty acid, the rate of synthesis decreased. This relationship was highly significant ($P < 0.001$), being linear, when the data were plotted on logarithmic co-ordinates (Fig. 1). A similar linear relationship ($P < 0.05$) was observed when insulin was infused. However, in the presence of the hormone the effect of increasing the concentration of serum non-esterified fatty acids on lipogenesis was less marked. At the highest concentration (1.8 μmol of fatty acid/ml of serum), the rate of synthesis in the presence of insulin was increased by approx. 100% over controls. This effect was highly significant, statistically ($P < 0.001$).

VLD-lipoprotein secretion

In both control and insulin-infused livers, the rate of VLD-lipoprotein triacylglycerol secretion was proportional to the serum non-esterified fatty acid concentration (Fig. 2). At all concentrations of perfusate non-esterified fatty acid, the rate of secretion (which was linear with respect to time) was significantly ($P < 0.001$) increased by insulin.

Fig. 1. Effect of serum non-esterified fatty acids and insulin on lipogenesis in perfused rat liver

Livers from fed rats were perfused with whole rat blood containing 5 mCi of $^3\text{H}_2\text{O}$ at a flow rate of 0.9–1.0 ml/min per g of liver. Non-esterified fatty acid (oleate) was infused to maintain the constant concentrations shown. Rates of lipogenesis were determined as described in the text and the data are represented as logarithms. Other additions were: ○, none (control); ●, insulin. Effect of non-esterified fatty acids: $P < 0.001$ (control); $P < 0.05$ (insulin). Difference between slopes of regression lines: $P < 0.01$.

Fig. 2. Effect of serum non-esterified fatty acids and insulin on VLD-lipoprotein-triacylglycerol fatty acid secretion in perfused rat liver

Livers from fed rats were perfused with whole rat blood at a flow rate of 0.9–1.0 ml/min per g of liver. Non-esterified fatty acid (oleate) was infused to maintain the constant concentrations shown. VLD-lipoprotein-triacylglycerol fatty acid secretion was determined as described in the text. Other additions were: ○, none (control); ●, insulin. Effect of non-esterified fatty acids: $P < 0.001$ (control); $P < 0.05$ (insulin). Difference between slopes of regression lines: $P = \text{not significant}$. Distance between regression lines: $P < 0.001$.

After approx. 40 min of perfusion, equilibration of existing liver triacylglycerol pools with newly-esterified fatty acids is complete (Topping & Mayes, 1972). The specific radioactivity of VLD-lipoprotein triacylglycerols, with respect to either serum non-esterified fatty acids or fatty acids synthesized *de novo*, is then constant with time. In this steady state the rate of secretion of newly-formed fatty acids was $1.5 \mu\text{mol}$ of triacylglycerol fatty acid/h per g of liver at the lowest concentration of serum non-esterified fatty acid employed, decreasing to 0.5 at the highest concentration of infused oleate (Fig. 3). Thus, in control livers, some 30% of VLD-lipoprotein-triacylglycerol fatty acids were synthesized *de novo* at $0.3 \mu\text{mol}$ of non-esterified fatty acid/ml of serum, decreasing to approx. 5% of total secretion at $1.8 \mu\text{mol}$ of non-esterified fatty acid/ml of serum. The infusion of insulin reduced substantially the inhibitory effect of long-chain fatty acid on the secretion of newly-synthesized triacylglycerol fatty acids ($P < 0.001$). In the presence of the hormone, the contribution of synthesis *de novo* to total triacylglycerol fatty acid secretion decreased from 35% to 11% as the serum non-esterified fatty acid concentration was increased from 0.3 to $1.8 \mu\text{mol/ml}$.

Cholesterogenesis

The rate of cholesterol biosynthesis in control livers was unaffected by the variations in the concentration of serum non-esterified fatty acid with a mean ($\pm \text{s.e.m.}$) of $153 \pm 21 \text{ nmol/h}$ per g of liver. In livers infused with insulin, synthesis was increased but was similarly unaffected by the concentration of serum non-esterified fatty acids and the mean rate was $400 \pm 32 \text{ nmol/h}$ per g of liver. This stimulatory effect of the hormone was highly significant statistically.

Perfusate glucose

In the absence of infused fatty acid, perfusate glucose concentrations stabilized at $7\text{--}10 \mu\text{mol/ml}$, 45–90 min after zero time (Fig. 4). With increasing concentrations of serum non-esterified fatty acids the steady-state concentration increased to $11\text{--}13 \mu\text{mol}$ of glucose/ml. Insulin infusion abolished this effect of non-esterified fatty acid, steady-state glucose concentrations remaining constant at approx. $7 \mu\text{mol/ml}$ over the range of fatty acid concentrations studied.

This steady state is an equilibrium between glucose release by the liver and erythrocyte glycolysis (Topping & Mayes, 1972, 1976b). Calculation of rates of hepatic glucose output showed that this

Fig. 3. Effects of serum non-esterified fatty acids and insulin on the secretion of newly-synthesized fatty acids in VLD-lipoprotein triacylglycerols in perfused rat liver. Livers from fed rats were perfused with whole rat blood containing 5 mCi of $^3\text{H}_2\text{O}$ at a flow rate of 0.9–1.0 ml/min per g of liver. Non-esterified fatty acid (oleate) was infused to maintain the constant concentrations shown. Rates of lipogenesis and the incorporation of newly-synthesized fatty acids into VLD-lipoprotein-triacylglycerol fatty acid in the steady-state were determined as described in the text. The data are represented as logarithms. Other additions were: ○, none (control); ●, insulin. Effect of non-esterified fatty acid: $P < 0.001$, control; not significant, insulin. Difference between slopes of regression lines: $P < 0.05$.

averaged $15.7 \pm 1.7 \mu\text{mol}$ of glucose/h per g of liver for all perfusions combined.

The concentration of liver glycogen at the end of perfusion was not affected significantly by infusion of either oleate or insulin and averaged $19.8 \pm 1.4 \text{ mg/g}$ of liver wet. wt.

Perfusate lactate concentration

As for perfusate glucose, concentrations of lactate at zero time did not differ significantly between individual perfusions. However, the steady-state concentrations attained after 45–90 min were positively related to the non-esterified fatty acid concentration (Fig. 5). Perfusate [lactate] was not

Fig. 4. Effect of serum non-esterified fatty acids and insulin on blood glucose concentrations in perfused rat liver

Livers from fed rats were perfused with whole rat blood at a flow rate of 0.9–1.0 ml/min per g of liver. Non-esterified fatty acid (oleate) was infused to maintain the constant concentrations shown. Blood glucose was determined as described in the text. Other additions were: ○, none (control); ●, insulin. Effect of non-esterified fatty acids: $P < 0.001$, control; not significant, insulin. Difference between slopes of regression lines: $P < 0.001$.

Fig. 5. Concentrations of perfusate lactate in perfused livers from fed rats

Livers from fed rats were perfused with whole rat blood at a flow rate of 0.9–1.0 ml/min per g of liver. Non-esterified fatty acid (oleate) was infused to maintain the constant concentrations shown. Perfusate lactate was determined as described in the text. Other additions were: ○, none (control); ●, insulin. Effect of non-esterified fatty acids: $P < 0.05$, control + insulin, combined.

altered by addition of insulin and, in the steady state, hepatic uptake (allowing for erythrocyte glycolysis) was similar in all perfusions with a mean value of

$35.6 \pm 1.9 \mu\text{mol/h}$ per g of liver. This uptake balanced, approximately, the glucose output referred to above.

Discussion

The inverse logarithmic relationship that we observed previously between serum long-chain fatty acid concentrations and hepatic lipogenesis (Mayes & Topping, 1974) was confirmed in the present studies and is of considerable importance as a potential short-term regulatory mechanism in the conversion of carbohydrate into fatty acids. The observations are explicable in several ways, e.g. inhibition of acetyl-CoA carboxylase (EC 6.4.1.2) by acyl-CoA (Tubbs & Garland, 1963; Bortz & Lynen, 1963), inhibition by acyl-CoA of citrate transport out of the mitochondrion (Halperin *et al.*, 1972) or conversion of pyruvate dehydrogenase (EC 1.2.4.1) into the inactive form by increased concentrations of fatty acid (Wieland *et al.*, 1972). This latter effect is considered to be mediated via increases in the mitochondrial ratios $[\text{acetyl-CoA}]/[\text{CoA}]$, $[\text{NADH}]/[\text{NAD}^+]$ and $[\text{ATP}]/[\text{ADP}]$ by long-chain fatty acids (Siess & Wieland, 1976). Any of these mechanisms could account for the diminution in the conversion of acetyl-CoA into fatty acid by oleate.

In the present experiments insulin opposed the antilipogenic effects of exogenous oleate and stimulated both the export of VLD lipoproteins and the contribution of newly-synthesized fatty acids to lipoprotein triacylglycerol secretion. It has previously been observed that insulin stimulates the esterification and inhibits the oxidation of endogenous fatty acids or serum non-esterified fatty acids and increases their export in VLD lipoproteins (Poledne & Mayes, 1970; Topping & Mayes, 1972). We have sought to explain these findings in terms of an inhibition of hepatic lipolysis. It was proposed that the liver contains a lipase, essentially similar to the hormone-sensitive cyclic AMP-dependent lipase present in adipose tissue, which insulin inactivates and glucagon stimulates. This enzyme was postulated to act on a small metabolically active pool of triacylglycerols, which is the immediate precursor for VLD-lipoprotein secretion and is distinct from that which forms the bulk of liver triacylglycerols (Topping & Mayes, 1972). Lipolysis of this pool would inhibit VLD-lipoprotein export and the consequent rise in intracellular $[\text{acyl-CoA}]$ would favour enhanced fatty acid oxidation and depress lipogenesis. Conversely, inhibition of the lipase would result in increased esterification and VLD-lipoprotein release. More recently we have demonstrated in perfused liver that insulin directly increases the activity of mitochondrial *sn*-glycerol 3-phosphate acyltransferase and decreases perfus-

ate non-esterified fatty acids in the absence of exogenous oleate (Bates *et al.*, 1977). Thus, at a given concentration of infused fatty acids, insulin would most likely lower the intracellular concentration of acyl-CoA and consequently diminish the inhibition of lipogenesis by the mechanisms discussed above. Such an effect would appear to be greater at elevated concentrations of non-esterified fatty acid when their contribution to the cellular economy is correspondingly higher. It must also be considered that the hormone might regulate biosynthesis of fatty acids by covalent changes of acetyl-CoA carboxylase. Geelen *et al.* (1978) showed in isolated hepatocytes that increased lipogenesis due to insulin was correlated with an enhancement of acetyl-CoA carboxylase. Whereas some of this increase might be ascribed to changes in the intracellular concentrations of allosteric effectors, Witters *et al.* (1979) have demonstrated a decrease in the proportion of the dephosphorylated form of the enzyme and shown that the insulin-stimulated enzyme is resistant to inhibition by palmitoyl-CoA.

Inhibition of hepatic lipolysis would also account for the increased contribution of fatty acids synthesized *de novo* to VLD-lipoprotein triacylglycerol secretion. It has been shown that when non-esterified fatty acid oxidation is inhibited either by insulin (Topping & Mayes, 1972) or physiological concentrations of fructose (Topping & Mayes, 1976b), VLD-lipoprotein release is enhanced. The proportional contribution of exogenous fatty acids to triacylglycerol release is also increased, probably by diminution of the exchange of newly-formed triacylglycerols with the more-slowly-turning-over pool, which represents the bulk of liver acylglycerols (Topping & Mayes, 1972, 1976b). Similarly, in the present experiments insulin could minimize the intracellular transfer of fatty acids synthesized *de novo*.

In the present study we observed that the synthesis of cholesterol by perfused rat liver was independent of the rate of infusion of oleate but was stimulated by insulin. The former finding contrasts with the data of Goh & Heimberg (1976), who found that cholesterogenesis rose with increasing concentrations of perfusate non-esterified fatty acids. The discrepancy possibly reflects the use by these authors of a blood-free perfusate in contrast with our use of undiluted blood. The stimulation of cholesterogenesis by insulin is presumably similar to its effect on fatty acid biosynthesis, i.e. an immediate alteration in enzyme activity rather than by formation of new enzyme protein. Hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34), considered to be a key enzyme in the regulation of cholesterol biosynthesis, is rapidly increased in activity by insulin in both normal and diabetic animals (Laksh-

manan *et al.*, 1973; Nepokroeff *et al.*, 1974). As in the case of lipolysis, effects of insulin on sterol biosynthesis may be explained by a cyclic AMP-dependent mechanism. Thus exogenous cyclic AMP (or dibutyryl cyclic AMP) inhibits hepatic cholesterologenesis (Bricker & Levey, 1972; Capuzzi *et al.*, 1974). Insulin is known to counteract effects of cyclic AMP, and Keith *et al.* (1979) have recently described interconversion of hydroxymethylglutaryl-CoA reductase between active and inactive forms in a manner analogous to acetyl-CoA carboxylase.

In confirmation of other reports (Mortimore, 1963; Haft, 1967; Storer *et al.*, 1980, 1981; Topping *et al.*, 1981), insulin was observed to lower equilibrium perfusate glucose concentrations. The rise in blood [glucose] due to inhibition of glycogen synthase (EC 2.4.1.11) and activation of phosphorylase (EC 2.4.1.1) after hormone-induced increases in liver cyclic AMP is well documented (Exton & Park, 1972). As insulin opposes increases in liver cyclic AMP, it is possible that the hormone lowered perfusate [glucose] via this mechanism by opposing changes in enzymic activity. Additionally, it is possible that alterations in ionic balance may have effected the observed metabolic changes. Attention is being drawn to the fact that a number of hormones appear to act on hepatic carbohydrate metabolism (and on lipogenesis) by altering the hepatocellular distribution of cations, notably Ca^{2+} (Exton, 1979; Hems, 1979). In perfused liver insulin stimulates (Storer *et al.*, 1980, 1981) and glucagon inhibits (Saggerson *et al.*, 1981) bile flow, both changes reflecting alterations in hepatocellular ionic balance. Therefore either mechanism may be invoked to account for the metabolic changes attending insulin infusion.

Perfusion with increased concentrations of serum non-esterified fatty acids raised the equilibrium concentrations of lactate in livers perfused with and without insulin. In control perfusions concentrations of glucose also increased, but steady-state rates of lactate uptake and glucose output were unaffected by serum non-esterified fatty acid concentration. There are conflicting reports in the literature as to whether fatty acids significantly alter gluconeogenesis in perfused liver. Williamson *et al.* (1966) have reported a stimulatory effect of oleate on glucose synthesis from lactate, whereas Exton & Park (1967) observed no change. The present data confirm previous observations (Mayes, 1970; Mayes & Topping, 1974) in livers of fed rats perfused with whole blood that infusion of oleate did not alter gluconeogenesis from lactate. It is pertinent to consider that in both the fed and starved states, the intestines *in vivo* are a net producer of lactate (Topping & Mayes, 1971). This has led to the suggestion (Topping *et al.*, 1979) that whole-blood

perfusates (containing actively glycolysing erythrocytes) are particularly useful in that they supply both O_2 and lactate under conditions close to the physiological. The latter is of importance as it is now recognized to be a major metabolic fuel of the liver in the fed state (Clark *et al.*, 1974; Salmon *et al.*, 1974). Its availability is governed by the activity of pyruvate dehydrogenase and the changes in perfusate lactate presumably reflect inhibition of this enzyme by increased concentrations of serum non-esterified fatty acids (Wieland *et al.*, 1972).

Conclusions

The data obtained in these experiments show clearly that, although insulin affects many of the metabolic processes of the liver without exogenous oleate, stimulatory effects of the hormone on lipogenesis were greatest at elevated concentrations of serum non-esterified fatty acids. This may partially explain the inability to some authors (e.g. Salmon & Hems, 1975) to detect any effect of insulin in the absence of infused fatty acid. However, it is also likely that discrepant effects of the hormone *in vitro* are also a consequence of the use of perfusion media other than blood of full haematocrit (Storer *et al.*, 1980; Topping *et al.*, 1981).

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