Glucagon inhibits triacylglycerol synthesis in isolated hepatocytes by lowering their glycerol 3-phosphate content

Peter E. DECLERCQ, Luc J. DEBEER and Guy P. MANNAERTS

Afdeling Farmakologie, Fakulteit Geneeskunde, K.U. Leuven Campus Gasthuisberg, B-3000 Leuven, Belgium

(Received 21 December 1981/Accepted 6 January 1982)

Triacylglycerol synthesis by glycogen-depleted hepatocytes from fed rats that have low glycerol 3-phosphate contents was stimulated by the addition of glycerol 3-phosphate precursors. Glucagon decreased triacylglycerol synthesis only when it also lowered glycerol 3-phosphate content. The hyperbolic-like relationship between glycerol 3-phosphate content and rates of triacylglycerol synthesis was identical in the absence or presence of glucagon, indicating that the glucagon effect on triacylglycerol synthesis was not mediated through changes in enzyme activities of the esterification pathway but through changes in cellular glycerol 3-phosphate content.

Several laboratories have shown that glucagon stimulates the oxidation and decreases the esterification of fatty acids in the perfused rat liver (Heimberg *et al.*, 1969) and in isolated rat hepatocytes (Christiansen, 1977; McGarry *et al.*, 1978; Beynen *et al.*, 1980). Since fatty acid oxidation and esterification constitute a branch-point, the effect of the hormone may be exerted either on one arm of the branch-point or in a reciprocal manner on both arms simultaneously. Through the work of McGarry and his co-workers (for review, see McGarry & Foster, 1980), it has become clear that glucagon stimulates fatty acid oxidation by lowering the concentration of malonyl-CoA, the first committed intermediate in lipogenesis and a potent inhibitor of carnitine palmitoyltransferase I, the enzyme controlling fatty acid entry into mitochondria. The decrease in malonyl-CoA and lipogenesis in glucagon-treated cells appears to result from the inhibition of glycolysis and of acetyl-CoA carboxylase.

It has not been established, however, whether glucagon exerts a separate control on esterification. A 10–20% decrease in glycerol 3-phosphate acyltransferase activity has been described in homogenates or microsomal fractions prepared from hepatocytes incubated with glucagon or dibutyryl cyclic AMP (Sugden *et al.*, 1980) and from livers perfused with dibutyryl cyclic AMP (Soler-Argilaga *et al.*, 1978). Haagsman *et al.* (1981) observed a decrease in diacylglycerol acyltransferase activity after incubation of hepatocytes with glucagon. In contrast with these findings, Soler-Argilaga *et al.* (1978) found an increased activity of this enzyme after perfusion of livers with dibutyryl cyclic AMP.

Several authors have demonstrated that glucagon lowers the glycerol 3-phosphate content of isolated hepatocytes and have raised the question as to what extent this decrease in glycerol 3-phosphate might be responsible for the reduction in fatty acid esterification (Christiansen, 1979; Beynen *et al.*, 1980; Lund *et al.*, 1980; Sugden *et al.*, 1980; Williamson *et al.*, 1980). In these studies, however, it was not established whether glycerol 3-phosphate availability was in fact limiting fatty acid esterification. We have determined the concentration range within which glycerol 3-phosphate limits esterification in isolated hepatocytes (Debeer *et al.*, 1981; Declercq *et al.*, 1982). In the present paper we demonstrate that glucagon exerts a separate (from oxidation) control on esterification in isolated hepatocytes. This control appears to be mediated by changes in glycerol 3-phosphate and perhaps dihydroxyacetone phosphate content.

Experimental

Male Wistar rats weighing 150–200 g and maintained on a standard laboratory diet were used. Hepatocytes were isolated as described previously (Mannaerts *et al.*, 1979). To lower their glycogen and glycerol 3-phosphate content, the hepatocytes were suspended in Krebs–Henseleit bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932), containing 2.6% defatted albumin and pre-incubated at 37°C for 90 min with intermittent washings at 30, 60 and 90 min (Declercq *et al.*, 1982). The washed cells were then resuspended in the same albumin-containing buffer and incubated in the presence of

0.3 mM- or 1 mM-[1-¹⁴C]palmitate (sp. radioactivity 1 Ci/mol). Incubations were stopped after 8 or 20 min with either HClO₄ for the determination of glycerol 3-phosphate (Debeer *et al.*, 1981) and radioactive acid-soluble oxidation products (Mannaerts *et al.*, 1979) or with chloroform/methanol (2:1, v/v) for the measurements of incorporation of radioactivity in tri- and di-acylglycerols (Mannaerts *et al.*, 1979). Under the various experimental conditions employed the contents of glycerol 3-phosphate and of radioactivity in diacylglycerols reached similar values at 8 and 20 min, indicating that the esterification pathway was operating under steady-state conditions. Therefore in Fig. 1, glycerol 3-phosphate content and radioactivity in diacylglycerols are expressed as means of the values measured at both time points. Triacylglycerol synthesis is expressed as rates of palmitate incorporation between the same two time points. In a separate series of experiments the dihydroxyacetone phosphate content of the cells was also measured. In

these experiments incubations were stopped after 15 min and glycerol 3-phosphate, dihydroxyacetone phosphate and incorporation of radioactivity in triacylglycerols were determined (Fig. 2). Dihydroxyacetone phosphate was measured by a fluorimetric adaptation of the method of Bücher & Hohorst (1970).

Results and discussion

Freshly isolated hepatocytes from overnight-starved animals incubated in albumin-containing Krebs–Henseleit buffer contain 0.1–0.2 μmol of glycerol 3-phosphate/ 10^8 cells. For hepatocytes from fed animals these values range from 0.4 to 0.7 $\mu\text{mol}/10^8$ cells. Below 0.3–0.4 $\mu\text{mol}/10^8$ cells, glycerol 3-phosphate availability seriously limits esterification, both in hepatocytes from fed (Declercq *et al.*, 1982) and starved rats (Debeer *et al.*, 1981). In preliminary experiments in which freshly isolated hepatocytes from fed animals were

Fig. 1. Incorporation of [1-¹⁴C]palmitate in triacylglycerols and [1-¹⁴C]palmitate content of diacylglycerols in isolated hepatocytes versus cellular glycerol 3-phosphate content

Isolated hepatocytes from fed rats were pre-incubated for 90 min to lower their glycerol 3-phosphate content and then incubated with various precursors of glycerol 3-phosphate in the presence of [1-¹⁴C]palmitate and 2 mM-(+)-octanoylcarnitine. (a) Shows incorporation of [1-¹⁴C]palmitate in triacylglycerols (TG). (b) Shows [1-¹⁴C]palmitate content of diacylglycerols (DG). Whereas the incorporation of [1-¹⁴C]palmitate in triacylglycerols (a) refers to the rate of diacylglycerols. Glycerol 3-phosphate precursors were: ○ and ●, none; △ and ▲, lactate plus pyruvate (2 mM; ratio 10:1); ▽ and ▼, glucose (10 mM); □ and ■, dihydroxyacetone (2 mM); ◇ and ◆, glycerol (1 mM). Open symbols are results in the absence of glucagon, closed symbols are results in the presence of 0.1 μM -glucagon. Results are means $\pm$ S.E.M. (indicated by the bars) for at least four experiments. Glucagon significantly ($P < \text{or} \leq 0.05$) lowered glycerol 3-phosphate contents together with palmitate incorporation in tri- and di-acylglycerols when glucose and dihydroxyacetone served as the precursors and, in the case of 1 mM-palmitate, also in the absence of precursor. The other differences were not statistically significant.

incubated with [$1\text{-}^{14}\text{C}$]palmitate, we observed that glucagon decreased the incorporation of radioactivity in triacylglycerols in only approximately half of the cell preparations. When glycerol 3-phosphate contents were measured it became apparent that glucagon suppressed triacylglycerol synthesis only in those cells in which it lowered glycerol 3-phosphate below $0.3\text{--}0.4\text{ }\mu\text{mol}/10^8$ cells. This seemed possible only in cell preparations in which glycerol 3-phosphate in the absence of glucagon was not too elevated. These observations strongly suggested that glucagon decreased triacylglycerol synthesis mainly through its effects on glycerol 3-phosphate contents. Freshly isolated hepatocytes were therefore pre-incubated for 90 min with intermittent washings to deplete their glycogen and lower their glycerol 3-phosphate content. After pre-incubation, glycerol 3-phosphate fell to $0.1\text{--}0.2\text{ }\mu\text{mol}/10^8$ cells. The cells were subsequently incubated with radioactive palmitate in the presence of various precursors of glycerol 3-phosphate (Fig. 1). (+)-Octanoylcarnitine, an inhibitor of carnitine palmitoyltransferase (McGarry & Foster, 1974), was also added to the incubations. By suppressing fatty acid oxidation

the inhibitor prevented the stimulation of fatty acid oxidation by glucagon (results not shown), thereby ruling out the possibility that a decrease in esterification produced by glucagon would be the result of its stimulatory effect on oxidation. In agreement with previous experiments (Debeer *et al.*, 1981), the Figure shows that rates of triacylglycerol synthesis steeply increased with increasing glycerol 3-phosphate content of the cells to reach a plateau at $0.3\text{--}0.4\text{ }\mu\text{mol}/10^8$ cells. A similar hyperbolic-like relationship held for glycerol 3-phosphate content and the amount of radioactivity present in diacylglycerols. The meaning of the intercept with the abscissa, which is obtained by extrapolation of the curves, has been discussed previously (Debeer *et al.*, 1981). With glucose or dihydroxyacetone as glycerol 3-phosphate precursors, glucagon significantly decreased glycerol 3-phosphate content together with the incorporation of palmitate in di- and triacylglycerols. However, in the presence of lactate plus pyruvate or glycerol, glucagon did not lower glycerol 3-phosphate and no significant effects on the incorporation of palmitate were noted. In the absence of (+)-octanoylcarnitine (results not

Fig. 2. Cellular contents of dihydroxyacetone phosphate and glycerol 3-phosphate and incorporation of [$1\text{-}^{14}\text{C}$] palmitate in triacylglycerols

Isolated hepatocytes from fed rats were pre-incubated for 90 min to lower their glycerol 3-phosphate content and then incubated with various precursors of glycerol 3-phosphate in the presence of 1 mM -[$1\text{-}^{14}\text{C}$]palmitate and 2 mM -(+)-octanoylcarnitine. (a) Shows dihydroxyacetone phosphate versus glycerol 3-phosphate content. If the values obtained with glycerol are omitted, a linear correlation between both phosphate esters is obtained ($P < 0.001$). (b) Shows incorporation of [$1\text{-}^{14}\text{C}$]palmitate in triacylglycerols (TG) versus cellular dihydroxyacetone phosphate content. Symbols are defined in the legend to Fig. 1, except: $\triangleleft$ and $\blacktriangleleft$, dihydroxyacetone (5 mM). Results are means $\pm$ s.e.m. (indicated by the bars) for experiments. Glucagon significantly ($P < \text{or} \ll 0.05$) lowered glycerol 3-phosphate content and palmitate incorporation in triacylglycerols when glucose or 2 mM -dihydroxyacetone served as precursors. With 5 mM -dihydroxyacetone phosphate there was a significant decrease in glycerol 3-phosphate, but not in triacylglycerol synthesis, since at both glycerol 3-phosphate values the plateau was reached. The other differences, including all changes in dihydroxyacetone phosphate, were not statistically significant.

shown), qualitatively similar results were obtained despite the variable effects on fatty acid oxidation seen when glucagon was added in combination with the various glycerol 3-phosphate precursors.

Acylation of dihydroxyacetone phosphate and subsequent reduction of acyldihydroxyacetone phosphate constitutes an alternate pathway for the synthesis of phosphatidate. The relative contribution to esterification of the glycerol 3-phosphate and dihydroxyacetone phosphate pathways remains controversial (see Hajra, 1977; Bell, 1980). In a separate series of experiments we therefore measured the cellular contents of glycerol 3-phosphate and dihydroxyacetone phosphate as well as incorporation of radioactive palmitate in triacylglycerols. The results of these experiments, also performed in the presence of (+)-octanoylcarnitine, are shown in Fig. 2. With all precursors except glycerol, dihydroxyacetone phosphate content of the cells was linearly related to their glycerol 3-phosphate content (Fig. 2a). The glycerol 3-phosphate/dihydroxyacetone phosphate ratio was approx. 3 (range 2.5–3.6). For glycerol this ratio was 6–9-fold greater. As a consequence, and except for glycerol, there was also a hyperbolic-like relationship between dihydroxyacetone phosphate content and rates of palmitate incorporation in triacylglycerols (Fig. 2b). As in the experiments of Fig. 1, glucagon significantly decreased glycerol 3-phosphate content and incorporation of radioactivity in triacylglycerols when glucose and 2 mM dihydroxyacetone served as precursors. Under these conditions there was also a decrease in dihydroxyacetone phosphate content, but differences were not statistically significant. Qualitatively similar results were obtained in the absence of (+)-octanoylcarnitine (results not shown). In this case, glycerol 3-phosphate/dihydroxyacetone phosphate ratios were in the range 3.2–5.0, and reached approx. 80 in the presence of glycerol, mainly as a result of higher glycerol 3-phosphate contents.

The concomitant changes in glycerol 3-phosphate levels and rates of triacylglycerol synthesis caused by glucagon clearly show that the hormone's effect on esterification is mediated by alterations in the intracellular concentration of the glycerol phosphate. An additional effect through parallel changes in the concentration of dihydroxyacetone phosphate cannot be entirely excluded. Importantly, Fig. 1 further reveals that all experimental points, whether obtained in the absence or presence of glucagon, belong to the same curve. This indicates that the kinetic parameters of the esterification pathway were not altered by glucagon. Therefore, the changes in enzymic activities described after treatment of

isolated liver preparations with glucagon or dibutyryl cyclic AMP cannot be of major significance for the operation of the overall pathway in the intact hepatocyte.

The present experiments lead us to conclude that in isolated liver preparations glucagon inhibits triacylglycerol synthesis by limiting the availability of substrates, glycerol 3-phosphate (and perhaps dihydroxyacetone phosphate) by its effects on the glycolytic/gluconeogenic pathway and acyl-CoA by its stimulatory effect on mitochondrial fatty acid oxidation.

We thank Mr. L. Govaert for expert technical assistance. This work was supported by Grant OT/VII/29 from the 'Onderzoeksfonds K. U. Leuven' and Grant 3.0031-79 from the 'Fonds voor Geneeskundig Wetenschappelijk Onderzoek'. P. E. D. is 'Aspirant van het Nationaal Fonds voor Wetenschappelijk Onderzoek'.

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