The effects of glucocorticoids on insulin-stimulated lipogenesis in primary cultures of rat hepatocytes

John M. AMATRUDA,* Susan A. DANAHY and Cecilia L. CHANG

Department of Medicine, Division of Endocrinology and Metabolism, University of Rochester School of Medicine, 601 Elmwood Avenue, Rochester, NY 14642, U.S.A.

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We used primary cultures of rat hepatocytes to evaluate the effects of glucocorticoids on insulin-responsive hepatic lipogenesis. The data indicate that hepatocytes incubated for 20 h with dexamethasone ($0.1 \mu\text{M}$) alone are profoundly resistant to the ability of insulin to stimulate lipogenesis acutely. In contrast, primary cultures of hepatocytes incubated with dexamethasone plus insulin are hyper-responsive to the ability of insulin to stimulate lipogenesis chronically. This potentiation of insulin action by a glucocorticoid occurs at physiological concentrations of the two hormones. Exposure to dexamethasone plus insulin for more than 4 h is required for the two hormones to enhance insulin action either by overcoming the insulin resistance induced by dexamethasone alone or by stimulating insulin action induced by insulin alone. Despite the marked potentiation of insulin action, hepatocytes exposed to dexamethasone plus insulin are less sensitive to insulin, as demonstrated by a shift to the right in the dose–response curve for insulin-stimulated lipogenesis. The resistance of hepatocytes to the acute effects of insulin after exposure to dexamethasone alone and the potentiation of insulin action and decreased sensitivity to insulin after exposure to insulin plus dexamethasone are all mediated by post-insulin-binding events. These studies demonstrate potentiation of insulin action in the liver by physiological concentrations of glucocorticoids and may have physiological significance for the regulation of normal hepatic lipogenesis, for the hyperlipidaemia observed with the pharmacological use of glucocorticoids, and for disease states in man associated with hyperinsulinaemia and hypercortisolism.

Increased concentrations of glucocorticoids in humans and animals are associated with enhanced hepatic rates of lipogenesis and increased hepatic and plasma lipids (Baker *et al.*, 1948; Steinberg *et al.*, 1952; Soffer *et al.*, 1961; Stern *et al.*, 1973; Casaretto *et al.*, 1974; Reaven *et al.*, 1974; Diamant & Shafir, 1975; Bagdade *et al.*, 1976a,b; Kirk *et al.*, 1976). In addition, physiological concentrations of glucocorticoids in conjunction with insulin are required for the enhanced lipogenesis associated with refeeding after starvation (Berdanier & Shubeck, 1979; Bouillon & Berdanier, 1980). The relative roles of these two hormones in this hyperlipogenic response are unknown. It has been suggested that the increased insulin concentrations associated with glucocorticoid excess, or a synergism between

glucocorticoids and insulin, as in refeeding, may mediate the enhanced hepatic lipogenesis (Kirk *et al.*, 1976; Berdanier & Shubeck, 1979). If so, this would represent a situation of differential responsiveness to glucocorticoids, since glucocorticoids induce insulin resistance for some biological responses in the liver when administered *in vivo* or *in vitro* (Caro & Amatruda, 1982). Although the exact mechanism of this insulin resistance is also unknown, studies in our laboratory with freshly isolated hepatocytes from glucocorticoid-treated animals and primary cultures of hepatocytes exposed to dexamethasone ($0.1 \mu\text{M}$) indicate that the insulin resistance in the liver with regard to aminoisobutyrate uptake is due to post-binding mechanisms (Caro & Amatruda, 1982). The direct effects of glucocorticoids on the liver are to increase insulin binding and to induce resistance to the ability of

* To whom reprint requests should be addressed.

insulin both to stimulate amino acid uptake and to down-regulate its receptors (Caro & Amatruda, 1982).

To define further the effects of glucocorticoids on another metabolic process in the liver and to establish the nature of the interrelationships between insulin and glucocorticoids on this process, we have evaluated lipogenesis in primary cultures of hepatocytes exposed to dexamethasone and insulin, separately or together. Primary cultures of hepatocytes were used because they allow us to evaluate the effects of these hormones singly and combined without the complicating changes in substrate and hormonal milieu that occur *in vivo*.

The data indicate that dexamethasone alone leads to an insulin-resistant state with regard to lipogenesis much the same as we have previously demonstrated for amino acid uptake (Caro & Amatruda, 1982). The combination of dexamethasone plus insulin, however, leads to a marked enhancement of insulin-stimulated lipogenesis. Thus, under conditions where insulin is present, glucocorticoids are permissive for stimulation of lipogenesis by insulin, whereas under conditions of insulin lack glucocorticoids induce or aggravate insulin resistance.

Experimental

Male Sprague-Dawley rats (200–300g) fed *ad libitum* were used for all experiments. The isolation of liver cells and preparation of primary cultures of rat hepatocytes on collagen-coated plates in serum-free medium was as previously described (Caro & Amatruda, 1980a,b; Cech *et al.*, 1980; Amatruda *et al.*, 1982). Any additions to the culture medium were added at the time of the first change of medium, 3–4 h after plating. Insulin was added again 16 h later and 4 h after this the cells were washed at 37°C with Hanks-Hepes [4-(2-hydroxymethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.4, containing 8 mM-glucose and albumin (10 mg/ml).

To measure lipid synthesis, [^{14}C]acetate (5 mM; 2.2 $\mu\text{Ci/ml}$) was added to the culture plates containing the above Hanks-Hepes buffer and the incubation was continued at 37°C for 2 h. The plates were then washed in cold phosphate-buffered saline, pH 7.4, and the cellular lipids were extracted by the method of Bligh & Dyer (1959) as previously reported (Amatruda *et al.*, 1982). In some experiments lipid synthesis was measured by the incorporation of $^3\text{H}_2\text{O}$ (0.6 mCi/ml) plus 5 mM unlabelled acetate (Amatruda *et al.*, 1982). Binding of ^{125}I -labelled insulin was measured as previously reported (Caro & Amatruda, 1980a,b; Cech *et al.*, 1980; Amatruda *et al.*, 1982) and expressed as specific binding. Dexamethasone was dissolved in water, filtered on Whatman no. 1 filter paper, and the

concentration was measured spectrophotometrically at 250 nm. A stock solution of 10 μM was prepared and diluted in culture medium.

$^3\text{H}_2\text{O}$ (1 Ci/g) and [^{14}C]acetic acid, sodium salt (1.9 Ci/mol), were purchased from New England Nuclear, Boston, MA, U.S.A.; crude collagenase (CLS II; batch no. 40-162P; 138 units/mg) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; dexamethasone and all amino acids were from Sigma Chemical Co., St. Louis, MO, U.S.A.; and serum-free medium (Waymouth's MB 752/1) and bovine albumin (fraction V) were from Grand Island Biological Co., Grand Island, NY, U.S.A. Crystalline pig insulin was kindly provided by Dr. Ronald Change of Eli Lilly and Co., Indianapolis, IN, U.S.A.

Results

Previous studies in our laboratory demonstrated that the exposure of primary cultures of hepatocytes to glucocorticoids leads to insulin resistance with regard to amino acid uptake (Caro & Amatruda, 1982). The present studies evaluating lipogenesis resulted in similar findings. The acute (2 h) lipogenic response to insulin of primary cultures of hepatocytes from normal rats was evaluated after a 16 h exposure to dexamethasone (0.1 μM). These data are illustrated in Fig. 1 and demonstrate a profound

Fig. 1. Acute lipogenic response to insulin of primary cultures of rat hepatocytes incubated for 20 h with no additions (○) or in the presence of dexamethasone (●).

For full details see the Experimental section. The data represent means $\pm$ S.E.M. for four separate experiments. Basal lipogenesis measured in the absence of insulin for 2 h is 12.3 ± 1.9 and 13.7 ± 3.5 nmol of [^{14}C]acetate incorporated into lipids/mg of cellular protein in control and dexamethasone-treated cultures respectively.

state of insulin resistance after the exposure of hepatocytes to dexamethasone as compared with control cultures. This decrease in responsiveness is significant at all insulin concentrations ($P < 0.05$ to $P < 0.025$). Hepatocytes exposed to dexamethasone have no detectable lipogenic response to insulin at hormone concentrations of 0.1 and 1 nM. At higher concentrations (10 and 100 nM) the insulin response of cultures treated with dexamethasone is only 8% and 4% that of control cultures respectively (Fig. 1). To validate these findings and to determine if alterations in substrate pool sizes could be affecting our interpretation of the insulin responsiveness of dexamethasone-treated cells, we evaluated the acute lipogenic response to insulin, using $^3\text{H}_2\text{O}$ and unlabelled sodium acetate (5 mM). In two experiments the acute lipogenic response to insulin (10 nM) in cultures exposed for 20 h to dexamethasone (0.1 μM) was only 35% of that of control cultures. These data indicate that resistance to the acute effects of insulin are present when utilizing both $^3\text{H}_2\text{O}$ and $[1\text{-}^{14}\text{C}]\text{acetate}$ as substrates, but that under these conditions dexamethasone may also be having an effect on the pool size of lipogenic precursors.

Since the lipogenic response to insulin is so low in dexamethasone-treated cells (Fig. 1), a concentration of insulin eliciting a half-maximal response (ED_{50}) cannot be accurately determined. However, in the control cultures the ED_{50} for insulin is 0.4 nM, which is similar to that previously reported from our laboratory (Caro & Amatruda, 1980a,b; Cech *et al.*, 1980; Amatruda *et al.*, 1982). Under the conditions of the present studies, insulin binding is increased, as we have previously reported (results not shown). (Caro & Amatruda, 1982), indicating that the dexamethasone-induced insulin resistance is due to post-binding mechanisms.

In contrast with the acute response to insulin, when primary cultures of hepatocytes are incubated in the presence of dexamethasone (0.1 μM) plus various concentrations of insulin for 20 h lipogenesis is markedly enhanced (Fig. 2). At 10 nM-insulin the enhancement of lipogenesis by dexamethasone plus insulin is 3.6-fold (4.9-fold at 100 nM-insulin) greater than that observed with insulin alone (Fig. 2). This enhancement of lipogenesis is significant at all insulin concentrations ($P < 0.05$ to $P < 0.0125$ by paired *t* test). These data were also confirmed with $^3\text{H}_2\text{O}$. At 10 nM-insulin plus 0.1 μM -dexamethasone, insulin-stimulated lipogenesis was enhanced 4.6-fold over that observed with insulin alone ($P < 0.005$). Although this enhancement of lipogenesis occurs at physiological concentrations of insulin, dexamethasone also has a significant effect on insulin sensitivity. The ED_{50} concentrations of insulin are 0.66 and 2.1 nM in the control and dexamethasone-treated cells respec-

Fig. 2. Insulin-stimulated lipogenesis in primary cultures of hepatocytes exposed to various concentrations of insulin alone (●) or plus 0.1 μM -dexamethasone (○) for 20 h

Lipogenesis from $[1\text{-}^{14}\text{C}]\text{acetate}$ is measured over 2 h as described in the Experimental section. The data represent means $\pm$ S.E.M. for six separate experiments. Basal lipogenesis (in 2 h) is 18.3 ± 3.1 nmol/mg of protein in the absence of any additions and 14.2 ± 1.6 nmol/mg of protein in the presence of 0.1 μM -dexamethasone alone.

tively ($P < 0.025$ by paired *t* test). This significant decrease in insulin sensitivity (Fig. 2) in the presence of an increase in insulin binding in previously published studies (Caro & Amatruda, 1982) again indicates that post-binding mechanisms can regulate both the sensitivity and responsiveness to insulin (Amatruda *et al.*, 1982). In addition, the present data indicate that these two parameters can be regulated in opposite directions, i.e. cells treated with dexamethasone plus insulin have a decrease in sensitivity and an increase in responsiveness to insulin.

To determine the dose-response relationship for the enhancement of lipogenesis by dexamethasone, we incubated primary cultures of hepatocytes for 20 h in the presence of various concentrations of dexamethasone (1 nM–0.1 μM) and a fixed concen-

tration of insulin (10 nM). Lipogenesis was then evaluated. A significant glucocorticoid effect was observed at 1 nM-dexamethasone (Fig. 3). At this concentration, dexamethasone in the presence of insulin led to a 25% enhancement of lipogenesis compared with insulin alone ($P < 0.05$ by paired t

Fig. 3. Lipogenesis in primary cultures of hepatocytes after a 20 h exposure to various concentrations of dexamethasone alone (●) or plus 10 nM-insulin (○). Lipogenesis is measured from [$1\text{-}^{14}\text{C}$]acetate as described in the Experimental section. The data represent means $\pm$ S.E.M. for three separate experiments.

test). The enhancement by dexamethasone of insulin-stimulated lipogenesis was 109% ($P < 0.005$) and 143% ($P < 0.001$) at 10 nM- and 0.1 μM -dexamethasone respectively, with an ED_{50} of 5.5 nM-dexamethasone. In these experiments, dexamethasone had no effect on basal lipogenesis, i.e. in the absence of insulin. Assuming a glucocorticoid potency 37.5 times that of cortisol, a significant glucocorticoid effect is observed at a dexamethasone concentration equivalent to 1.5 μg of cortisol/100 ml (1 nM-dexamethasone), a half-maximal effect at a dexamethasone concentration equivalent to 8.25 μg of cortisol/100 ml and a maximal increase in effect at a dexamethasone concentration equivalent to 15 μg of cortisol/100 ml, well within the physiological range for basal plasma cortisol concentrations in non-stressed man.

We next evaluated the time course of the dexamethasone effect on lipogenesis. Primary cultures of hepatocytes were incubated with insulin alone for 0, 2, 4, 12 and 20 h and lipogenesis from [$1\text{-}^{14}\text{C}$]acetate was evaluated over a 2 h period. As illustrated in Fig. 4, insulin action is maximal by 2 h of preincubation ($P < 0.1$ for 2 versus 4 h). With more prolonged preincubation with insulin, no further increase in insulin's effect was observed. In additional experiments, primary cultures of hepatocytes were incubated with dexamethasone for 20 h and insulin was added for the full 20 h incubation or for the final 12, 4, 2 or 0 h. Lipogenesis was then evaluated from [$1\text{-}^{14}\text{C}$]acetate. As illustrated in Fig. 4, as compared with insulin alone, cultures incubated with dexamethasone for 20 h were resistant to insulin's effect on lipogenesis for 2 and 4 h ($P < 0.01$). By 12 h insulin is able to overcome this resistance, and insulin-stimulated lipogenesis is enhanced 2.2-fold in the presence of dexamethasone for 20 h and insulin for 12 h. When both insulin and dexamethasone are included for 20 h there is a 3.4-fold enhancement of insulin action. Thus dexamethasone-induced insulin resistance is present only within 4 h of insulin exposure. After this time, the two hormones act together to enhance insulin responsiveness. In primary cultures of hepatocytes incubated with insulin for 20 h, dexamethasone has no effect on insulin-stimulated lipogenesis for the first 4 h of exposure to dexamethasone, i.e. cultures are incubated with 10 nM-insulin for 20 h and 0.1 μM -dexamethasone is added at 16 h and 18 h of incubation or at 4 and 2 h before measurement of [^{14}C]acetate incorporation into lipids (Fig. 4). When dexamethasone is added for the final 12 h and the entire 20 h of culture, the data are identical with those for the cultures in which insulin is added after dexamethasone. Thus exposure to insulin plus dexamethasone must be for greater than 4 h for the two hormones to enhance insulin action either by overcoming the insulin resistance induced by dexa-

Fig. 4. Time course of the effect of insulin and dexamethasone plus insulin on lipogenesis

Primary cultures of hepatocytes were incubated with insulin (10nM) alone for 0, 2, 4, 12 or 20h and lipogenesis was measured over 2h (●), or with dexamethasone (0.1 μ M) for 20h and insulin (10nM) for the last 0, 2, 4, 12 or 20h of the dexamethasone incubation (○), or with insulin (10nM) for 20h and dexamethasone (0.1 μ M) for the last 0, 2, 4, 12 or 20h of the insulin incubation (□). The data represent means of three experiments. Basal lipogenesis in the absence of any additions is 15.7 ± 2.3 , 16.4 ± 2.8 and 16.0 ± 1.1 nmol/2h per mg of protein in the three groups of experiments respectively. Panel (a) represents the increase in lipid synthesis above basal owing to insulin and dexamethasone plus insulin. Panel (b) represents the same data expressed as a percentage of the maximal effect observed in panel (a).

methasone alone or by stimulating insulin action induced by insulin alone. Fig. 4(b) illustrates these time-course data as a percentage of the maximal effect observed; clearly the maximal effect of insulin alone is reached by 2–4 h, whereas in the presence of dexamethasone the time of appearance of the maximal insulin effect is markedly retarded.

Discussion

The role of glucocorticoids in the regulation of hepatic lipogenic enzymes and lipogenesis has been studied in intact animals and in the perfused rat liver (Altman *et al.*, 1951; Diamant & Shafir, 1975; Kirk *et al.*, 1976). This effect of glucocorticoids was first demonstrated by Altman *et al.* (1951), who showed that pharmacological amounts of insulin and glucocorticoids act synergistically to enhance lipogenesis in the perfused liver. Subsequent studies indicated that the administration of glucocorticoids to rats increases the activity of hepatic acetyl-CoA carboxylase and fatty acid synthetase as well as lipogenesis from [14 C]acetyl-CoA (Diamant & Shafir, 1975). These effects were not present in alloxan-diabetic rats, suggesting that insulin is a necessary mediator of the glucocorticoid effect. Additional studies in starved-refed rats indicate that the increase in glucose 6-phosphate dehydrogenase and malic enzyme activities which is present in normal rats is not demonstrable in adrenalectomized, streptozotocin-diabetic and adrenalectomized-diabetic rats (Berdanier & Shubeck, 1979). Treatment with glucocorticoids, insulin, and both hormones in the respective groups restores the hepatic-enzyme overshoot associated with refeeding (Berdanier & Shubeck, 1979). From those studies it has been suggested that insulin may be responsible for the regulation of substrate flux into lipogenic pathways, and glucocorticoids and insulin may be responsible for enzyme protein synthesis *de novo* (Berdanier & Shubeck, 1979). In addition, in perfused livers from adrenalectomized rats, lipogenesis from $^3\text{H}_2\text{O}$ and ^{14}C -labelled precursors is decreased (Kirk *et al.*, 1976). Cortisol addition to the perfusion has no stimulatory effect on lipogenesis, whereas fatty acid synthesis is restored to normal in perfused livers from adrenalectomized rats within 5 h after treatment of the animal with cortisol *in vivo*. These studies suggested that both insulin and glucocorticoids are necessary for normal hepatic lipogenesis. Glucocorticoid excess in both animals and man leads to hepatic lipid accumulation (Baker *et al.*, 1948; Steinberg *et al.*, 1952; Soffer *et al.*, 1961; Reaven *et al.*, 1974) and increased synthesis and secretion of very-low-density lipoproteins (Stern *et al.*, 1973; Casaretto *et al.*, 1974; Bagdade *et al.*, 1976a,b) and in some cases low-density lipoproteins (Bagdade *et al.*, 1976a). Taken together, the above

studies suggest that glucocorticoids may be necessary for maximum expression of insulin-stimulated lipogenesis in the liver and indicate that glucocorticoid excess is associated with increased hepatic lipogenesis.

Our studies confirm and extend these observations. First, as we have previously shown for aminoisobutyrate uptake (Caro & Amatruda, 1982), glucocorticoids in the absence of insulin lead to a profound resistance to the lipogenic effects of subsequently added insulin (Fig. 1). This resistance is due to post-binding mechanisms, since glucocorticoids alone increase insulin binding (Caro & Amatruda, 1982). Thus glucocorticoid-induced insulin resistance can now be generalized to at least two biological responses in the liver. Secondly, the glucocorticoid-induced insulin resistance is reversed after 4 h exposure to insulin in the presence of glucocorticoids (Fig. 4). After this the two hormones act together to potentiate insulin's stimulation of lipogenesis. This potentiation is also a post-binding enhancement of insulin action, since insulin binding in the presence of insulin plus dexamethasone is the same as that in the presence of dexamethasone alone (Caro & Amatruda, 1982). Thirdly, the potentiation of insulin action by dexamethasone occurs at glucocorticoid concentrations equivalent to physiological concentrations of cortisol in man (Fig. 3). The onset of the effect is at an equivalent cortisol concentration of $1.5 \mu\text{g}/100 \text{ ml}$ and the maximal rate of increase at a cortisol equivalent of $1.5\text{--}15 \mu\text{g}/100 \text{ ml}$. This suggests that the effect may be physiologically meaningful. Fourthly, the glucocorticoid effect is observed at physiological concentrations of insulin with an insulin dose-response relationship which is similar to that for insulin alone (Fig. 2). There is, however, a significant rightward shift in the dose-response curve for insulin-stimulated lipogenesis in the presence of dexamethasone (Fig. 2). In conjunction with our previous data, indicating that insulin binding is equally increased in the presence of dexamethasone and dexamethasone plus insulin (Caro & Amatruda, 1982), these data again suggest that alterations in insulin sensitivity in addition to insulin responsiveness can be mediated by post-binding mechanisms (Amatruda *et al.*, 1982).

Finally, our data may have physiological significance for the regulation of normal hepatic lipogenesis and for disease states in man. Since the enhancement of insulin-stimulated lipogenesis is observed at concentrations of glucocorticoids equivalent to physiological values in man, it is likely that the two hormones act together to regulate normal hepatic lipogenesis and perhaps the hyperlipidaemia associated with increased plasma insulin (Olefsky *et al.*, 1974). Also, the hepatic steatosis and hyperlipidaemia associated with exogenous or endogenous glucocorticoid excess (Baker *et al.*, 1948;

Steinberg *et al.*, 1952; Soffer *et al.*, 1961; Stern *et al.*, 1973; Casaretto *et al.*, 1974; Reaven *et al.*, 1974; Bagdade *et al.*, 1976a,b) can now be explained by an enhancement of insulin-stimulated lipogenesis by glucocorticoids. Since glucocorticoid excess results in increased plasma insulin, the two hormones together would act additively in the liver to enhance lipogenesis. In fact, increased synthesis and secretion of very-low-density lipoproteins have been shown in states of glucocorticoid excess (Bagdade *et al.*, 1976a,b; Stern *et al.*, 1973). This, in conjunction with increased lipoprotein lipase activity (Stern *et al.*, 1973; Pykalisto *et al.*, 1975), might increase adipose-tissue fat stores, leading to enlargement of fat-cells, and subsequent insulin resistance of adipocytes (Czech *et al.*, 1977). This indirect induction of insulin resistance in adipocytes might be additive to other effects of glucocorticoids in these cells to decrease the activity of lipogenic enzymes (Diamant & Shafir, 1975) and glucose transport (Livingston & Lockwood, 1975). Thus glucocorticoid potentiation of insulin action in the liver in conjunction with increased insulin concentrations may explain many of the observed metabolic alterations associated with hypercortisolism.

Further studies are required to elucidate the mechanism(s) by which glucocorticoids enhance insulin-stimulated hepatic lipogenesis. It is possible, however, that glucocorticoids modulate the synthesis of hepatic lipogenic enzymes and insulin modulates the synthesis and activity of these enzymes.

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