Altered regulation of glycogen metabolism by vasopressin and phenylephrine in hepatocytes from insulin-resistant obese (*fa/fa*) rats

Role of protein kinase C

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The hormonal control of glycogen synthase and phosphorylase interconversion was investigated in hepatocytes isolated from lean and genetically obese (*fa/fa*) rats. In cells from obese animals, the inactivation of synthase by 4 β -phorbol 12 β -myristate 13 α -acetate (PMA), phospholipase C, vasopressin and the α_1 -adrenergic agonist phenylephrine was markedly impaired, and the property of PMA to counteract phosphorylase activation by phenylephrine was attenuated. The maximal response of phosphorylase activation to phenylephrine and vasopressin was increased in obese-rat hepatocytes, but the sensitivity to these hormones was similar to that in lean-rat hepatocytes. These observations indicate that the defect in protein kinase C that we reported previously in heart of insulin-resistant *fa/fa* rats [van de Werve, Zaninetti, Lang, Vallotton & Jeanrenaud (1987) *Diabetes* 36, 310–319] is probably also expressed in liver.

INTRODUCTION

Insulin resistance is expressed in the mature genetically obese (*fa/fa*) rat at the level of several tissues, including muscle, adipose tissue and liver (reviewed in Jeanrenaud *et al.*, 1985). We previously observed that protein kinase C was deficient in heart from these obese animals and that phorbol ester administration *in vivo* aimed at down-regulating this kinase was associated with insulin resistance (van de Werve *et al.*, 1987).

The present work explores the possibility that the hormonal regulation of glycogen metabolism by protein kinase C is impaired in hepatocytes from *fa/fa* rats. We found marked alterations in synthase inactivation and phosphorylase activation by hormones and agents known to activate protein kinase C. Our results suggest that a defect in protein kinase C activation might be present in insulin-resistant liver, similarly to that observed in heart (van de Werve *et al.*, 1987).

EXPERIMENTAL

Handling of animals and sampling of livers

Well-fed lean (*FA/fa?*) and genetically obese (*fa/fa*) female rats were purchased from Charles River (Canada). At the time of the experiments they were 10 weeks old. In some experiments the animals were deprived of food overnight (18:00–10:00 h), but had free access to water. Each group of animals was anaesthetized with pentobarbital (0.1 mg/g body wt., intraperitoneally) and the liver was sampled as described by van de Werve & Jeanrenaud (1987).

Preparation and incubation of hepatocytes

Hepatocytes were prepared (van de Werve, 1980) from both groups of animals (fed *ad lib.* or fasted), and were incubated at 37 °C in Krebs–Henseleit medium as indicated. At different incubation times, samples (100 μ l) of cell suspension (50 mg/ml) were frozen in liquid N₂ and stored at –65 °C pending further determinations.

Enzymes

Phospholipase C (*Clostridium perfringens*, type X) was purchased from Sigma. Glycogen synthase and phosphorylase activities were assayed at 30 °C in homogenates of thawed cell suspensions as described by Hue *et al.* (1975).

RESULTS

Inactivation of glycogen synthase by PMA and phospholipase C

Phorbol esters are structural analogues of diacylglycerol, the presumed physiological activator of protein kinase C, and are widely used to activate protein kinase C directly in intact cells. In fasted-lean-rat hepatocytes, the phorbol ester PMA provoked a dose-dependent inactivation of glycogen synthase, which was markedly impaired in cells from obese rats (Fig. 1, left panel). In hepatocytes from fed lean rats, PMA still inactivated synthase significantly, but to a lesser extent than in the fasted condition, and was without effect in the obese group (Fig. 1, middle panel). Similar results (Fig. 1, right panel) were obtained with exogenous phospholipase C, which increases cell diacylglycerol and presumably protein kinase C (Blackmore *et al.*, 1986).

These results thus suggest that protein kinase C activation might be deficient in hepatocytes from obese rats. The high basal levels of synthase *a* in hepatocytes from obese and lean rats (see 100 % values in legends to Figs. 1 and 2) that we observed previously (van de Werve *et al.*, 1987; van de Werve, 1990) could be indicative of decreased basal protein kinase C as well. They could not be attributed to changes in total (*a* + *b*) synthase activity, which was not significantly different in both groups in the same nutritional condition (van de Werve, 1990).

Inactivation of glycogen synthase by vasopressin and α_1 -adrenergic (phenylephrine) stimulation

Since inactivation of glycogen synthase by direct activation of protein kinase C by PMA and exogenous phospholipase C

Fig. 1. Effect of PMA and of phospholipase C on glycogen synthase α levels in hepatocytes from fed and overnight-fasted lean (Δ) and obese ($\blacktriangle$) rats

Hepatocytes were preincubated for 30 min with 50 mM-glucose to activate glycogen synthase. The cells were then further incubated for 10 min with or without (control) the indicated concentrations of PMA or phospholipase C (50 m-units in 1 ml). Values, expressed as a percentage of the control (100%), are means for five different hepatocyte preparations; * values differed by $P < 0.01$, by Student's t test for paired samples (control versus PMA or phospholipase C) and were considered significant. All values (lean versus obese) except at [PMA] below 50 ng/ml (fasted) and 100 ng/ml (fed) differed by $P < 0.05$ (unpaired t test). Absolute (100%) values of synthase α (m-units/g of liver at 30 °C) were 300 ± 28 and 960 ± 76 in hepatocytes from fasted lean and obese rats and 272 ± 28 and 368 ± 18 in those from fed lean and obese rats.

Fig. 2. Concentration-dependence of glycogen synthase inactivation in response to vasopressin and phenylephrine in hepatocytes from overnight-fasted lean and obese rats

Same legend as in Fig. 1, except that vasopressin or phenylephrine was added to the cells and that means are for six different hepatocyte preparations in each group. *Values differed significantly by $P < 0.025$, by using Student's t test (paired samples, control versus vasopressin and phenylephrine). All values (lean versus obese) at [vasopressin] above 10^{-10} M and at [phenylephrine] above 10^{-8} M differed by $P < 0.05$ (unpaired t test). Absolute (100%) values of synthase α (m-units/g of liver at 30 °C) were, in lean- and obese-rat hepatocytes respectively, 380 ± 28 and 960 ± 76 for the left panel and 380 ± 47 and 825 ± 162 for the right panel.

seemed impaired in hepatocytes from obese animals, it was decided to study the effect of hormones acting via endogenous phospholipase C. Such activation of protein kinase C by Ca^{2+} -mobilizing hormones has indeed been demonstrated previously (Hernández-Sotomayor & García-Sáinz, 1988). Fig. 2 shows the

dose-dependence of synthase inactivation by vasopressin and by the α_1 -adrenergic agonist phenylephrine in fasted-rat hepatocytes: the sensitivity of synthase inactivation to vasopressin (left panel) and to phenylephrine (right panel) was significantly decreased in liver cells from *fa/fa* rats.

Fig. 3. Concentration-dependence of phosphorylase activation in response to vasopressin in hepatocytes from fed and overnight-fasted lean (○) and obese (●) rats

Hepatocytes were preincubated for 30 min with 10 mM-glucose to inactivate phosphorylase. The cells were then further incubated for 5 min with increasing concentrations of vasopressin, and samples were taken for phosphorylase measurements. Values are means $\pm$ S.E.M. for six different cell preparations in each group. Values were not statistically different (lean versus obese).

Activation of phosphorylase by vasopressin and α_1 -adrenergic (phenylephrine) stimulation

Genetically obese rodents have been reported to lack (*ob/ob* mice; Assimacopoulos-Jeannet *et al.*, 1983) or to have decreased (*db/db* mice and *fa/fa* rats; Cantau *et al.*, 1984) liver V_1 vasopressin receptors. If impaired synthase inactivation by vasopressin (Fig. 2) could be attributed to a receptor defect, we should expect another parameter affected by vasopressin (phosphorylase activation) to be deficient as well. This was not the case, since phosphorylase activation by vasopressin was enhanced in hepatocytes from fed as well as from fasted obese rats (Fig. 3). Similarly, the activation of phosphorylase in response to phenylephrine was also increased in hepatocytes from fasted obese compared with lean rats (Fig. 4). The K_a values of phosphorylase activation by both hormones were, however, not different between lean and obese rats. Total phosphorylase ($a+b$) was not different between the two groups of animals (van de Werve, 1990).

Counteraction by PMA of the activation of phosphorylase by α_1 -adrenergic (phenylephrine) stimulation

PMA is known to antagonize α_1 -adrenergic activation of glycogenolysis (Corvera & García-Sáinz, 1984) and phosphorylase (Cooper *et al.*, 1985; Lynch *et al.*, 1985; van de Werve *et al.*, 1985). Accordingly, preincubation of fasted-lean-rat hepatocytes for 5 min with an optimal concentration (1 μ g/ml) of PMA completely blocked phosphorylase activation by phenylephrine (Fig. 4, left panel). In contrast, the anti-phenylephrine action of the phorbol ester was attenuated in hepatocytes from obese animals (Fig. 4, right panel). Fig. 5 shows the dose-dependency

Fig. 4. Concentration-dependence of phosphorylase activation in response to phenylephrine and counteraction by PMA in hepatocytes from overnight-fasted lean and obese rats

Hepatocytes from lean (left panel) and obese (right panel) rats were preincubated for 30 min with 10 mM-glucose to inactivate phosphorylase and further incubated for 5 min in the presence (●) or absence (○) of 1 μ g of PMA/ml. The cells were then incubated for another 5 min after addition of increasing concentrations of phenylephrine, at which time samples were taken for phosphorylase measurements. Values are means $\pm$ S.E.M. for four different cell preparations in each group: *statistical significance for values (control or PMA versus phenylephrine, paired *t* test) differing at least by $P < 0.05$.

Fig. 5. Concentration-dependence of counteraction by PMA of phosphorylase activation in response to phenylephrine in hepatocytes from overnight-fasted lean and obese rats

Hepatocytes from lean (○) and obese (●) rats were preincubated for 30 min with 10 mM-glucose to inactivate phosphorylase, and were further incubated for 5 min in the absence or presence of the indicated PMA concentrations. Then 3 μ M-phenylephrine was added and cells were sampled 3 min later for the determination of phosphorylase α .

of the effect: at 100 ng/ml, PMA completely suppressed α_1 -adrenergic activation (expressed as 100% in the absence of PMA) of phosphorylase in the lean group, whereas optimal inhibition did not exceed 40% in the obese group.

DISCUSSION

Impaired interconversion of glycogen synthase and phosphorylase in obese-rat hepatocytes by activators of protein kinase C

The original observation by Roach & Goldman (1983) that PMA inactivates glycogen synthase in fasted-rat hepatocytes was confirmed here. This effect was much less pronounced in hepatocytes from fed rats (Fig. 1, middle panel). The most likely explanation for this decreased response is that protein kinase C is inhibited by glycogen (Ahmad *et al.*, 1984). One could then argue that the loss of response to PMA in obese-rat hepatocytes could be due to the higher glycogen content of livers of fasted obese than lean rats (van de Werve, 1990). However, this possibility is unlikely, given that synthase was still inactivated by PMA, though to a lesser extent, in hepatocytes from fed lean rats (Fig. 1, middle panel), which contain at least 3 times more glycogen than do fasted-obese-rat hepatocytes.

Purified brain protein kinase C phosphorylates liver glycogen synthase (Ahmad *et al.*, 1984; Imazu *et al.*, 1984), but does not modify its activity (Imazu *et al.*, 1984), thus suggesting that in the intact cell some intermediary step might be necessary for PMA inactivation of synthase. We cannot exclude that this putative step could be affected in obese-rat hepatocytes.

Exogenous phospholipase C, at the low concentration used, increases hepatocyte 1,2-diacylglycerol, but not *myo*-inositol 1,4,5-trisphosphate (InsP_3) (Blackmore *et al.*, 1986), and is

thus more likely to activate protein kinase C than to release intracellular Ca^{2+} . The absence of synthase inactivation by phospholipase C in obese-rat hepatocytes is thus another strong indication for impaired protein kinase C.

Role of protein kinase C in the actions of vasopressin and α_1 -adrenergic hormones on phosphorylase and synthase

Vasopressin and the α_1 -adrenergic component of adrenaline increase InsP_3 (Thomas *et al.*, 1984; Seyfred & Wells, 1984; Charest *et al.*, 1985), which mobilizes Ca^{2+} from intracellular stores (Burgess *et al.*, 1984; Joseph *et al.*, 1984), and 1,2-diacylglycerol (Thomas *et al.*, 1983; Hughes *et al.*, 1984), which activates protein kinase C (Nishizuka, 1984). Vasopressin forms InsP_3 (and the accompanying diacylglycerol) in large excess over what is required for maximal Ca^{2+} mobilization (Thomas *et al.*, 1984), whereas α_1 -adrenergic agents induce smaller increases in InsP_3 (Williamson *et al.*, 1985; Charest *et al.*, 1985), even though both hormones increase Ca^{2+} at the same rate and to the same extent (Charest *et al.*, 1983, 1985; Williamson *et al.*, 1985). This means that only a small increase in InsP_3 is required to provoke a maximal Ca^{2+} response. Omission of extracellular Ca^{2+} prevents the effect of vasopressin on phosphorylase, but not on synthase (Bouscarel & Exton, 1986; Blackmore *et al.*, 1986), suggesting that Ca^{2+} is more important for phosphorylase activation than for synthase inactivation by vasopressin. Protein kinase C rather than Ca^{2+} might then be important for the inactivation of synthase by vasopressin. In support for our hypothesis of deficient protein kinase C, the inactivation of synthase was severely impaired (Fig. 2), whereas phosphorylase activation by vasopressin was barely affected in hepatocytes from obese rats (Fig. 3).

The results with phenylephrine suggest that protein kinase C activation is implicated in both phosphorylase activation and synthase inactivation, because these responses were altered in hepatocytes from fasted obese rats (Figs. 2 and 4). The enhancement of phenylephrine activation of phosphorylase in obese-rat hepatocytes can indeed be explained by a protein kinase C defect. Protein kinase C activation is expected to produce a negative feedback on α_1 -adrenergic activation of phosphorylase (Cooper *et al.*, 1985; Lynch *et al.*, 1985; van de Werve *et al.*, 1985; and Fig. 4 of the present work). Since the phenylephrine signal comprises protein kinase C activation as an intrinsic component, one might expect that in protein kinase C-deficient hepatocytes the effect of phenylephrine on phosphorylase would be amplified, which is the case. Moreover, prior stimulation of protein kinase C with PMA demonstrated a lesser inhibition of subsequent activation of phosphorylase by phenylephrine in obese-rat hepatocytes (Figs. 4 and 5). Other work also suggests that impaired protein kinase C enhances the effect of Ca^{2+} -mobilizing hormones because of the loss of the intrinsic negative feedback in the action of these hormones. A role of a negative feedback of protein kinase C on the α_1 -adrenergic stimulation of ureagenesis was demonstrated by García-Sáinz & Hernández-Sotomayor (1987), using inhibitors of protein kinase C to block the action of agents inducing α_1 -adrenergic refractoriness. In another study (Stassen *et al.*, 1989) an enhanced response to Ca^{2+} -mobilizing hormones was elicited in vascular smooth-muscle cells where protein kinase C had been down-regulated.

In conclusion, a possible alteration in protein kinase C-mediated processes in hepatocytes from *fa/fa* rats was searched for by using different approaches and was evidenced both by the inactivation of glycogen synthase and by the activation of phosphorylase. Further studies will question the hormonal activation of protein kinase C in hepatocytes from *fa/fa* rats and attempt to establish a relationship of a potential defect in this

enzyme with insulin resistance. An alteration of particular isoenzymes of protein kinase C is not excluded, because these forms seem to have different properties which might correspond to specific cellular functions (Nishizuka, 1988).

The technical assistance of C. Camenzind, F. Monsch and D. Savard-Trouvé is gratefully acknowledged. Preliminary experiments were performed at the Laboratoires de Recherches Métaboliques, University of Geneva, and were supported by Swiss National Science Foundation grant 3.822.086 and by a grant-in-aid from Nestlé S.A. (Vevey, Switzerland). This work was supported by grants DG 353 and DG 356 from the Medical Research Council of Canada.

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Received 1 December 1989/17 April 1990; accepted 1 May 1990