Lack of vasopressin receptors in liver, but not in kidney, of *ob/ob* mice

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The activity of phosphorylase *a* was measured in isolated hepatocytes from fed lean and *ob/ob* mice after addition of vasopressin, angiotensin, phenylephrine and glucagon. The binding of these hormones to purified liver plasma membranes was also determined. (1) In hepatocytes of *ob/ob* mice, no increase in phosphorylase *a* was measured after addition of vasopressin, whereas the other hormones promoted an increase in the activity of the enzyme. (2) No specific vasopressin receptors could be measured on purified liver plasma membrane of *ob/ob* mice. A decrease in the number of receptors for angiotensin and glucagon, without modification of the affinity, was also observed. (3) No restoration of the number of vasopressin receptors was observed in liver of *ob/ob* mice starved for 3 days or in younger (5–6 weeks) animals. (4) Vasopressin receptors and vasopressin-stimulated adenylate cyclase, measured on purified kidney medulla membranes, were similar in both lean and *ob/ob* mice. The data indicate a selective lack of vasopressin receptors and metabolic response in liver of the *ob/ob* mouse.

In rat and mouse liver, vasopressin, angiotensin and α_1 -adrenergic agents are thought to elicit most of their metabolic responses (i.e. glycogenolysis, inhibition of lipogenesis, decreased ketogenesis) through changes in cytosolic $[Ca^{2+}]$ (Stubbs *et al.*, 1976; Keppens *et al.*, 1977; Sugden *et al.*, 1980). Previous data suggest that the liver of the obese (*ob/ob*) mouse is resistant to the action of vasopressin; for example, in perfused livers of *ob/ob* mice, vasopressin failed to inhibit lipogenesis (Hems & Ma, 1976). This defect was not reversed when the animals were food-restricted for 1 month. In isolated hepatocytes of *ob/ob* mice, no action of vasopressin on glucose production, ketogenesis and oxidation and esterification of oleate was observed; furthermore, food restriction did not restore any response of these parameters to vasopressin (Edwards *et al.*, 1981).

The lack of responsiveness of hepatocytes from *ob/ob* mice to vasopressin suggests either a lack of receptors for the hormone or a deficiency in the coupling of the receptors to the mechanisms promoting the metabolic response.

To investigate this question, binding of vasopressin to purified membranes from lean and *ob/ob* mice was measured. The action of vasopressin on phosphorylase *a* was also determined in isolated hepatocytes from lean and *ob/ob* mice and compared with the actions of angiotensin, phenylephrine and glucagon.

The results obtained show that hepatocytes from *ob/ob* mice do not respond to vasopressin because they lack the receptor for this hormone. In contrast, the plasma-membrane receptor is present in kidney. Alterations in the sensitivity and receptor number for other hormones, i.e. angiotensin and glucagon, are also observed in hepatocytes from *ob/ob* mice.

Materials and methods

Materials

Collagenase, unlabelled [arginine]vasopressin (grade VI), angiotensin, bovine serum albumin, ATP and cyclic AMP were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phosphocreatine (disodium salt), creatine kinase, GTP and guanosine 5'-[β,γ -imido]triphosphate were provided by Boehringer (Mannheim, Germany); [α - ^{32}P]ATP (15–25 Ci/mmol) and cyclic [3H]AMP were purchased from New England Nuclear (Boston, MA, U.S.A.); and [3H]prazosin was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Glucagon was supplied by Novo Laboratories (Bagsvaerd, Denmark), [lysine]vasopressin by Bachem (Budendorf, Switzerland), phentolamine (Regitine) by Ciba (Basel, Switzerland) and prazosin by Pfizer (Karlsruhe, Germany).

Animals

Male C57 BL/6J *ob/ob* mice and their lean

controls (+/+) were obtained from Jackson Memorial Laboratories, Bar Harbor, ME, U.S.A. They were maintained at a constant temperature with a fixed 12h-light/12h-dark cycle, and fed *ad libitum* with standard laboratory chow. At the time of the experiment the animals were either 5–6 or 12–14 weeks old.

Isolation of hepatocytes

Hepatocytes from fed lean and *ob/ob* mice were prepared between 09:00 and 10:00h by collagenase digestion (Le Cam *et al.*, 1976; Le Cam & Freychet, 1977). Viability of the cells was 90–96% for hepatocytes from lean mice and 85–90% for hepatocytes from *ob/ob* mice. Cells were both preincubated (30min) and incubated in Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing 3% (w/v) dialysed bovine serum albumin and 10mM-glucose.

For measurement of phosphorylase *a*, batches of cells were immediately cooled in liquid N₂, and later thawed in 3 vol. of 100mM-NaF/20mM-EDTA/0.5% glycogen in 100mM-glycylglycine buffer, pH 7.4 (Stalmans *et al.*, 1974). Phosphorylase *a* activity was determined as described by Stalmans & Hers (1975).

Purification of liver and kidney membranes

Purified plasma membranes were prepared from whole liver by the procedure of Neville (1968) (up to step 11) and then stored in liquid N₂. Recovery and purification of membranes were quantified by using the specific activity of 5'-nucleotidase (Newby *et al.*, 1975). It was similar for purified liver plasma membranes of lean and *ob/ob* mice.

The method of purification of mouse renal medullo-papillary membrane has been described (Bockaert *et al.*, 1973; Rajerison *et al.*, 1974). Adenylate cyclase activity was assayed on purified kidney membranes immediately after preparation. The membranes (20–50 µg of protein) were preincubated for 15 min at 30°C with the hormones, and the reaction was initiated by the addition of [α -³²P]ATP and stopped 6 min later. Labelled cyclic AMP was separated (Salomon *et al.*, 1974) and counted for radioactivity.

Radiolabelled ligands

[Lysine]vasopressin labelled with ³H on the tyrosine residue in position 2 ([³H]vasopressin) was prepared as described by Pradelles *et al.* (1972), purified by affinity chromatography (Camier *et al.*, 1973) and stored in liquid N₂. The tritiated peptide was tested for its biological activity by comparing its characteristics with those of the unlabelled material submitted to the same purification procedure. Both labelled and unlabelled peptides behaved identically. The specific radioactivity of the labelled hormone

was 8.5 Ci/mmol. [³H]Angiotensin was prepared as described by Morgat *et al.* (1970). The labelled product was purified by high-pressure liquid chromatography to a specific radioactivity of 14 Ci/mmol. Biological activity of the tritiated hormone was identical with that of the unlabelled peptide submitted to the same purification procedure. [¹²⁵I]-Iodinated glucagon was prepared and purified as described by Desbuquois (1975). Its specific radioactivity was 500 Ci/mmol.

Binding assays

Binding of [³H]vasopressin to purified liver membranes was performed as described by Cantau *et al.* (1980), that of [³H]angiotensin as described by Keppens *et al.* (1982), and that of [¹²⁵I]-labelled glucagon as described by Butlen *et al.* (1980). [³H]Prazosin binding was taken as an estimation of α_1 -adrenergic receptors and was measured by the procedure described by Hoffman *et al.* (1981). [³H]Vasopressin binding to partially purified kidney medulla membranes was performed immediately after membrane preparation (Bockaert *et al.*, 1973; Rajerison *et al.*, 1974).

Results

*Effect of vasopressin, angiotensin, phenylephrine and glucagon on the activity of phosphorylase *a* in isolated hepatocytes of lean and obese mice*

Dose-response curves of the effects of vasopressin, angiotensin, phenylephrine and glucagon on phosphorylase *a* activity in hepatocytes from 12–14-week-old fed lean and obese mice are illustrated in Fig. 1. Phosphorylase *a* activity was measured 2 min after the addition of each hormone. No response to vasopressin is observed in hepatocytes from obese mice, even at very high concentrations (100 nM). This lack of response at 2 min is also observed after a longer incubation (results not shown), indicating that the response to vasopressin is not delayed, but is suppressed. Starving the *ob/ob* mice for 72 h does not restore the response to vasopressin (Table 1). A small, not statistically significant, response to vasopressin is, however, observed in hepatocytes from 5–6-week-old *ob/ob* mice (Table 1). Phenylephrine promotes a comparable dose-response in hepatocytes from lean and obese mice, whereas the response to angiotensin displays a decrease in both sensitivity and maximal response (Fig. 1) and the response to glucagon shows a decrease in sensitivity only.

Receptors for glycogenolytic hormones on purified liver plasma membranes from lean and ob/ob mice

Specific vasopressin receptors were measured on purified liver membranes from lean mice (Fig. 2). Determination of the dose-dependence of hormone

Fig. 1. Dose-response curves of the effect of vasopressin, angiotensin, glucagon and phenylephrine on phosphorylase α activity in hepatocytes of fed lean (—) and *ob/ob* (---) mice

Hepatocytes were prepared and incubated as described in the Materials and methods section. Phosphorylase α was measured 2 min after addition of the hormone. Values are means $\pm$ S.E.M. for three to five hepatocyte preparations: * $2P \leq 0.05$.

binding indicated the presence of a single population of sites with a maximal binding capacity of 1 pmol/mg of protein (Table 2) and a dissociation constant of 21 nM. The latter value is higher than that previously determined on rat liver membranes (4.5 nM) (Cantau *et al.*, 1980). No specific binding of [3 H]vasopressin could be detected on purified plasma membranes prepared from *ob/ob*-mouse livers, even at very high vasopressin concentration (60 nM). No binding sites could be observed in liver membranes of 72 h-starved *ob/ob* mice. When liver plasma membranes from younger *ob/ob* mice were used, less than 3% of binding sites to vasopressin measured in lean mice could be detected. The apparent affinity constant of [3 H]angiotensin bind-

ing to liver plasma membranes was identical for lean and *ob/ob* mice ($K_d = 3$ nM) (Table 2), whereas the total number of binding sites was decreased by 50% in liver membranes of *ob/ob* mice. [3 H]Prazosin, used to label α_1 -receptor sites, exhibited the same binding constants in both groups of mice. The concentration of glucagon leading to half-maximal inhibition of [125 I]-labelled-glucagon binding was identical for lean and *ob/ob* mice (Table 2), indicating a similar affinity of the receptor for glucagon. The difference in glucagon binding measured with a constant concentration of [125 I]-labelled glucagon (0.25 nM) very likely reflects a decreased maximal binding capacity in liver membranes of *ob/ob* mice as compared with controls.

Table 1. *Effect of maximal concentration of vasopressin, angiotensin, phenylephrine and glucagon on phosphorylase a activity in hepatocytes of 5–6-week-old and of 72 h-starved ob/ob mice*

Hepatocytes were prepared and incubated as described in the Materials and methods section. Phosphorylase *a* was measured 2 min after addition of the hormone. Values are means $\pm$ S.E.M. for two or three hepatocyte preparations: * $2P \leq 0.01$ by unpaired Student's *t* test.

Conditions	Phosphorylase <i>a</i> (units/g)	
	5–6-week-old	72 h-starved
Control	11.5 $\pm$ 1.1	9.0 $\pm$ 1.6
Vasopressin (10 nM)	13.3 $\pm$ 1.3	10.4 $\pm$ 1.6
Angiotensin (10 nM)	21.0	17.4 $\pm$ 1.2*
Phenylephrine (10 μ M)	17.5	16.1 $\pm$ 0.4*
Glucagon (1 nM)	23.5	24.4 $\pm$ 2.9*

Fig. 2. *Dose-dependence of specific [3 H]vasopressin binding to purified liver membranes of fed lean (Δ) and obese ($\blacktriangle$) ob/ob mice*

Specific [3 H]vasopressin binding was measured as described in the Materials and methods section. Values are means of three independent experiments. Insert: Scatchard plots of dose-dependent binding.

Receptors for vasopressin on purified kidney medulla membranes from lean and ob/ob mice

[3 H]Vasopressin binding and adenylate cyclase activation by vasopressin were determined on kidney

medulla membranes prepared from obese and lean mice. As shown in Fig. 3, dose-dependent [3 H]-vasopressin binding and adenylate cyclase activation by vasopressin were similar in both groups. Adenylate cyclase activation by guanosine 5'-[β , γ -imido]triphosphate, alone or in combination with vasopressin, as well as activation by NaF, were also similar in both groups (Fig. 3).

Discussion

The above data demonstrate that, in *ob/ob* mice, hepatic phosphorylase is not activated by vasopressin, and that [3 H]vasopressin does not specifically bind to purified liver membranes. The characteristics of the [3 H]vasopressin-binding sites detected on liver membranes from lean mice are comparable with those of the binding sites previously characterized on rat hepatocytes and rat liver membranes and identified as the physiological receptors responsible for vasopressin-induced glycogenolysis (Cantau *et al.*, 1980). These two observations suggest that, in *ob/ob* mice, the hepatic resistance to vasopressin (as previously described and confirmed in the present study) is a consequence of an absence of vasopressin receptors. This lack of hepatic vasopressin binding, in *ob/ob* mice, is unlikely to be the result of a 'down-regulation' process owing to increased blood vasopressin, since renal vasopressin receptors, which were shown in the rat to be sensitive to down-regulation (Rajerison *et al.*, 1974), appear essentially unchanged in *ob/ob* mice as compared with control lean mice. Liver vasopressin receptors are absent from younger *ob/ob* mice (5–6 weeks) and from mice starved for 3 days, a condition known to reverse many of the defects observed in these mice (Le Marchand *et al.*, 1977).

The present data do not allow a distinction to be made between a genetic defect in liver vasopressin receptors or a progressive disappearance of the receptors early in life. However, the presence of the *ob/ob* mutation clearly has quite different consequences on hepatic and renal vasopressin receptors. This might indicate different control and regulatory mechanisms for vasopressin receptors, according to the type of transduction process: adenylate cyclase activation for V_2 -type renal receptors and Ca^{2+} mobilization for V_1 -type hepatic receptors (Michell *et al.*, 1979; reviewed by Jard, 1983).

The present data also show an alteration in the number of angiotensin and glucagon receptors in *ob/ob* mice. The 50% decrease in receptor number is accompanied by a significant increase in the A_{50} (concn. giving half-maximal response) values for phosphorylase activation and a decrease in the magnitude of the maximal response. Previous studies on liver angiotensin receptors in the rat indicate the existence of a large receptor reserve (half-maximal response is obtained at a fractional

Table 2. Affinity constant and number of receptor sites for [^3H]vasopressin, [^3H]prazosin, [^3H]angiotensin and [^{125}I]-labelled glucagon in purified liver plasma membranes of lean and *ob/ob* mice

K_D or half-inhibition values are expressed in nM, and maximal binding (B_{max}) or B_0 (with 0.2 nM- ^{125}I -labelled glucagon) in pmol/mg of protein. Abbreviation: NM, not measurable. Results are means $\pm$ s.e.m. for three to five membrane preparations: * $2P \leq 0.02$, and † $2P \leq 0.001$, compared with lean mice.

	[^3H]Vasopressin		[^3H]Prazosin		[^3H]Angiotensin		^{125}I -labelled glucagon	
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}	Half inhibition	B_0
Lean mice	21.05 $\pm$ 4.5	1.03 $\pm$ 0.14	0.57 $\pm$ 0.14	0.375 $\pm$ 0.065	3.13 $\pm$ 0.6	0.468 $\pm$ 0.038	0.50 $\pm$ 0.10	0.625 $\pm$ 0.015
<i>ob/ob</i> mice	NM	NM	0.78 $\pm$ 0.13	0.422 $\pm$ 0.027	2.81 $\pm$ 0.81	0.217* $\pm$ 0.055	0.64 $\pm$ 0.15	0.358† $\pm$ 0.025

Fig. 3. Adenylate cyclase activity after addition of vasopressin, and [^3H]vasopressin binding to purified kidney medulla membranes of lean (○) and *ob/ob* (●) mice

Adenylate cyclase activity (a) was determined as indicated in the Materials and methods section. Basal activities were 230 and 216 pmol/6 min per mg of protein. Values after addition of NaF (10 mM) were 575 and 430 pmol/6 min per mg. After addition of guanosine 5'-[β,γ -imido]triphosphate (10 μM) they were 1175 and 976 pmol/6 min per mg. Values after addition of guanosine 5'-[β,γ -imido]triphosphate (10 μM) + vasopressin (10 μM) were 1283 and 1065 pmol/6 min per mg for purified kidney medulla membranes of lean and *ob/ob* mice respectively. [^3H]Vasopressin binding (b) was performed as described in the Materials and methods section. K_d and B_{max} values as estimated from Scatchard plots were 4.20 nM, 130 fmol/mg of protein for lean mice and 1.95 nM, 125 fmol/mg of protein for *ob/ob* mice respectively. All values are means of three independent determinations.

occupancy of less than 0.5%) (Keppens *et al.*, 1982). In such a situation the expected consequence of a progressive decrease in receptor number would be a gradual increase in the A_{50} value, followed by a decrease in the magnitude on the maximal response. Indeed, if the total number of receptors is decreased, the same concentration of occupied receptors will correspond to a higher fractional receptor occupancy, i.e. will be obtained at higher hormonal concentration. A lower maximal response will occur when the highest concentration of occupied receptors is below the value needed to elicit a maximal response. Therefore our observation of a decreased efficiency of glucagon and angiotensin in activating

phosphorylase does not necessarily imply the presence of any further alterations in the cascade of events leading to the activation of phosphorylase. The observation that the sensitivity to phenylephrine is normal in *ob/ob* mice with respect to receptor number and phosphorylase activation favours the above conclusion. The decrease in sensitivity and maximal response of phosphorylase *a* to angiotensin could also be explained by a modification in the number of high-affinity low-capacity receptors measured with ^{125}I -labelled angiotensin (Campanile *et al.*, 1982).

These data suggest that hepatocytes from *ob/ob* mice selectively lack the vasopressin receptor, but

that the receptors for other hormones acting via changes in cytosolic Ca^{2+} are present. The coupling of these receptors to the mechanisms promoting the metabolic response is also present, since the activity of phosphorylase α is increased by these hormones.

A relationship between this lack of vasopressin receptors and obesity is difficult to assess.

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