Effects of Insulin on Lipolysis and Lipogenesis in Adipocytes from Genetically Obese (*ob/ob*) Mice

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A method for the preparation of isolated adipocytes from obese mice is described. Similar yields of adipocytes (50–60%), as judged by several criteria, are obtained from obese mice and lean controls. Few fat-globules and no free nuclei were observed in cell preparations, which are metabolically active, respond to hormonal control and appear to be representative of intact adipose tissue. Noradrenaline-stimulated lipolysis was inhibited by insulin, equally in adipocytes from lean and obese mice. Inhibition in obese cells required exogenous glucose, and the insulin dose–response curve was shifted to the right. Basal lipogenesis from glucose was higher in adipocytes from obese mice, and the stimulatory effect of insulin was greater in cells from obese mice compared with lean controls. A rightward shift in the insulin dose–response curve was again observed with cells from obese animals. This suggests that adipose tissue from obese mice is insulin-sensitive at the high blood insulin concentrations found *in vivo*. The resistance of obese mice to the hypoglycaemic effect of exogenous insulin and their impaired tolerance to glucose loading appear to be associated with an impaired insulin response by muscle rather than by adipose tissue.

Several metabolic differences, including hyperglycaemia and hyperinsulinaemia, have been observed between genetically obese (*ob/ob*) mice and their lean littermates (Bray & York, 1971). Obese mice are resistant to the hypoglycaemic effect of exogenous insulin (Shull & Mayer, 1956), and their tolerance to glucose loading is impaired (Chlouverakis *et al.*, 1970). This suggests that the uptake of glucose by adipose tissue and muscle, which are normally responsive to insulin, is impaired.

Insulin has no effect on theophylline-stimulated lipolysis in pieces of adipose tissue from obese mice, whereas it shows an inhibitory effect in lean controls (Yen & Steinmetz, 1972a).

Fatty acid biosynthesis from glucose *in vivo* is increased in adipose tissue of obese mice compared with lean mice (Hems *et al.*, 1975), and the incorporation of [¹⁴C]glucose into triacylglycerol is reported to be greater in pieces of adipose tissue in obese mice, when related to cell numbers (Jamdar *et al.*, 1976). In contrast, in pieces of adipose tissue, on a tissue nitrogen basis, Christophe *et al.* (1961) found that triacylglycerol biosynthesis was lower in obese mice than their lean counterparts, even in the presence of added insulin.

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Shepherd *et al.* (1977) and Carnie & Smith (1978) have drawn attention to the difficulties that arise when pieces of adipose tissue from obese mice are used for metabolic studies on the basis of tissue weight, tissue protein or tissue DNA. The difficulties associated with the use of adipose-tissue pieces do not occur with isolated adipocytes, which have not been previously used to investigate the effects of insulin on lipolysis in obese mice. Chang *et al.* (1975) reported that very few intact adipocytes were obtained after collagenase treatment of epididymal fat-pads from obese mice. They concluded that fat-cells from obese mice are extremely fragile, releasing fat-globules that could be mistaken for adipocytes into the preparation medium. They did not give a detailed account of the conditions used. Abraham & Beloff-Chain (1971) prepared adipocytes from obese mice with collagenase in an albumin-free medium, and obtained variable cell yields and degrees of cell lysis. They suggested that large adipocytes are particularly susceptible to lysis. Clearly the cell population in adipocyte preparations from obese mice cannot be representative of the population of the intact tissue unless large cells survive lysis and comparable yields of adipocytes are obtained from obese and lean mice.

The present paper presents evidence that good yields of intact metabolically active adipocytes can

be prepared from lean and obese mice, and considers the effects of insulin on lipolysis and lipogenesis in these cells.

Experimental

Materials

Animals, saline medium and silicone-treated glassware were as previously described (Carnie & Smith, 1978). D-[U- 14 C]Glucose (333 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Insulin (22.4 i.u./mg, where 1 i.u. is equivalent to 0.042 mg; BDH) was dissolved in 0.03 M-HCl at a concentration of 1 mg/ml and diluted in Krebs-Henseleit saline to the concentration required in assays. All other chemicals were of the highest grade available.

Preparation of isolated adipocytes

Isolated adipocytes were prepared and counted as previously described (Carnie & Smith, 1978), except that centrifugation of the suspension at 250g for 1 min was replaced by centrifugation with an integrated field-time of 80g-min at r_{av} . 11 cm. This gave a clean preparation with a slightly higher yield, by further reducing damage to adipocytes during centrifugation.

Attempts to produce adipocytes from obese mice by the method of Rodbell (1964) gave preparations containing many broken cells. Exploratory experiments were undertaken to devise a method for the preparation of good yields of viable cells. The method described above differs from that of Rodbell (1964), particularly in three respects: only one centrifugal step is involved, the centrifugal force applied is greatly reduced, and, after being washed, the adipocytes are isolated by flotation without centrifugation.

Determination of total lipid in fat-pads and isolated cells

For obese mice, one epididymal fat-pad, and for lean mice, two pads (one from each of two animals) were homogenized in 20 ml of chloroform/methanol (2:1, v/v) per g of tissue in a Teflon/glass homogenizer of the Potter-Elvehjem type.

Isolated adipocytes were prepared from the remaining fat-pads by the routine method, except that the cells were finally suspended in approx. 1 and 3 ml of saline medium for cells from lean and obese mice respectively. The final volume of the suspension from obese mice was measured. To all of the suspension from lean mice and to 1 ml samples of the suspension from obese mice, 8 ml of chloroform/

methanol was added, and the mixtures were vortex-mixed and left to extract overnight at room temperature. The chloroform/methanol was separated from the aqueous phase.

The chloroform/methanol extracts from fat-pads and isolated adipocytes were washed once with half their volume of chloroform/methanol/0.9% (w/v) NaCl (3:48:47, by vol.) (Folch *et al.*, 1957), evaporated in a stream of N_2 and dried to constant weight *in vacuo* over $CaCl_2$. The total weight of lipid extracted from the cell suspensions was then calculated as a percentage of the total lipid from the corresponding fat-pad or -pads.

Determination of ATP citrate lyase activity in fat-pads and isolated cells

For obese mice, one epididymal fat-pad was homogenized in 5 ml of 0.25 M-sucrose/g of tissue. For lean mice two pads (one from each of two animals) were homogenized in 4 ml of 0.25 M-sucrose at 5°C.

Isolated adipocytes were prepared from the remaining fat-pads by the routine method, except that the cells were finally suspended in 5 ml of 0.25 M-sucrose/g of original tissue and 4 ml of 0.25 M-sucrose/g for cells from obese and lean mice respectively, before homogenization at 5°C.

Homogenates from pad and cell preparations were centrifuged at 38000g for 30 min at 5°C. ATP citrate lyase (EC 4.1.3.8) in the supernatants was assayed by the method of Kornacker & Ball (1965).

Determination of lactate dehydrogenase activity in fat-pads and isolated cells

One fat-pad from each animal, lean or obese, was cut into pieces of about 100 mg. Each weighed piece was sonicated in 1 ml of saline medium with an MSE-Mullard 60 W ultrasonic drill at maximum output for 3 min, and centrifuged at 12000g for 12 min. Sonication and centrifugation were carried out at 5°C.

The remaining fat-pad from each animal was used to make adipocytes by the routine method, except that the final volumes of the cell suspensions were 2 ml for lean mice and 4 ml for obese mice. Then 1 ml samples of the cell suspensions were sonicated and centrifuged as described above.

After centrifugation, the fat-cakes were removed from the pad and cell preparations, and the fat-free aqueous layers made to 3 ml with 0.05 M-potassium phosphate buffer, pH 7.5. Lactate dehydrogenase (EC 1.1.1.27) in the phosphate buffer extracts was assayed in the direction of pyruvate reduction essentially as described by Schwartz & Bodansky (1966).

Effect of insulin on lipolysis in isolated adipocytes

The effect of insulin on noradrenaline-stimulated lipolysis in isolated adipocytes was determined as follows. Isolated adipocytes were incubated in saline medium, with or without glucose, at a final concentration of 10mM, together with noradrenaline bitartrate (2µg/ml in distilled water) as previously described (Carnie & Smith, 1978), except that insulin (10µl), at final concentrations indicated in Figs. 1(a) and 1(b), was added to each 25ml flask. In control flasks 10µl of saline medium replaced insulin. Fatty acids from incubated cells were measured by the method of Laurell & Tibbling (1967).

Effect of insulin on lipogenesis in isolated adipocytes

The effect of insulin on lipogenesis from glucose in isolated adipocytes was determined as follows. Isolated cell suspension (400µl), saline medium with glucose at a final concentration of 10mM (600µl), D-[U-¹⁴C]glucose (20µCi/ml in distilled water; 100µl) and insulin (10µl) at final concentrations indicated in Fig. 2, were added to 25ml flasks, gassed with O₂/CO₂ (19:1) and incubated with shaking at 37°C for 1 h. To obtain basal rates, insulin was replaced by saline medium. After incubation, 1ml of 1M-H₂SO₄ was added to each flask, and the contents were transferred with 5ml of chloroform/methanol (2:1, v/v) to glass-stoppered tubes and vortex-mixed. After being left overnight at room temperature the aqueous layer in each tube was discarded, and the lower chloroform layer was washed twice with 3ml of chloroform/methanol/1.0M-H₂SO₄ (3:48:47, by vol.) (Folch *et al.*, 1957), and transferred to glass scintillation vials. After evaporation to dryness at 50°C, and addition of 5ml of scintillation fluid [2,5-diphenyloxazole (3g/l) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.1g/l) in scintillation-grade toluene], the radioactivity was determined. Efficiency of counting (about 80%) was determined by using an internal standard ([¹⁴C]toluene), and no extra quenching was observed with the addition of incubation samples, provided that scintillation fluid was added immediately after evaporation was complete. If vials were allowed to remain at 50°C for longer periods, a yellow deposit occasionally formed on addition of scintillation fluid and strong quenching was observed.

Incubation extracts dissolved in chloroform/methanol (2:1, v/v) were investigated by t.l.c. The separated components, detected by radioautography, were analysed for radioactivity and stained for lipid with Bromothymol Blue. Except for a small amount of material remaining at the origin, identified as glucose, all of the labelled material was lipid. Control flasks with H₂SO₄ added before the cells gave a small but constant count rate, which represented glucose carried over by the extraction procedure,

and was never more than 10% of the total rate of incorporation. This control value was subtracted from basal and insulin-stimulated rates, which were linear for at least 1 h for cells from both lean and obese mice.

Results and Discussion*Yield and viability of adipocytes*

Adipocytes were examined under light microscopy with and without Methylene Blue staining. Freshly prepared adipocytes from lean and obese mice had a similar appearance, except for size, and nuclei were clearly visible even without staining. Free nuclei were not observed. The average diameter of cells from obese mice was approximately twice that of cells from lean mice. Cells had the typical 'signet ring' appearance of adipocytes with flattened nuclei and a thin film of cytoplasm surrounding the central fat-globule. Very few free fat-globules were apparent in preparations from both lean and obese mice. They may be clearly distinguished from adipocytes owing to their lack of nuclei and the fact that they lose their spherical appearance when attached to a cover slip. The DNA contents of adipocytes from lean and obese mice are very similar on a per-cell basis (Carnie & Smith, 1978) and are in good agreement with values reported for other somatic cells in the mouse (Vendrely, 1955), confirming the microscopic evidence that few fat-globules were present in adipocyte preparations. In preparations left at room temperature for 1 h or longer, lysing of individual cells could be observed to take place and the proportion of fat-globules was increased.

The yields of isolated adipocytes based on the recoveries of total lipid, ATP citrate lyase and lactate dehydrogenase activities of intact fat-pads for two separate experiments in each case were respectively 48 and 55%, 49 and 56%, and 53 and 55% for lean mice. The corresponding values for obese mice were 49 and 60%, 48 and 51%, and 55 and 56%. Thus yields of 50–60% were obtained for all three parameters with cells from lean and obese mice. ATP citrate lyase and lactate dehydrogenase are located in the cytosol and are readily released from damaged cells. The fact that cell yields based on recovery of total lipid were very similar to those obtained with the enzymes can only be interpreted in terms of a recovery of intact cells with little or no loss of cell contents. A higher percentage recovery of partly damaged cells could not be ruled out from the results for the enzyme activities alone. Similar recoveries of cells from lean and obese mice make it unlikely that an unrepresentative sample of the total population of the intact tissue is obtained in our cell preparations.

Cell yields obtainable in adipocyte preparations do not appear to have been published for any animal.

Yields of liver parenchymal cells from at least three animals and of rat heart cells have been determined. Yields of 50% for liver parenchymal cells from the rat (Berry, 1974), of 30% for liver parenchymal cells from the guinea pig (Elliott & Pogson, 1977) and of 25% for liver parenchymal cells from the mouse (Crisp & Pogson, 1972) have been obtained, after perfusion of liver with media containing collagenase. Lower yields are obtained when liver slices are used as the starting material with the rat (La Brecque & Howard, 1976) or sheep (Ash & Pogson, 1977). Farmer *et al.* (1977) report yields of 35–50% for rat heart cells after perfusion of intact heart. Our yields for adipocytes are therefore comparable with the most favourable yields so far reported for isolated cells from liver and heart.

Isolated adipocytes are sensitive to the lipolytic hormone noradrenaline (Carnie & Smith, 1978). The maximal lipolytic effect of noradrenaline is similar for cells from lean and obese mice, but is greatly increased in cells from obese but not lean mice, in the presence of glucose. The present paper demonstrates that adipocytes from lean and obese mice are metabolically active with respect to lipogenesis, and are responsive to insulin with respect to inhibition of lipolysis and stimulation of lipogenesis. Adipocytes prepared in a medium in which albumin is replaced with Ficoll type 70 have the same appearance as those prepared in the medium containing albumin. Cells prepared in Ficoll medium show a stimulation of alanine formation in the presence of glutamate, and the stimulation is similar on a per-cell basis with adipocytes from lean and obese mice (J. A. Carnie, D. G. Smith & M. Mavris-Vavayannis, unpublished work). Snell & Duff (1977) have demonstrated a stimulation of alanine formation by glutamate in isolated fat-pads from the rat.

Effects of insulin on lipolysis and lipogenesis in isolated adipocytes

The effect of insulin on noradrenaline-stimulated lipolysis in adipocytes from lean and obese mice is shown in Fig. 1(a). With adipocytes from lean mice, increasing concentrations of insulin progressively inhibit lipolysis up to an insulin concentration of $45 \mu\text{i.u./ml}$, and this inhibition is reversed at insulin concentrations above this value. It is interesting that the insulin concentration causing maximal inhibition of lipolysis corresponds closely to the concentration in plasma ($35 \mu\text{i.u./ml} \pm 10 \text{ s.e.m.}$) determined by Abraham *et al.* (1971) for fed lean mice of the same strain and age. The reversal of inhibition at high insulin concentrations has been previously reported with rat adipocytes (Siddle & Hales, 1974). By contrast adipocytes from obese mice are highly resistant to the inhibitory effect of insulin on lipolysis.

Fig. 1. *Effect of insulin on noradrenaline-stimulated lipolysis in isolated adipocytes*

The effects of various concentrations of insulin on noradrenaline-stimulated lipolysis in adipocytes prepared from lean ($\circ$) or obese (Δ) mice were determined as described in the Experimental section. In (a) the incubation medium was glucose-free and in (b) the incubation medium was 10 mM with respect to glucose. Zero-time blanks were subtracted from values for incubated samples to take account of the small amount of fatty acids bound to albumin in the saline medium. Results are means for five incubations and bars indicate the s.e.m.

Fig. 1(b) demonstrates the effect of insulin on noradrenaline-stimulated lipolysis in the presence of 10 mM-glucose. Glucose does not alter the dose-response curve for insulin with adipocytes from lean mice, but with cells from obese mice the resistance to the inhibitory effect of insulin is abolished. Moreover, the dose-response curve of insulin-inhibited lipolysis is shifted to the right compared with that obtained with cells from lean controls, and the maximal inhibition of lipolysis is caused at an insulin concentration of $1500 \mu\text{i.u./ml}$. This again corresponds closely to the plasma insulin concentration ($1160 \mu\text{i.u./ml} \pm 460 \text{ s.e.m.}$) obtained by Abraham

et al. (1971) for obese mice of the same strain and age. Reversal of inhibition occurred at insulin concentrations above 1500 μ -i.u./ml. Thus with adipocytes from obese mice, higher insulin concentrations are required to produce comparable anti-lipolytic effects.

In the absence of glucose, insulin appears to act on the lipolytic process at two points: inhibition of adenylate cyclase and stimulation of cyclic AMP phosphodiesterase (Lambert *et al.*, 1978). In the presence of glucose, insulin can also apparently inhibit fatty acid release by accelerating re-uptake of fatty acids, through the provision of *sn*-glycerol 3-phosphate by increasing glucose transport and metabolism. Since the activity of the particulate phosphodiesterase from adipocytes of obese mice is stimulated by insulin (Malbon & Fain, 1977), an inhibition of lipolysis might be expected in the absence of glucose. The inhibition of lipolysis in the presence of glucose may in part be apparent, being due to increased uptake of fatty acids, but the extent of the effect together with the absence of an effect with lean mice suggests that a true inhibition of lipolysis is also occurring. The mechanism of the effect of glucose in eliciting sensitivity to insulin in cells from obese mice is not known, but it is clear that high glucose and high insulin concentrations are required to produce a significant inhibition of lipolysis. Since obese mice are hyperglycaemic and hyperinsulinaemic, it is reasonable to conclude that lipolysis in adipose tissue of these animals is subject to control by insulin *in vivo*.

Yen & Steinmetz (1972a) were unable to test the anti-lipolytic effect of insulin on hormone-stimulated lipolysis with fat-pieces from obese (*ob/ob*) mice, since their preparations responded poorly to adrenaline. This may have been due to lack of penetration of the hormone into tissue pieces, since maximal effects of noradrenaline on lipolysis with isolated adipocytes are achieved at concentrations 0.5% of those required when tissue pieces are used (Carnie & Smith, 1978). Yen & Steinmetz (1972a) observed that theophylline-stimulated lipolysis in pieces of adipose tissue from obese mice was not affected by insulin, whereas the anti-lipolytic effect of insulin was apparent in tissue from lean controls. The apparent lack of an anti-lipolytic effect in obese mice could be due to the absence of exogenous glucose in their assay system, since, at the concentration of insulin they employed (2000 μ -i.u./ml), we have demonstrated the anti-lipolytic effect of insulin (Fig. 1b).

The incorporation of radioactive label from D-[U- 14 C]glucose into extracted total lipid was assayed as a measure of lipogenesis from exogenous glucose in isolated adipocytes, although this measurement does not distinguish between fatty acid biosynthesis and triacylglycerol biosynthesis. Fig. 2

Fig. 2. Effect of insulin on lipogenesis in isolated adipocytes. The effects of various concentrations of insulin on lipogenesis in adipocytes prepared from lean ($\circ$) or obese ($\bullet$) mice were determined as described in the Experimental section. Results are means for six incubations and bars indicate the S.E.M.

shows that the basal rate of lipogenesis from glucose is 2.6-fold higher in cells from obese mice than in cells from lean mice, and insulin at a concentration of 9000 μ -i.u./ml (the highest tested) stimulates lipogenesis 3-fold and 4-fold in cells from lean and obese mice respectively. Even at this very high insulin concentration, maximal stimulation of lipogenesis was not achieved, particularly with adipocytes from obese mice.

Batchelor *et al.* (1975) obtained a higher basal rate of lipogenesis with cells from obese mice than with lean controls. In their experiments insulin at a concentration of 500 μ -i.u./ml in the presence of 0.4 mM-glucose failed to stimulate lipogenesis in cells from obese mice. However, we have observed that lowering the exogenous glucose concentration reduces the effect of insulin at low insulin concentrations in cells from the obese mouse, but has little effect at high concentrations. To demonstrate clearly the effect of insulin on lipogenesis in adipocytes a glucose concentration of about 10 mM is required, together with a range of insulin concentrations.

Yen & Steinmetz (1972b) measured the incor-

poration of glucose into lipid in pieces of adipose tissue from lean and obese mice. Insulin did not stimulate lipogenesis in the preparations from obese mice. Christophe *et al.* (1961) and Chlouverakis & White (1969), also using pieces of adipose tissue, obtained lower basal rates of lipogenesis and low levels of stimulation of lipogenesis by insulin with preparations from obese mice. These results may be explained by the penetration barriers present in tissue pieces compared with the accessibility to substrates in isolated cell suspensions.

Irrespective of the mechanism responsible for the shift in the dose-response curves for the antilipolytic and lipogenic effects of insulin on adipocytes from obese mice, it is clear that these cells are not insulin resistant at the high insulin concentrations that occur *in vivo*. The insulin resistance commonly associated with obese mice is therefore apparent rather than real, and the paradox of obesity associated with resistance to a hormone that stimulates lipogenesis is explained.

Le Marchand-Brustel *et al.* (1978) observed a rightward shift in the insulin dose-response curve for deoxyglucose uptake in isolated soleus muscle from obese mice compared with lean controls. However, even at high concentrations the stimulatory effect of insulin was much less marked with muscle from obese mice compared with lean mice. Basal unstimulated deoxyglucose uptake was identical for lean and obese mice, but at concentrations of insulin producing maximal stimulation the rate of uptake in muscles from lean mice was double that obtained with obese mice. We have observed higher basal rates of lipogenesis with adipocytes from obese mice, and at high insulin concentrations a greater stimulation of lipogenesis compared with lean controls. We conclude that the resistance to the hypoglycaemic effect of exogenous insulin and the impaired tolerance to glucose loading in obese mice are due to a diminished response to insulin in muscle but not in adipose tissue.

It has been shown that noradrenaline-stimulated lipolysis is not impaired in isolated adipocytes from obese mice, and the concentration of serum fatty acids is increased 4-fold in lean and obese mice after short cold exposure (Carnie & Smith, 1978). Thus the failure of obese mice to survive cold exposure (Davis & Mayer, 1954) cannot be due to a failure to mobilize fatty acids, but may be due to a diminished capacity of muscle to take up or utilize fatty acids released in response to stress.

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