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Insulin depletion leads to adipose-specific cell death in obese but not lean mice

(obesity/adipocyte/*ob*/*ob* mouse/*db*/*db* mouse/apoptosis)

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ABSTRACT **Mutation of the obese gene produces obesity, hyperinsulinemia, and compensatory "overexpression" of the defective gene. As insulin activates obese gene expression, it seemed possible that hyperinsulinemia might be responsible for overexpression of the gene. To address this question we rapidly neutralized circulating insulin by injection of an insulin antibody. Unexpectedly, insulin depletion in obese (*ob*/*ob* or *db*/*db*) mice caused massive adipose RNA degradation confirmed by histological analysis to result from adipocyte cell death by a largely necrotic mechanism. This effect was not observed in lean littermates and was completely corrected by coadministration of insulin. Comparison of multiple tissues demonstrated that the effect was restricted to adipose tissue. Insulin depletion in obese mice by administration of streptozotocin also led to cell death, but this death was less extensive and appeared to be apoptotic in mechanism. Thus insulin may promote the survival side of the physiological balance between adipocyte survival and death.**

Obesity is a major health problem in western societies. The excessive accumulation of body fat constitutes a risk factor for noninsulin dependent diabetes mellitus, cardiovascular and cerebrovascular disease, and a number of other pathological conditions (1). Recently, signaling systems have been discovered that serve to regulate body weight and fat accumulation. Leptin, the product of the *obese* gene, suppresses food consumption and increases energy expenditure (2).

Loss of leptin signaling, due to mutation of the obese gene (as in the *ob*/*ob* mouse) or of the leptin receptor (as in the *db*/*db* mouse) leads to massive obesity, hyperglycemia and hyperinsulinemia as in type II diabetes, as well as a 10- to 20-fold over-expression of the leptin message, albeit a defective message (2). Previous findings showed that insulin promotes expression of the *obese* gene both *ex vivo* and *in vivo* (2) (3). To investigate the possibility that the hyperinsulinemia that accompanies obesity in *ob*/*ob* mice might be responsible for the "overexpression" of the *obese* gene in these mice, we injected anti-insulin antibody to rapidly lower the blood insulin level. Unexpectedly, acute neutralization of blood insulin *in vivo* with a specific anti-insulin antibody resulted in massive adipocyte cell death by necrotic and apoptotic mechanisms. Surprisingly, adipocyte cell death did not occur in normal lean littermates. Moreover, cell death was adipose tissue-specific and did not occur in muscle, liver, spleen, or brain. This result suggests that the hyperinsulinemia associated with obesity may serve to balance the anti-adipogenic signal and promote adipocyte survival.

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EXPERIMENTAL PROCEDURES

Mice. Seven-week-old C57BL/6, C57BL/6J *ob*/*ob*, and C57BLS/J *db*/*db* mice were obtained from The Jackson Laboratory and housed under pathogen-free conditions for 1–3 weeks before use. All procedures were performed in accordance with Johns Hopkins University School of Medicine institutional guidelines for animal welfare.

Decomplementation analysis was performed as described (4) by i.p. administration of 20 anti-complementarity units/kg of body weight cobra venom anti-complementarity protein (Sigma) 16 hr before antibody injection. Streptozotocin was administered as a 200 mg/kg i.v. injection and animals were analyzed 24 hr after injection.

Glucose Tolerance Test. After an initial blood sample, C57BL/6J mice were injected i.p. with 2 g/kg glucose together with anti-insulin antibody or a nonimmune serum control. Serial blood samples were drawn from the tail vein and serum glucose levels were determined by using a hexokinase glucose assay kit (Sigma).

Excision of Tissue and Histology. Mice were anesthetized with inhaled metofane and killed by decapitation. The tissues were excised, and those intended for histology were fixed in neutral buffered formalin (Baxter Healthcare, Deerfield, IL). The remainder of the tissue was frozen in liquid nitrogen and stored at −80°C for use in RNA or DNA purification. For light microscopic histology, epididymal fat pads were embedded in paraffin after 24 hr of buffered formalin fixation. Tissue sections (4 μm) were stained with hematoxylin and eosin for analysis.

Glucose Uptake. Two-day postconfluent 3T3-L1 preadipocytes (5) were induced to differentiate in DMEM supplemented with 10% fetal bovine serum, 167 nM insulin, 1 μM dexamethasone, and 170 μg/ml isobutylmethylxanthine (6) for 2 days followed by 2 days with fetal bovine serum and insulin and 6 days with fetal bovine serum alone. For glucose uptake studies (7), 3T3-L1 adipocytes were washed three times in serum-free DMEM and incubated for 2 hr in the absence of serum. Cells were then washed three times with Krebs-Ringer phosphate buffer (pH 7.4), and incubated for 10 min at 37°C in the presence of insulin, in 1 ml of Krebs-Ringer phosphate, followed by addition of 2-deoxy-D-[1-^{14}C]glucose (1 μCi brought to 200 μM with unlabeled deoxyglucose; 1 Ci = 37 GBq) for an additional 10-min incubation. Cells were washed with ice-cold PBS, and lysed in 1% SDS, 0.5 M NaOH, and uptake of 2-deoxy-D-[1-^{14}C]glucose was determined.

RNA Isolation and Northern Blot Analysis. Total RNA was purified by the single-step guanidine isothiocyanate method (8). RNA was electrophoresed in formaldehyde containing gels and blotted on Hybond-N membrane (Amersham) by capillary transfer as described (9). Immobilized RNA was

Abbreviation: TNFα, tumor necrosis factor α.

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visualized by staining for 5 min with 0.05% methylene blue in 0.5 M sodium acetate, followed by destaining with water.

DNA Laddering Analysis of Adipose Tissue. Genomic DNA was isolated from snap frozen mouse tissues as described (10) and electrophoresed on a 2% agarose gel. DNA was capillary blotted to Hybond-N membrane and visualized by Southern blot analysis (11, 12) by using random primed mouse genomic DNA as a probe.

RESULTS

To functionally neutralize the hyperinsulinemia of the obese (*ob/ob*) mice, a Guinea pig anti-insulin (porcine)antibody was selected. It should be noted that many commercially available antibodies, raised against insulin from other sources, react poorly with mouse insulin. To assess the antibody's ability to functionally sequester insulin, the antibody was first tested for its ability to inhibit insulin-stimulated 2-deoxyglucose uptake by fully differentiated 3T3-L1 adipocytes. Serum starved-adipocytes were shown to have a low basal uptake rate that was stimulated 8–12-fold by the addition of 5 nM insulin (Fig. 1*A*). Increasing concentrations of the antibody were found to inhibit over 95% of this activation. Having demonstrated the activity of the antibody in cell culture, its effectiveness was tested *in vivo*. Lean C57BL/6 mice injected either with the antibody or vehicle (control) were subjected to an i.p. glucose challenge after which their serum glucose levels were monitored. The control mice, while evincing a transient increase in serum glucose level, exhibited rapid restoration of glucose level to normal (Fig. 1*B*). The antibody treated mice, however, showed impaired glucose tolerance, indicating an inability of secreted insulin to compensate normally for the increased glucose levels due to sequestration of the hormone by the antibody. It was also found that administration of the antibody to fed animals leads to increased serum glucose level in the absence of a glucose challenge (data not shown).

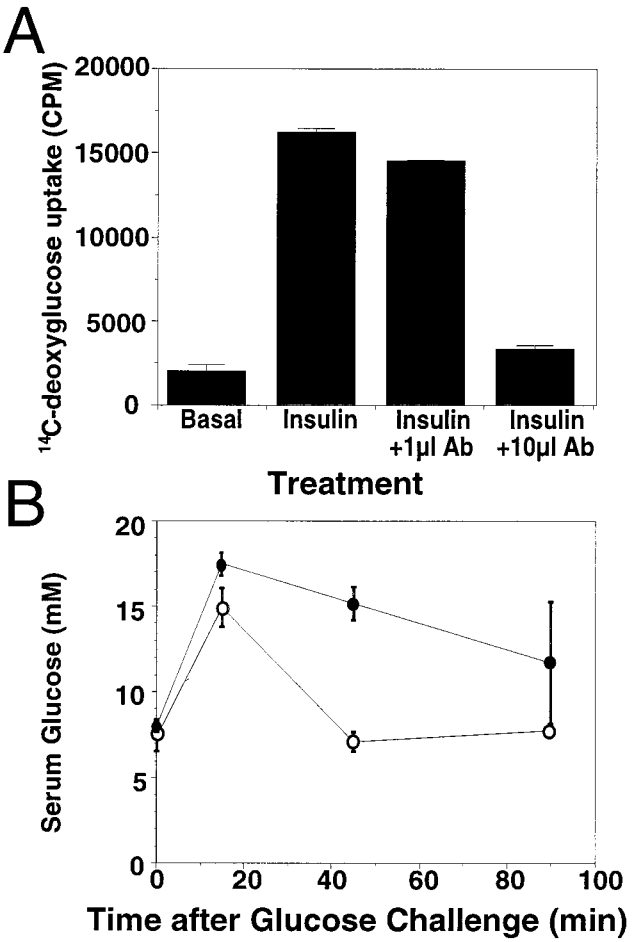

FIG. 1. Functional neutralization of insulin by antibody. (*A*) Cultured 3T3-L1 adipocytes were assayed for their rate of 2-deoxyglucose uptake in the absence or presence of 5 nM porcine insulin (Lilly), alone, or in combination with guinea pig anti-porcine insulin (Sigma). Values represent mean ± SEM. (*B*) After an initial blood sample, C57BL/6J mice ($n = 3$) were injected i.p. with 2 g/kg glucose along with 50 μl anti-insulin antibody (●) or a nonimmune control serum (○). Serial blood samples were drawn from the tail vein and serum glucose levels were determined by using a hexokinase glucose assay kit (Sigma) Values represent mean ± SEM.

The effect of acute insulin depletion on the adipose tissue of obese mice was then determined. For this purpose, *ob/ob* and *db/db* obese mice that carry an inactivating mutation in the leptin protein or receptor, respectively (13, 14), were selected. These mice demonstrate early onset obesity accompanied by hyperinsulinemia and noninsulin dependent diabetes mellitus. *ob/ob* mice and their lean littermates were treated with a nonimmune control or the anti-insulin antibody via two i.v. injections at three hr intervals. After 6 hr, adipose tissue was excised and snap frozen. Electrophoresis and capillary blotting of total cellular RNA derived from these tissues, preparatory to analysis, revealed that, unlike that from the nonimmune treated mice, >90% of the total RNA from the antibody treated *ob/ob* mice was degraded to low molecular weight fragments. This is illustrated in Fig. 2*A* by the virtually complete loss of RNA (28S ribosomal RNA shown). Subsequent analysis demonstrated that adipose tissue RNA fragmentation also occurred in antibody-treated obese *db/db* mice. This response was indicative of massive cell death, suggesting that insulin promoted adipocyte survival and that disruption of the insulin signal rendered the adipocytes susceptible to cytotoxic activities. Although this degradation was consistently observed, the extent of degradation often varied from experiment to experiment.

In contrast to the obese mice, insulin depletion had no such effect on antibody treated lean animals, suggesting that the cytotoxic signal(s) was the result of the obese or the diabetic state. To ensure that the effect of the antibody was mediated by its effects on insulin, the ability of the antibody to cause RNA degradation was evaluated when coinjected with insulin. Insulin was found to completely block the cytotoxic effects of the antibody (Fig. 2*B*), indicating that acute insulin sequestration is the mode of antibody action.

To extend this observation, we evaluated the effect of insulin depletion on other tissue types by using RNA integrity as a global indicator of cell death (see below). RNA was isolated from multiple tissues of control or antibody-treated *db/db* (Fig. 2*C*) or *ob/ob* (not shown) mice, including liver and skeletal muscle, the other major insulin-regulated tissues, and brain and spleen. None of these tissues exhibited RNA degradation due to acute insulin depletion in mice that had undergone massive adipocyte cell death.

The cell death indicated by the RNA degradation is supported by histological analysis of adipose tissue from antibody treated obese animals (Fig. 3 *B–D*) as compared with tissue from control treated mice (Fig. 3*A*). Eight hr after a single injection of antibody, large gaps between cells were evident in the tissue where adipocytes had been destroyed. Furthermore, there were widespread nuclear and cellular morphological changes indicative of dead or dying cells (Fig. 3*C*). The tissue showed scattered foci of acute inflammation with adipocyte injury and associated lipid-laden macrophages. Importantly, the acute inflammatory response was restricted to adipocytes; with no visible effect on vascular or stromal cells. No vasiculitis or thrombosis was identified within the adipose tissue, indicating that cell death was not resulting from impaired blood flow. The restriction of injury to adipocytes within the adipose

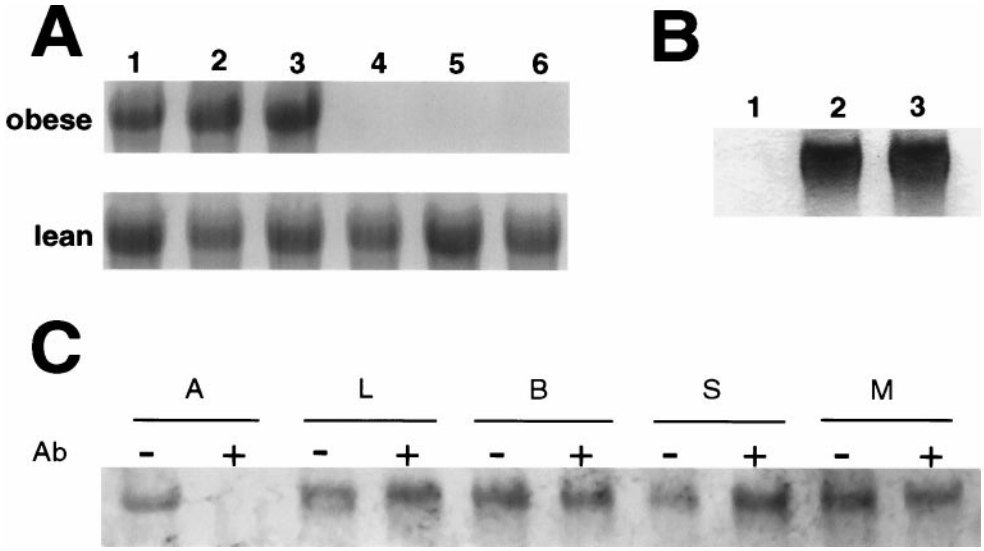

FIG. 2. Effect of insulin depletion on tissue total RNA in obese and lean. (*A*) Male C57BL/6J *ob/ob* or lean mice were given two i.v. injections of 100 μl of anti-insulin antibody (4–6) or nonimmune control serum (1–3) at 0 and 3 hr. At 6 hr, mice were killed and epididymal white adipose tissue excised and snap frozen. (*B*) *ob/ob* mice were given a single i.p. injection of 100 μl of anti-insulin antibody alone (1) or in combination with 20 (2) or 60 μg (3) of porcine insulin. After 4 hr, adipose tissue was excised and snap frozen. (*C*) C57BLS/J *db/db* mice were given a single i.p. injection of 100 μl of antibody or a saline control. Eight hr postinjection, adipose (A), liver (L), brain (B), spleen (S), and skeletal muscle (M) tissues were isolated and snap frozen. In all cases, RNA was extracted from the isolated tissues by the guanidinium thiocyanate method, separated by formaldehyde/agarose gel electrophoresis, capillary blotted, and stained with methylene blue (18). The 28S ribosomal RNA band is shown.

tissue supports the evidence from the tissue panel indicating an adipocyte-specific effect.

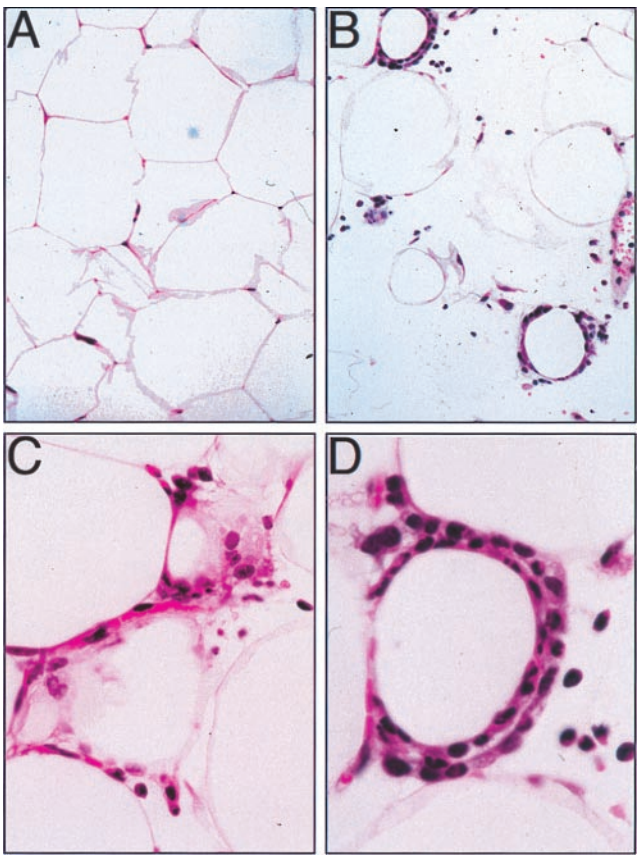

FIG. 3. Histological analysis of adipose tissue from control and anti-insulin antibody injected obese mice. Epidiymal adipose tissue was excised from control (*A*) or antibody-treated (100 μl i.p.) (*B–D*) male C57BLS/J *db/db* mice eight hr postinjection. After routine overnight fixation in neutral buffered formalin and paraffin embedding, 4-μm sections were prepared and stained with hematoxylin and eosin for light microscopy.

The inflammatory response observed in adipose tissue after anti-insulin antibody administration raised the possibility that the adipocyte cell death results from antibody binding to cell surface-associated insulin (bound to insulin receptors) and mediating cell lysis via complement fixation. It is unclear why such an effect would be specific for adipocytes as other tissues (e.g., muscle and liver) also display a high surface density of insulin receptors. However, to evaluate this possibility, *ob/ob* mice were depleted of complement by treatment with cobra venom anti-complementarity protein (4). Decomplementation had no effect on antibody-induced cell death (Fig. 3), indicating that complement is an unlikely mediator of this phenomenon.

As the primary defect of *ob/ob* and *db/db* obese animals is in leptin signaling, leptin was tested for its ability to acutely prevent this effect. Recombinant leptin (10 mg/kg body weight, Amgen) was administered to *ob/ob* mice 1 hr before anti-insulin antibody injection and RNA was isolated from adipose tissue 3 hr later. Leptin was found to have no effect on antibody-induced cell death (data not shown).

One of the signaling cascades activated by insulin is the mitogen-activating protein kinase pathway (15). Activation of this pathway has been shown to protect cells from apoptosis (16) and insulin itself has been shown to have anti-apoptotic effects in some cell types (17). Furthermore, at elevated concentrations, insulin can activate the insulin-like growth

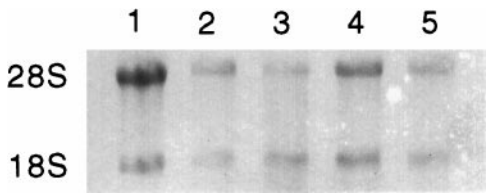

FIG. 4. Effect of complement depletion on adipocyte cell death in obese mice in response to anti-insulin antibody. C57BL/6 *ob/ob* mice were injected i.p. with either saline (2–3) or 20 units/kg body weight cobra venom anti-complementarity protein (1, 4, 5) 16 hr before injection with either saline (1) or anti-insulin antibody (2–5). After 5 hr, epidydimal fat was excised and RNA was purified, electrophoresed, blotted, and stained with methylene blue.

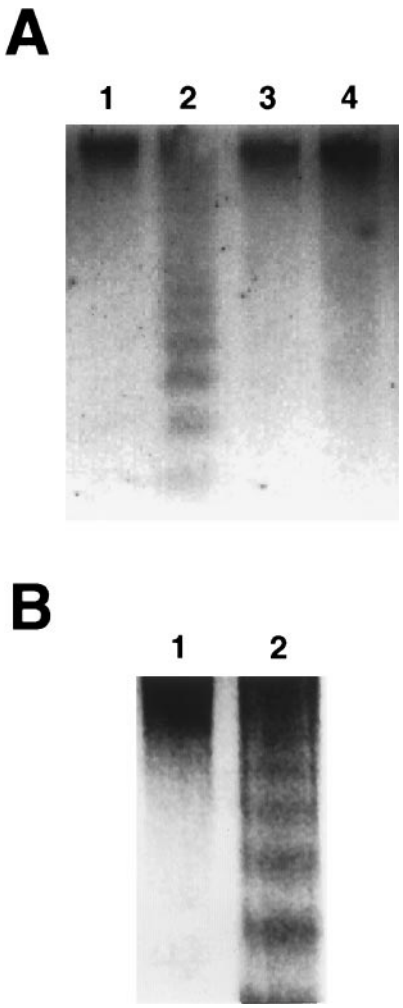

FIG. 5. DNA laddering analysis of adipose tissue DNA from insulin-depleted obese mice. (*A*) Genomic DNA was isolated from adipose tissue of control (3, 4) or anti-insulin antibody treated (1, 2) C57BL6 *ob/ob* mice described in Fig. 2*A*. (*B*). Male C57BL/6J *ob/ob* mice were injected with 200 mg/kg streptozotocin (2) or saline vehicle (1) as an i.v. bolus in 100 μl, adipose tissue was excised after 24 hr, and genomic DNA was prepared. DNA ladder was visualized by Southern blot analysis with a total mouse DNA probe.

factor receptor (18), a canonical anti-apoptotic signaling molecule (16). Conversely, tumor necrosis factor α (TNFα), which is overexpressed by adipocytes in obese individuals, promotes apoptosis of adipocytes (19). With this in mind, genomic DNA from the adipose tissue of obese mice injected with the anti-insulin antibody was analyzed for DNA laddering, a phenomenon typically associated with apoptosis. Although minimal DNA laddering was observed in many antibody-treated obese adipose samples (a representative sample is shown in Fig. 5*A*), the laddering was not always observed, and did not correlate with the extent of cell death indicated by the magnitude of RNA degradation. This finding is consistent with the histological analysis that indicated that the cell death is primarily necrotic, rather than apoptotic. It should be noted that after antibody treatment the adipose tissue undergoes rapid degradation, thus it remains possible that adipocytes are undergoing a more significant degree of apoptosis, but that this degradation interferes with its detection. Analysis of multiple tissues demonstrated that DNA laddering, when it occurred, appeared only in the adipose tissue of antibody treated mice, supporting the observation that this is an adipose specific event.

Necrosis and apoptosis differ significantly in that apoptosis is an energy requiring process. Recent evidence suggests that cell death under conditions of insufficient ATP production may default to a necrotic process (20). It is possible that a sudden complete loss of insulin signaling may trigger changes in adipocyte metabolism that result in such energy depletion and consequently necrotic cell death. However, under circumstances where insulin depletion is less precipitous, apoptosis might be favored. To test this possibility, *ob/ob* mice were given an i.v. injection of streptozotocin, a toxin that selectively destroys the β-cells of the pancreas (21) and results in a relatively slow decline in insulin levels. By 24 hr postinjection, adipose tissue is undergoing significant lipolysis and animals demonstrate signs of ketoacidosis. DNA laddering analysis of adipose tissue from these animals indicated significant apoptosis (Fig. 5*B*); however, the tissue lacked the massive RNA degradation associated with the anti-insulin-induced cell death (not shown). It should be noted that these animals were significantly compromised by secondary effects of the diabetes and the extent to which these effects influenced adipocyte death is unknown.

DISCUSSION

In light of the detrimental effects of obesity, it is not surprising that the body has developed numerous mechanisms to counter excess adipose accumulation. Many of these factors, including leptin and TNFα, are secreted by the adipocyte in proportion to adiposity and are greatly overexpressed in obesity (22). TNFα not only opposes the recruitment/differentiation of new adipocytes but also leads to dedifferentiation of mature adipocytes; furthermore, in many cell types TNFα can induce cell death. Thus some mechanism allows excess adipose tissue to persist in spite of these adipolytic signals. Obesity is usually accompanied by hyperinsulinemia and often by noninsulin dependent diabetes mellitus. Insulin is a strong adipogenic signal and is here shown to exert a protective effect on adipocytes in the obese state. Hyperinsulinemia may therefore act to preserve adipose tissue in the obese state.

The reason for the specificity of adipocyte cell death caused by acute insulin deficiency, i.e., only affecting adipocytes and only in the obese state, is unknown but may result from the compromised regulatory state of the obese adipocyte. Some aspects of metabolism in these cells are insulin resistant as a result of defects in the insulin signaling pathway (23). Furthermore, they secrete cachectic factors like TNFα that promote adipocyte loss and oppose insulin action (24, 25). In addition, in the diabetic state, numerous global hormones such as glucagon, which promotes an increased cAMP level in adipocytes, and glucocorticoids are abnormally elevated (26). Both elevated cAMP and glucocorticoid promote cell death in our hands in primary *ob/ob* adipose explants (T.M.L. and M.D.L., unpublished results).

Adipose tissue and muscle are the only tissues that express significant levels of the insulin stimulated glucose transporter. Removal of insulin may lead to a rapid drop in glucose uptake in these tissues and may result in energy depletion, a condition for which muscle is better equipped to compensate. In addition, inhibition of glucose transport or a rapid drop in glucose influx has been reported to promote cell death in some systems (27).

The drop in insulin also results in a rapid mobilization of fatty acids. High local concentrations of fatty acid can have cytotoxic effects on adipocytes. However, this is an unlikely mechanism for this effect as nicotinic acid, which inhibits lipolysis in adipocytes, appears to exacerbate the effect rather than correcting it (T.M.L. and M.D.L., unpublished results). This result is more consistent with the energy depletion model as inhibition of lipolysis would be expected to further restrict energy production.

The regulation of adipose mass in the normal individual represents a balance between adipogenic and lipolytic/cachectic signals. In the obese state, the magnitude of both the lipogenic and lipolytic signals is dramatically increased. Thus, although the removal of the adipogenic signal in the normal state would merely result in a metabolic shift, in the obese state, this may result in a massive shift of that balance and consequently cell death.

Although dramatic adipocyte cell death was only observed in obese mice, it is possible that insulin plays a subtler role in the regulation of adipocyte death even in normal individuals. Normal mice treated with streptozotocin lose the ability to secrete insulin and become diabetic (21). This is accompanied by a rapid loss of adipose tissue that has generally been attributed to lipolysis. However, in the light of this effect, the more prolonged loss of insulin in this condition may also result in adipocyte cell death. Adipocyte cell death has also been shown to occur during human weight loss. Recent reports have demonstrated that adipocyte loss can occur by apoptotic cell death and have suggested apoptosis as a mechanism in weight loss (28, 29). Among the other changes that occur during weight loss is a significant reduction in serum insulin and this may play a role in the adipocyte loss.

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