Transferrin and Iron Contribute to the Lipolytic Effect of Serum in Isolated Adipocytes

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Previous reports have demonstrated that normal serum can increase the rate of adipocyte lipolysis in vitro. However, the nature of the lipolytic activity has remained obscure. We have investigated the lipolytic activity of human serum using isolated rat adipocytes. Human serum resulted in a dose-dependent stimulation of lipolysis (glycerol release) in adipocytes, with a half-maximal effective dose of 0.05% serum and with maximal stimulation with 1% serum. The effect of serum on glycerol release was rapid (within 30 min), and the effect was reversible. Partial purification of this lipolytic activity using gel filtration and ion-exchange chromatography demonstrates that a protein of ~80 kDa contributes to the lipolytic activity. Human transferrin mimicked the activity of partially purified serum, resulting in a maximal 50% increase in basal lipolysis. In addition, ferrous sulfate heptahydrate induced a biphasic increase in the rate of lipolysis, with a maximal increase of 50% at ~0.6 μg/ml iron. Inhibitors of protein kinase A (H89) and mitogen-activated protein kinase (PD98059) did not block the effect of serum on lipolysis, whereas the free radical scavenger *N*-acetyl-L-cysteine completely inhibited the effect. These findings suggest that the stimulatory effect of serum on lipolysis is in part mediated by iron, probably through a prooxidant mechanism. *Diabetes* 53:2535–2541, 2004

Adipocyte lipolysis is the major source of circulating free fatty acids (FFAs) in the postprandial state. Furthermore, excess FFA production is increasingly recognized as a major contributor to the medical consequences of being overweight or obese. FFAs increase hepatic glucose output (1,2) and inhibit glucose uptake by skeletal muscle (3), thus contributing to the insulin resistance syndrome, which is a major risk factor for diabetes and contributes to the increased cardiovascular risk associated with obesity. FFAs also increase hepatic VLDL synthesis, contribute to hypertriglyceridemia and hence lower HDL, and may contribute to the development of hypertension (rev. in 4,5). For these reasons, identification of circulating factors that can affect adipocyte lipolysis may yield new insight into the etiology of insulin resistance and type 2 diabetes and related disorders.

It has been known for >30 years that the addition of normal serum to isolated adipocytes can increase the rate of lipolysis (6–9), demonstrating the presence of lipolytic factor(s) in serum. However, the underlying mechanism of this lipolytic effect of serum and the nature of the serum factor(s) responsible for the effect has remained obscure. Importantly, the activity was previously reported (8) to be partially but not completely removed by ultrafiltration, suggesting that there may be more than one component of this lipolytic activity.

In this report, we have characterized the lipolytic activity of human serum and determined that one component of this activity is likely transferrin and the associated iron. These effects of transferrin and iron account for ~50% of the lipolytic activity of serum. This finding may contribute to the recently identified strong relationship between iron stores, iron metabolism, and diabetes (10–12).

RESEARCH DESIGN AND METHODS

BSA was from Intergen (Purchase, NY), collagenase type 2 was from Worthington (Freehold, NJ), and human serum was from SeraCare Life Sciences (Oceanside, CA). All other reagents were from Sigma (St. Louis, MO) unless otherwise noted.

Male Sprague-Dawley rats were used for all experiments. Animals (180–240 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). They were maintained on a 12-h light-dark cycle and fed Prolab RMH 1,000 (PMI Nutrition, Brentwood, MO) and tap water ad libitum.

Adipocyte isolation. Animals were killed by CO_2 asphyxiation. Animal protocols were approved by Bassett Healthcare's Institutional Animal Care and Use Committee. Adipocytes were isolated from epididymal fat pads as previously described (13,14). Digestion was carried out at 37°C with constant shaking (140 cycles/min) for 26 min. Cells were filtered through nylon mesh (1 mm) and washed three times with incubation buffer (IB) containing 137 mmol/l NaCl, 5 mmol/l KCl, 4.2 mmol/l $NaHCO_3$, 1.3 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, 0.5 mmol/l $MgSO_4$, 0.5 mmol/l KH_2PO_4, and 20 mmol/l HEPES (pH 7.6), plus 1% BSA.

Adipocyte treatment. After isolation, adipocytes were resuspended 1:20 (wt/vol) in IB, and 1-ml aliquots were incubated with shaking (140 cycles/min) in 12 × 75-mm polypropylene tubes containing serum and/or other additions as indicated for 1 h (unless otherwise indicated) at 37°C.

Lipolysis assay. Lipolysis was measured by following glycerol release, as previously described, using a kit from Sigma (15).

Purification of serum lipolytic activity. For gel filtration chromatography, human serum was diluted 1:1 in HEPES-buffered saline (HBS), pH 7.6, and applied to a Sephadex G200 (Pharmacia, Uppsala, Sweden) column. Proteins were eluted with HBS at 5 ml/h and collected in 30-min fractions. For ion-exchange chromatography, human serum was diluted 1:1 in HBS and loaded onto a Q Sepharose fast flow column (Pharmacia). After loading, protein was eluted with a 150- to 600-mmol/l NaCl gradient at 18 ml/h and collected in 5-min fractions. Protein concentrations of fractions were determined by the method of Bradford (16) using a kit from BioRad (Hercules, CA).

SDS-PAGE and silver staining. SDS-PAGE gels were prepared using the BioRad Mini-Protean II Gel Electrophoresis kit according to the manufactur-

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FFA, free fatty acid; HBS, HEPES-buffered saline; HNE, 4-hydroxy-2-nonenal; IB, incubation buffer; LMF, lipid-mobilizing factor; MAP, mitogen-activated protein; NALC, *N*-acetyl-L-cysteine.

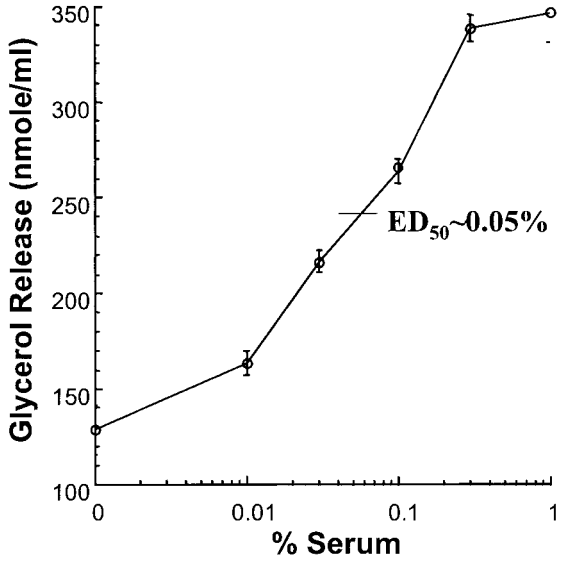

FIG. 1. Effect of human serum on lipolysis. Isolated adipocytes were incubated at 37°C with various concentrations of human serum, as indicated. After a 2-h incubation, glycerol release was measured. Data are mean ± SE (n = 3). ED_{50}, half-maximal effective dose.

er's protocol. Purified fractions (1 μg) were resolved on 7.5% SDS-PAGE gels and then silver stained using the ProteoSilver Silver Stain kit (Sigma).

Identification of oxidized proteins. Exposed carbonyls of oxidized cytosolic and membrane proteins were detected by Western analysis after derivatization with 2,4-dinitrophenylhydrazine using the Oxyblot Protein Oxidation Detection kit (Chemicon, Temecula, CA) according to the manufacturer's instructions.

RESULTS

Effect of serum on lipolysis. To investigate the effect of serum on lipolysis, isolated rat adipocytes were incubated for 2 h with normal human serum at concentrations ranging from 0 to 1% by volume. Glycerol release was then measured to determine the rate of lipolysis. As shown in Fig. 1, small amounts of serum more than doubled the rate of lipolysis in these cells, with a half-maximal effect occurring in the presence of only 0.05% serum. The effect was maximal in the presence of ~1% serum.

To determine the rate of onset of the serum effect, adipocytes were incubated with or without serum, and glycerol accumulation was measured over time (Fig. 2). Under these conditions glycerol release was linear with time for up to 3 h, indicating that the rate of lipolysis was constant. The rate of glycerol release was higher in the presence of either 0.1 or 1% serum at the earliest time measured (30 min). Furthermore, the higher rate of lipolysis was maintained for the 3-h duration of the experiment. This finding demonstrates that the onset of the lipolytic effect of serum is very rapid.

Studies were then performed to determine whether the effect of serum is reversible. In Fig. 3, adipocytes were treated with or without 1% serum for 1 h to allow maximum serum stimulation. The cells were then washed, and the rate of glycerol release was determined over the next 3 h. The serum-stimulated rate did not decline measurably for the first 30 min after serum was removed, but then returned to control values by 3 h, demonstrating that the lipolytic effect of serum is readily reversible.

Partial purification of serum lipolytic activity. In order to purify the serum lipolytic activity, several prelim-

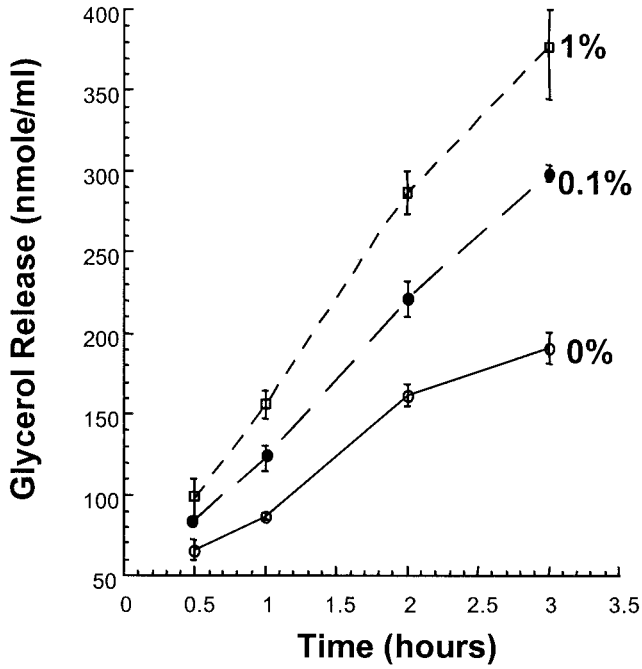

FIG. 2. Time course of the lipolytic effect of serum. Isolated adipocytes were incubated at 37°C with 0 (○), 0.1 (●), or 1% (□) human serum. At the indicated times, glycerol release was measured. Data are mean ± SE (n = 3).

inary studies were performed. First, the activity was not removed from serum after prolonged dialysis and was precipitable with polyethylene glycol, suggesting that the activity is due to one or more protein components of serum (data not shown). Second, ultrafiltration studies confirmed observations made by Curtis-Prior in 1973 (8)

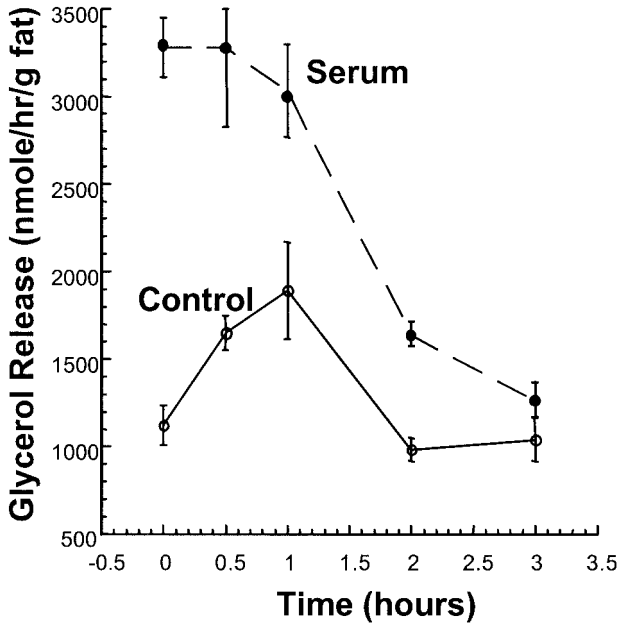

FIG. 3. Reversibility of the serum effect. Isolated adipocytes were incubated with (●) or without (○) 1% serum at 37°C for 1 h, and then an aliquot of media was taken for measurement of glycerol release. Cells were then washed into fresh medium and incubated for 30 min, and another aliquot was taken for measuring glycerol release. Media were replaced, and aliquots were taken at 1, 2, and 3 h. After 3 h, glycerol release was measured on all samples. Data are mean ± SE (n = 4).

that the lipolytic activity of the serum was divided between concentrate and ultrafiltrate, irrespective of the "cutoff " value of the membranes used. Similarly, the activity was partially precipitable with ammonium sulfate (data not shown). Together, these preliminary studies suggest that the lipolytic activity of serum is due to either multiple forms of lipolytic proteins or a small molecule that can bind to a protein without losing activity.

In order to purify the polyethylene glycol–precipitable serum activity further, human serum was diluted 1:1 in HBS and loaded onto a Sephadex G200 column (Fig. 4*A*). Fractions from the G200 column were assayed for lipolytic activity by measuring their effect on adipocyte glycerol release. Two major activity peaks were observed eluting from the G200 column. The first peak coincided with the column void volume, and a second major peak coeluted approximately with the immunoglobulins, but before albumin. The activity is, however, not attributable to immunoglobulins. First, the peak is quite broad. Furthermore, depletion of IgG from serum with protein A agarose did not deplete the activity, and IgG alone did not mimic the effect of serum (data not shown).

In an effort to achieve greater purification of the lipolytic protein, activity was purified from human serum using a Q Sepharose column (Fig. 4*B*). Protein was eluted using a 150- to 600-mmol/l NaCl gradient, with the major activity eluting at ~300 mmol/l NaCl. Two bands, as assessed by silver staining (Fig. 4*C*), with apparent molecular weights of 75 and 80 kDa correlated with the appearance of lipolytic activity in the Q Sepharose eluate. The other major band of ~66 kDa is likely to be albumin, the major serum protein.

Role of iron in serum lipolytic activity. Based on the molecular weight of the bands eluted from Q Sepharose, we hypothesized that the 80-kDa band is transferrin. This was investigated by the use of purified human transferrin. Treatment of isolated adipocytes with human transferrin resulted in a dose-dependent increase in lipolysis (Fig. 5). Transferrin at a concentration of 30 μg/ml resulted in an ~35% increase in the rate of lipolysis. This corresponds well with the concentration of transferrin that would be expected to result from the addition of 1% serum because the concentration of transferrin in normal serum is ~3 mg/ml. Therefore, transferrin likely is a component of the total lipolytic activity of normal serum.

We next evaluated whether iron in the form of ferrous sulfate could stimulate lipolysis in adipocytes (Fig. 6). Like transferrin, iron II resulted in a dose-dependent increase in lipolysis, with a maximum 50% increase over basal. Unlike transferrin, the effect of iron was biphasic, with the peak lipolytic concentration at 3 μg/ml ferrous sulfate heptahydrate (which corresponds to ~0.6 μg/ml elemental iron). Thus iron itself appears to be sufficient to stimulate lipolysis to the same extent as transferrin. Furthermore, the effect of iron was not additive to that of transferrin (data not shown), suggesting that iron and transferrin stimulate lipolysis through the same mechanism.

Potential mechanisms of serum lipolytic activity. To investigate the mechanism by which serum stimulates lipolysis, we incubated adipocytes with inhibitors of protein kinase A or mitogen-activated protein (MAP) kinase. In Fig. 7*A*, adipocytes were treated with 2% serum or with isoproterenol at a concentration chosen to stimulate lipolysis to a comparable rate (3 nmol/l isoproterenol). As expected, H89, an inhibitor of protein kinase A, completely

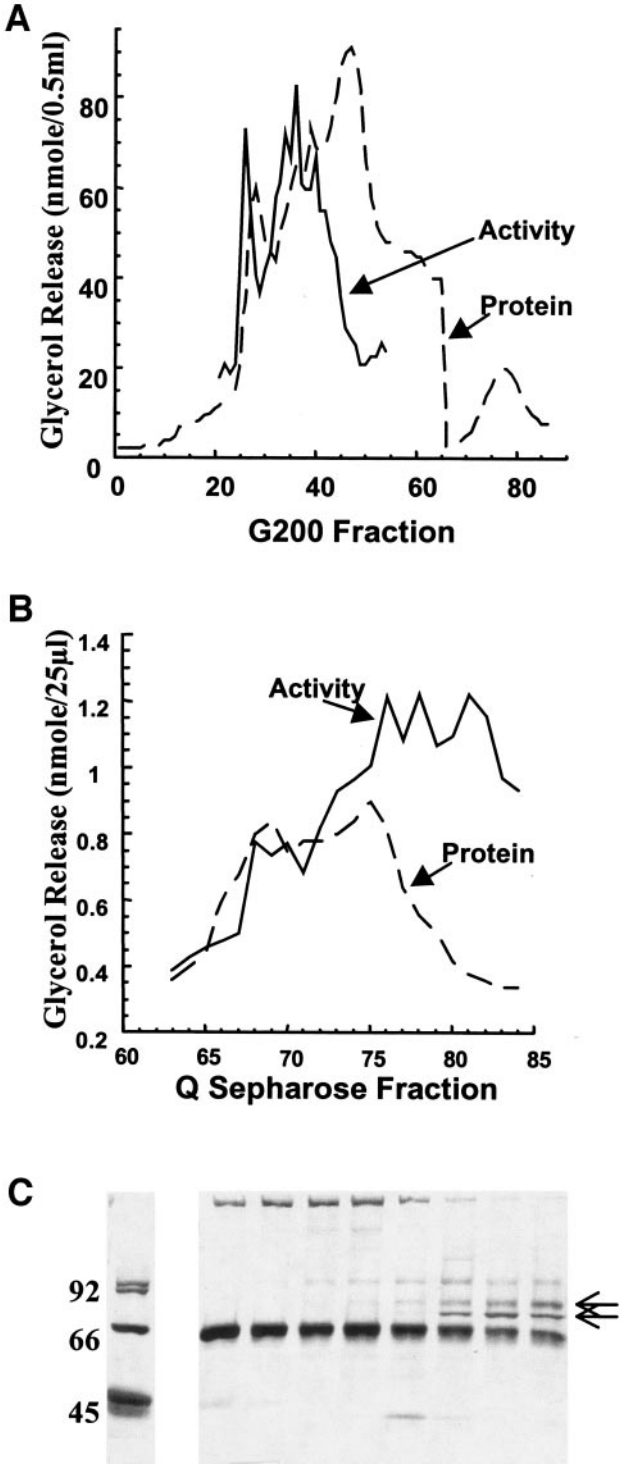

FIG. 4. Partial purification of serum lipolytic activity. *A*: Human serum was fractionated on a G200 column. Isolated adipocytes were incubated with 20 μl of G200 fractions at 37°C for 2 h, and glycerol release (in nanomoles per 0.5 ml) was measured. Lipolytic activity was plotted, with total protein represented by relative absorbance at A_{280nm}. *B*: Serum was fractionated on a Q Sepharose column using a 150- to 600-mmol/l NaCl gradient. Lipolytic activity from 20 μl of fractions was expressed as glycerol release (in nanomoles per 25 μl) and plotted with total protein represented by relative absorbance at A_{280nm}. *C*: Q Sepharose fractions (1 μg) were resolved using SDS-PAGE and the protein visualized by silver staining.

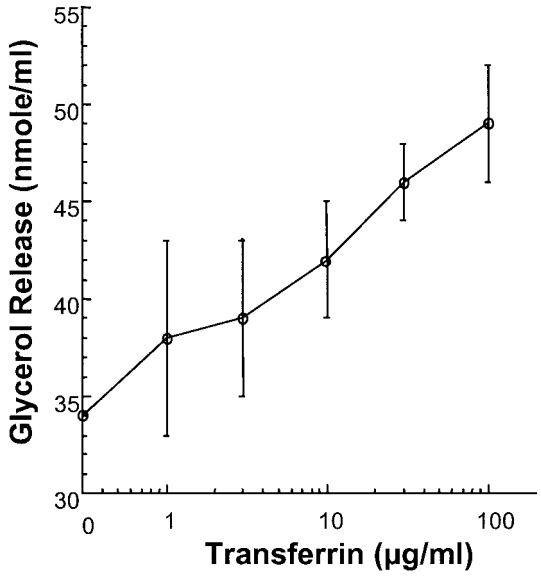

FIG. 5. Effect of human transferrin on lipolysis. Isolated adipocytes were incubated at 37°C for 1 h with various concentrations of human transferrin as indicated, and then glycerol release was measured. Data are mean ± SE (n = 3).

blocked the action of isoproterenol. However, H89 did not affect the lipolytic activity of serum. Similarly, the MAP kinase inhibitor, PD98059, did not prevent the effect of serum on lipolysis (Fig. 7*B*). Further experiments (not shown) demonstrated that the effect of serum was also not blocked by the protein kinase C inhibitor, staurosporine, or the phosphatidylinositol 3-kinase inhibitor, wortmannin. Therefore, the effect of serum does not seem to involve the cAMP, MAP kinase, protein kinase C, or phosphatidylinositol 3-kinase pathways.

Because iron appears to play a role in the serum stimulation, we hypothesized that the lipolytic activity might be due to the prooxidant effects of iron. Iron is known to catalyze a wide range of free radical species, including hydroxyl radicals, via the Fenton reaction. In Fig. 8 we measured the rate of serum-stimulated lipolysis in the presence or absence of the free radical scavenger, *N*-acetyl-L-cysteine (NALC). NALC (3 mmol/l) completely prevented the effect of serum on lipolysis, but did not affect the threefold stimulation by 3 nmol/l isoproterenol. Therefore, the lipolytic effect of serum may be mediated in part by prooxidant effects of iron and/or other components of serum. To further investigate this possibility, we oxidatively quantified modified proteins in control and serum-treated cells. Many types of oxidative damage to proteins result in the formation of carbonyl groups (17). We therefore treated extracts from control and serum-treated cells (described in RESEARCH DESIGN AND METHODS) to convert these carbonyl groups to their dinitrophenylhydrazine derivatives. These modified proteins were then detected by Western blot, using an antibody that recognizes the dinitrophenylhydrazine moieties. However, although we detected a variety of oxidatively damaged proteins, we were unable to detect any difference between serum- or transferrin-treated cells and untreated adipocytes (data not shown). This suggests that either the prooxidant effects of iron are not responsible for the lipolytic effect or that the differences were too subtle to detect using this method.

Hydroxyl radicals generated by the Fenton reaction can initiate lipid peroxidation. Lipid peroxidation has been reported (18) to increase the basal rate of lipolysis in adipocytes. 4-Hydroxy-2-nonenal (HNE), a byproduct of lipid peroxidation, has been reported (19) to be a signaling molecule in the regulation of mitochondrial function. Therefore, we determined the effect of HNE on lipolysis compared with serum (Fig. 9). This experiment revealed that low concentrations of HNE mimic the effect of serum on lipolysis. This finding provides further evidence that serum may act through a prooxidant mechanism by initiating lipid peroxidation.

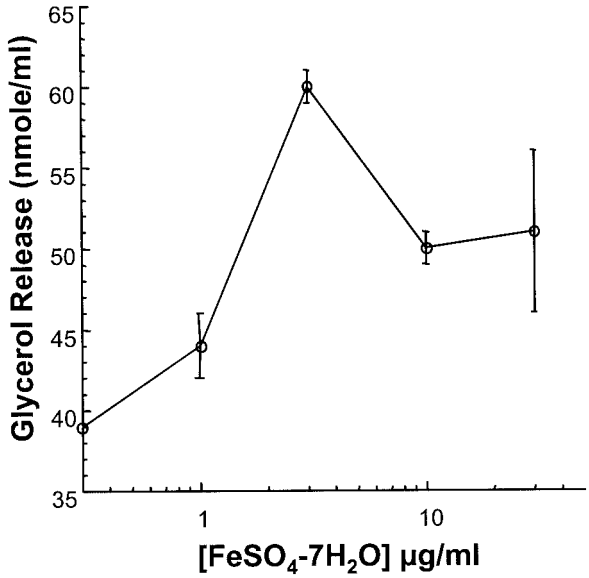

FIG. 6. Effect of iron on lipolysis. Isolated adipocytes were incubated at 37°C for 1 h with various concentrations of ferrous sulfate heptahydrate, and then glycerol release was measured. Ferrous sulfate heptahydrate contains 20.1% iron by weight. Data are mean ± SE (n = 3).

DISCUSSION

We have demonstrated that the addition of very small amounts of normal human serum to rat adipocytes results in a pronounced increase in the rate of lipolysis. Although this observation was first reported in the 1970s (6–9), earlier studies used much greater concentrations of serum. In addition, the underlying mechanism of this lipolytic effect of serum, and the nature of the serum factor(s) responsible for the effect, has remained obscure.

From the present studies it is clear that at least part of the lipolytic effect of serum is due to one or more high–molecular-weight proteins. This is evidenced by the partial purification of the activity by gel filtration and the fact that the activity survives dialysis. We have eliminated a number of proteins that could explain this effect. First, the effect is clearly not due to immunoglobulins because the effect was not mimicked by IgG, neither was it eliminated by treatment of the serum with protein A agarose.

Tisdale and colleagues (20,21) have described a "lipid-mobilizing factor" (LMF) that is produced by tumors, and we have considered the possibility that LMF is responsible for the lipolytic effect of serum. However, LMF is ~43 kDa, is heat labile, and its effect is blocked by the protein kinase

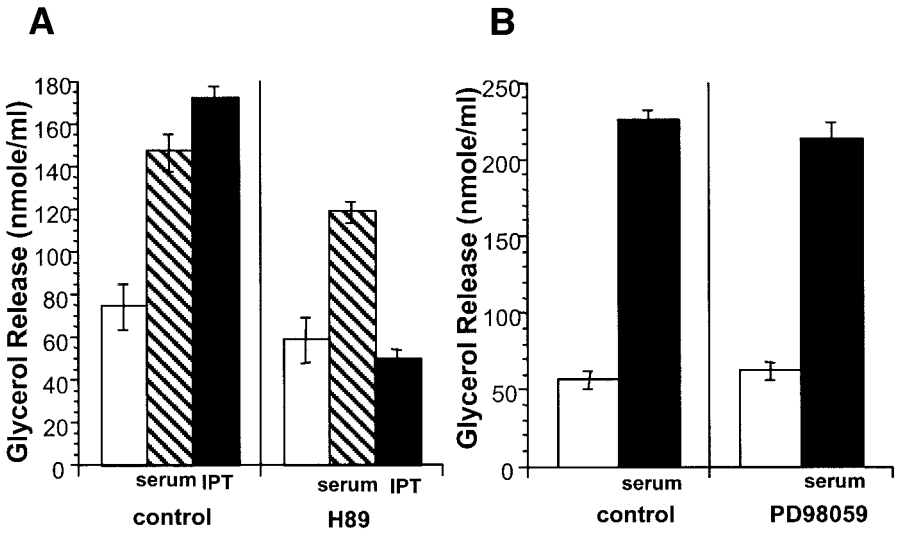

FIG. 7. Effect of inhibitors of protein kinase A (*A*) and MAP kinase (*B*). *A*: Isolated adipocytes were incubated at 37°C for 1 h with no additions (□), 2% human serum (serum, ▨), or 3 nmol/l isoproterenol (IPT, ■) in the presence or absence of 50 μmol/l H89. *B*: Isolated adipocytes were incubated at 37°C for 2 h with 1% serum (serum, ■) in the presence or absence of 100 μmol/l PD98059. After incubation, glycerol release was measured. Data are mean ± SE (n = 3).

A inhibitor, H89 (21). By contrast, the protein we are describing is larger, is very heat stable (data not shown), and its action is not prevented by H89. Another possibility would be cytokines such as tumor necrosis factor-α, but these are much smaller than the protein(s) we are describing. Furthermore, these cytokines circulate at very low concentrations in normal serum (22), and their action on lipolysis is very slow to develop (23–26). We therefore believe that the lipolytic action of serum is due to one or more large protein(s) that may be important in the regulation of fat cell metabolism.

The high molecular weight of the lipolytic activity raises the question of whether it would have access to the interstitial space. However, we have found that the effect of serum is maximal at a concentration of only 1%, i.e., when serum was diluted 1 to 100. This suggests that these factors circulate at ~100 times the maximally effective concentration for stimulation of adipocyte lipolysis. Therefore, it is likely that enough would find its way to the adipocytes to regulate their activity in vivo. For example, transferrin has been shown to be a component of the serum lipolytic activity and is known to have access to the interstitial space.

The importance of regulation of lipolysis in determining overall glucose metabolism is becoming increasingly recognized. In 1963, Randle et al. (3) proposed that glucose uptake in skeletal muscle is inhibited by FFAs. Although this was controversial for many years, there is now overwhelming evidence that circulating FFAs have many actions that oppose insulin in humans in vivo (27–32). In addition to the effect originally identified by Randle et al., and often referred to as the "glucose–fatty acid cycle," it is now established that FFAs increase hepatic glucose output (1,27,31,33) and can have effects on the pancreas (2,30).

The effects of FFAs on glucose metabolism are probably important in normal physiology. For example, during fasting, the ability of FFAs (and ketone bodies) to inhibit glucose metabolism probably "spares" glucose for those tissues that require glucose for survival, in particular the brain, erythrocytes, and kidney medulla. However, in obese subjects with excess adipose tissue stores, it is

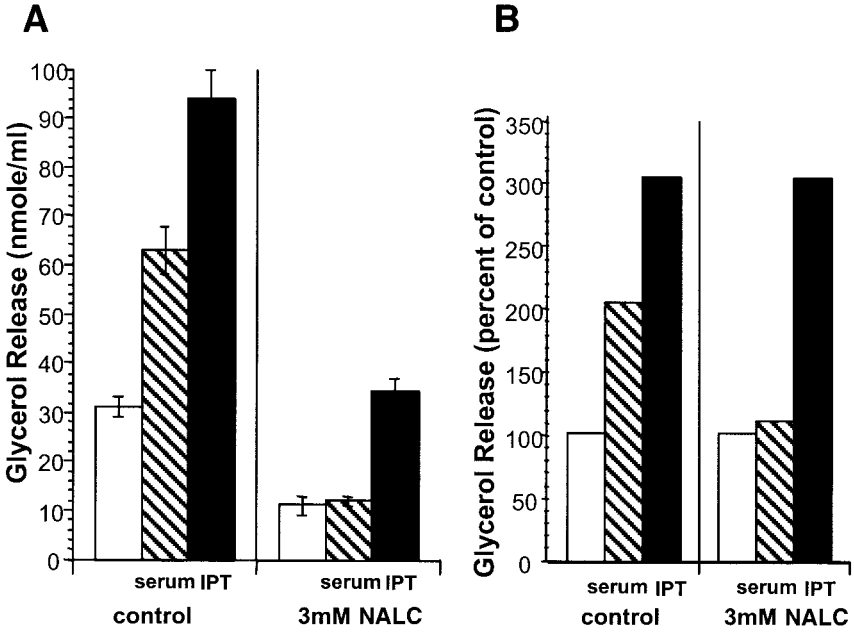

FIG. 8. NALC inhibits the effect of serum on lipolysis. Isolated adipocytes were incubated at 37°C for 1 h with no additions (□), 1% serum (▨), or 3 nmol/l isoproterenol (IPT, ■) in the presence or absence of 3 mmol/l NALC, and then glycerol release was measured. Data are mean ± SE (n = 3). *A:* Absolute rates of glycerol release. *B:* Data are expressed as percentage of the rate determined in the absence of serum or isoproterenol.

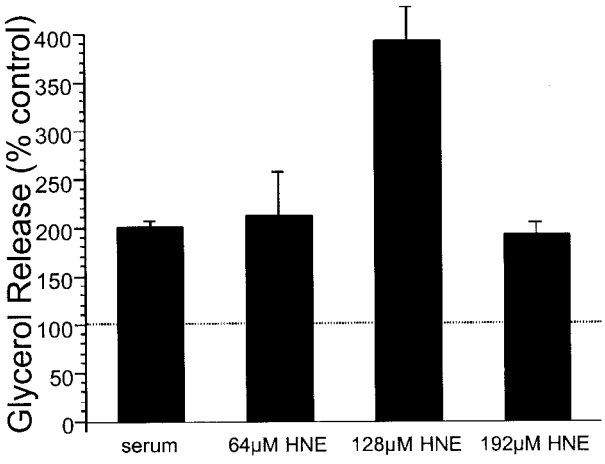

FIG. 9. Effect of the lipid peroxidation byproduct HNE on lipolysis. Isolated adipocytes were incubated at 37°C for 1 h with 1% serum or the indicated concentrations of HNE, and then glycerol released was measured. Averaged values (n = 3) from a representative experiment are graphed as a percentage of control ± SE.

likely that the resulting excess FFA is deleterious, especially with respect to insulin resistance and the development of type 2 diabetes. Therefore, an understanding of mechanisms responsible for the regulation of lipolysis may lead to new therapeutic approaches for the treatment of insulin resistance and type 2 diabetes and/or to prevention of diabetes in at-risk subjects. Therefore, determining the identity of serum lipolytic proteins, elucidating their mechanism of action, and determining whether their activities are altered in obese or diabetic individuals may lead to new insights into the mechanism or treatment of insulin resistance.

These findings demonstrate that ~50% of the lipolytic effect of serum can be accounted for by transferrin, an effect that can be mimicked by free iron. The mechanism by which iron stimulates lipolysis is unclear but may be related to its prooxidant effects. Iron can catalyze the formation of free radicals, including hydroxyl radicals, through the Fenton reaction. Hydroxyl radicals can initiate lipid peroxidation, which has been shown (18) to increase basal lipolysis. The free radical scavenger NALC completely prevented the stimulation of lipolysis by serum, suggesting that a prooxidant mechanism may be involved. Furthermore, NALC decreased basal lipolysis in these cells, suggesting a baseline level of oxidative stress that may be important in the regulation of lipolysis. However, we were unable to determine any difference in the concentration of oxidatively modified proteins between serum or transferrin-treated cells compared with control cells. This suggests that either the prooxidant effects of iron are not responsible for the lipolytic effect or that the differences were too subtle to detect by this method. Conversely, in support of the idea that a prooxidant mechanism is involved, we found that the lipid peroxidation byproduct HNE, which is believed to be a signaling molecule in certain systems (19), mimicked the effect of serum. Clearly, further work will be required to elucidate the relationship between the prooxidant activity of iron and its effect on lipolysis.

Our findings have eliminated most of the known signaling pathways responsible for regulation of lipolysis. First, the fact that the effect is not blocked by H89 (an inhibitor of protein kinase A) rules out involvement of cAMP and hence the classic pathway for β-adrenergic receptor–mediated stimulation. Similarly, the effect was not inhibited by staurosporine or wortmannin, effectively eliminating the involvement of protein kinase C or phosphatidylinositol 3-kinase. It is also possible that serum increases the concentration of one or more cytokines, many of which increase adipocyte lipolysis (34). However, the latter explanation is unlikely because the cytokines take several hours to stimulate lipolysis, whereas the effects we report occur much faster. Clearly more work will be needed to elucidate the mechanism(s) involved in the effects we report here.

Our findings demonstrate that both transferrin and free iron increase the rate of lipolysis in adipocytes. However, these effects were not additive, suggesting that they work through the same mechanism. Calculations from the published dissociation constant for iron binding to transferrin would predict that the concentration of free iron in normal serum would be on the order of 10^{-19} μg/ml (35). In our hands, ultrafilterable iron concentrations in serum samples were less than the lower limit of our assay (0.02 μg/ml), whereas the effective concentration of iron in our lipolysis assays was ~0.6 μg/ml. Therefore it is much more likely that iron bound to transferrin is the physiologically relevant form for stimulation of lipolysis. It is well established that adipocytes express transferrin receptors (36), and so it is likely that serum iron is taken up by adipocytes in its transferrin-bound form through receptor-mediated endocytosis.

There is much evidence in the literature (11) for a relationship between iron metabolism and type 2 diabetes. For example, blood letting has been shown (10) to improve insulin resistance and β-cell function in high-ferritin diabetes. Furthermore, a recent report from the Nurses' Health Study (12) has revealed a strong positive relationship between iron stores and risk of type 2 diabetes. Iron overload appears to decrease liver insulin sensitivity and skeletal muscle glucose metabolism (11), perhaps contributing to development of diabetes. The findings presented here suggest another mechanism by which excess iron may worsen insulin resistance, i.e., by increasing lipolysis, raising circulating FFAs, and consequently contributing to insulin resistance by the mechanisms described above.

In summary, these findings demonstrate that normal human serum contains high–molecular-weight components, including transferrin and associated iron, that increase the rate of lipolysis in isolated adipocytes. Further studies will be needed to elucidate the mechanism(s) by which transferrin and iron regulate lipolysis and to identify other lipolytic components of normal serum.

ACKNOWLEDGMENTS

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