Lipolysis is an important process determining fuel metabolism, and insulin regulates this process in adipose tissue. The aim of this study was to investigate the long-term effects of insulin, an insulin enhancer (rosiglitazone [RSG]), and insulin in combination with RSG on the regulation of lipolysis and lipogenesis in human abdominal subcutaneous fat. Lipolysis and lipogenesis were assessed by protein expression studies of hormone-sensitive lipase (HSL) (84 kDa) and lipoprotein lipase (LPL) (56 kDa), respectively. In addition, lipolytic rate was assessed by glycerol release assay and tumor necrosis factor (TNF)-α release measured by enzyme-linked immunosorbent assay (n = 12). In subcutaneous adipocytes, increasing insulin doses stimulated LPL expression, with maximal stimulation at 100 nmol/l insulin (control, 1.0 ± 0.0 [mean ± SE, protein expression relative to control]; 1 nmol/l insulin, 0.87 ± 0.13; 100 nmol/l insulin, 1.68 ± 0.19; P < 0.001). In contrast, insulin at the 100 nmol/l dose reduced the expression of HSL (100 nmol/l insulin, 0.49 ± 0.05; P < 0.05), while no significant reduction was observed at other doses. Higher doses of insulin stimulated both HSL (1,000 nmol/l insulin, 1.4 ± 0.07; P < 0.01) and LPL (control 1.00 ± 0.0; 1,000 nmol/l insulin, 2.66 ± 0.27; P < 0.01) protein expression. Cotreatment with RSG induced an increased dose response to insulin for LPL and HSL (P < 0.05); RSG alone also increased LPL and HSL expression (P < 0.05). Insulin stimulated TNF-α secretion in a dose-dependent manner (P < 0.01); the addition of RSG (10−8 mol/l) reduced TNF-α secretion (P < 0.05). In summary, chronic treatment of human adipocytes with insulin stimulates lipolysis and LPL protein expression. The addition of RSG reduced the lipolytic rate and TNF-α secretion. The increase in lipolysis is not explained by changes in HSL expression. These data, therefore, may explain in part why hyperinsulinemia coexists with increased circulating nonesterified free fatty acids and increased adiposity in obese and/or type 2 diabetic patients. Diabetes 51:1493–1498, 2002
triglyceride and NEFA concentrations (12). Previous in vitro studies have also shown that in murine 3T3-L1 adipocytes, RSG does not influence basal or catecholamine-stimulated lipolysis but can block tumor necrosis factor (TNF)-α-mediated NEFA release (13). The ability of TZDs to modulate the antilipolytic effect of insulin in human adipocytes in vitro is currently unknown.

In humans, lipogenesis is regulated by a number of enzymes, but, because the major determinant of lipogenesis is re-esterification of NEFAs after hydrolysis of circulating triglycerides, the activity of adipose tissue lipoprotein lipase (LPL) (56 kDa) is of primary importance. LPL activity is regulated by several hormones including catecholamines, insulin, and steroids, as well as by certain cytokines (8,14,15), and serves as an appropriate marker of the overall lipogenic capacity of adipose tissue.

The aim of this study was to define the effects of insulin on lipolysis and lipogenesis in cultured adipocytes prepared from human abdominal subcutaneous adipose tissue. The effects of chronic hyperinsulinemia were compared with acute actions of insulin. Second, we investigated the effects of rosiglitazone on lipolysis and lipogenesis and determined whether rosiglitazone may sensitize the adipocyte to the effects of insulin. To determine lipolysis, HSL protein expression was used in conjunction with direct measurements of lipolysis. LPL protein expression was measured as an index of lipogenic potential.

RESEARCH DESIGN AND METHODS

Subjects. Subcutaneous abdominal adipose tissue was obtained from 12 female nondiabetic subjects (age 46.2 ± 7.3 years [mean ± SD]; BMI 26.90 ± 3.93 kg/m²). All patients were undergoing elective surgery; samples were collected with the approval of the South Birmingham Ethics Committee. Subjects on endocrine therapy (e.g., steroids, HRT, thyroxine) or antihypertensive therapy and patients with malignant diseases were excluded.

Mature adipocyte isolation and cell culture. In brief, 10–20 g wet wt fresh abdominal subcutaneous adipose tissue was collected and processed to produce isolated mature adipocytes as previously described (16). Isolated mature adipocytes were subsequently cultured in flasks (25 cm²) in phenol red–free Dulbecco’s modified Eagle’s medium (DMEM):F12 medium containing 15 mmol/l glucose, penicillin (100 U/ml), and streptomycin (100 μg/ml). Compacted 1-ml aliquots of adipocytes (500,000 cells) were maintained in medium (5 ml) for 48 h and treated once with insulin alone (1–1,000 μmol/l) (human recombinant insulin expressed in Escherichia coli; Sigma, Poole, U.K.), RSG alone (10⁻⁸ to 10⁻¹⁰ mol/l) (GlaxoSmithKline, Harlow, U.K.), and insulin (1–1,000 nmol/l) in combination with RSG (10⁻⁸ mol/l). As controls, adipocytes were maintained in unsupplemented medium for 48 h. Viability of adipocytes was assessed using trypan blue (Sigma) as previously described (16). After treatment, the medium and adipocytes were separated by centrifugation (360g for 2 min). The medium was removed, aliquoted, and stored at −70°C.

Lipolysis studies on chronically treated adipocytes. The short-term influence of norepinephrine (Sigma) and insulin on lipolysis was assessed in cells that had been chronically treated with insulin or RSG for 47 h. Adipocytes treated as described above were washed with PBS and centrifuged (360g for 1 min). The adipocytes were then aliquoted into 25-cm² flasks with fresh medium, and the respective insulin and RSG treatments were restored. A parallel set of cells was incubated with norepinephrine (1 μmol/l) for 1 h. Thus all cell populations were incubated for a total of 48 h. The percentage of adipocyte lysis was taken into account when reseeding the cells for the further 1 h incubation. After treatment, culture medium and adipocytes were separated by centrifugation (360g for 2 min). Conditioned medium was aliquoted and stored at −70°C. In addition, the acute effect of insulin on lipolysis in the mature adipocytes was also examined. freshly isolated mature adipocytes were incubated with medium and treated with varying insulin doses (1–1,000 nmol/l) in the presence or absence of norepinephrine (1 μmol/l) for 1 h. After treatment, culture medium and cells were separated and stored as described above.

Effect of rosiglitazone (RSG) on adipocyte protein expression. After removal of the medium, boiling 4% SDS was added to the adipocytes. The suspension was then heated for 2 h at 95°C, until the adipocytes had dissolved (17). The resultant extracted proteins were stored at −70°C.

Protein assay. Protein concentrations were determined using the Bio-Rad detergent-compatible protein assay kit (Bio-Rad, Hemel Hempstead, U.K.) (18). Adipocyte protein samples were assessed to confirm that there was no significant effect of individual treatments on adipocyte protein content, which was further confirmed by the use of an actin antibody (Biogenesis, Poole, U.K.) in Western blotting (data not shown).

Western blotting. Western blot analysis was performed using a method previously described (19). In brief, after gel electrophoresis and electrobiasing, filters were incubated overnight at 4°C with continual motion, using specific primary antibodies (1:5,000 for HSL and 1:500 for LPL). Detection of HSL and LPL was achieved using horseradish peroxidase–conjugated secondary antibodies (Calbiochem, La Jolla, CA) diluted 1:60,000 and 1:2,500, respectively, in PBS with 0.05% Tween. A chemiluminescence detection system (ECL/ECL+; Amersham, Little Chalfont, U.K.) enabled visualization after exposure to X-ray film. Autoradiographs were quantified by densitometry using a Gelbase/Gelblot program (UVP, Cambridge, U.K.).

Lipolysis studies. Stored conditioned medium samples were used to assay glycerol production as a measure of lipolysis (micromoles per milliliter) using a commercially available colorimetric kit (Randox Laboratories, Antrim, U.K.).

TNF-α assessment. TNF-α levels were determined as a factor influencing lipolysis (20). A commercially available high-sensitivity enzyme-linked immunosorbent assay (ELISA)-based colorimetric kit was used to determine TNF-α secreted from adipocytes over a 48-h period (R&D Systems, Oxford, U.K.).

Statistical analysis. Statistical analysis was undertaken using ANOVA for comparison of control versus treatments. The threshold for significance was P < 0.05. Data in the text and figures are presented as means ± SE.

RESULTS

Effect of insulin alone and in combination with RSG on HSL expression. HSL protein expression was regulated by insulin in a biphasic manner, with a small increase detectable at 10 mol/l followed by a significant suppression at 100 nmol/l insulin (P < 0.05) (Fig. 1A). In adipocytes treated with high concentrations of insulin, HSL protein expression was significantly increased (P < 0.01) (Fig. 1A). The magnitude of the changes relative to control was small, however. RSG (10⁻⁸ mol/l) potentiated insulin-mediated increases in HSL protein expression. RSG increased insulin responsiveness, with a relative reduction in HSL expression observed at 10 nmol/l insulin plus RSG (10⁻⁸ mol/l), compared with 100 nmol/l when cells were treated with insulin alone (Fig. 1B). High concentrations of insulin in combination with RSG stimulated HSL expression, with a maximum increase of approximately sevenfold occurring at 1,000 nmol/l insulin (P < 0.05) (Fig. 1B).

Effect of insulin alone and in combination with RSG on LPL expression. Insulin produced a concentration-dependent increase in LPL protein expression, with significant elevation detectable at ≥100 nmol/l insulin (Fig. 2A). Insulin effects on LPL protein expression were modified by addition of RSG. Addition of RSG increased insulin responsiveness, with a maximal effect on LPL expression observed at 10–100 nmol/l insulin (Fig. 2B). Surprisingly, RSG abolished the ability of insulin at concentrations of ≥500 nmol/l to regulate LPL expression. An actin antibody was used as a marker to determine equal protein loading of samples by Western blotting. No significant variation in protein loading was observed with actin (data not shown).

Effect of rosiglitazone (RSG) on HSL and LPL expression. RSG (10⁻⁸ mol/l) alone stimulated HSL protein expression in adipocytes, with an increase sixfold above basal (Fig. 3A). In addition, RSG also stimulated LPL protein expression, with a ninefold stimulation above basal (Fig. 3B).
Effect of long-term insulin and RSG treatment on lipolysis activity. Adipocytes were assessed as >99% viable as previously described (16). Adipocyte size (89.46 ± 4.13 μm) was assessed, and no significant difference was observed with change in BMI or age (data not shown). The effect of long-term treatment with insulin (1–1,000 nmol/l), RSG (10^{-8} mol/l), and insulin (1–1,000 nmol/l) in combination with RSG (10^{-8} mol/l) on lipolytic activity in adipocytes was assessed using cumulative glycerol release over 48 h as an index of lipolytic activity. Lipolysis was significantly stimulated by both 500 and 1,000 nmol/l insulin in the presence or absence of RSG (10^{-8} mol/l) (Table 1). Additionally, 100 nmol/l insulin alone also appeared to stimulate lipolysis, whereas RSG did not alter the lipolytic rate (Table 1).

Effect of long-term insulin exposure on norepinephrine-stimulated lipolysis. The long-term effect of insulin on lipolysis was assessed over a 1-h period, measuring lipolysis for 1 h from 47 h. Adipocytes were treated with insulin, RSG, and both in combination over 1 h in the presence or absence of norepinephrine (1 μmol/l), and the assay was terminated at 48 h. Lipolysis was significantly increased by insulin at 500 and 1,000 nmol/l in the presence or absence of norepinephrine (Table 2). Furthermore, insulin at a dose of 100 nmol/l in the presence of norepinephrine stimulated lipolysis, whereas no stimulatory effect was observed at the same insulin dose in the absence of norepinephrine. Assessment of adipocytes co-treated with insulin and RSG (10^{-8} mol/l) indicated that both 500 and 1,000 nmol/l insulin stimulated lipolytic rate.
in the presence or absence of norepinephrine (Table 2). RSG alone (10⁻⁸ to 10⁻¹⁰ mol/l) did not significantly alter glycerol release (data not shown). The acute effects of insulin dose (1–1,000 nmol/l) in the presence of norepinephrine reduced lipolysis in a dose-dependent manner, whereas insulin in the absence of norepinephrine had no significant effect on lipolytic rate (data not shown). These results reaffirmed previous acute lipolysis studies (4,21–23).

**The effect of insulin and RSG on TNF-α secretion.** In adipocytes, TNF-α secretion was stimulated by insulin in a dose-dependent manner, with maximal stimulation observed at 1,000 nmol/l insulin (Table 3). RSG alone did not stimulate TNF-α secretion above control. The addition of RSG (10⁻⁸ mol/l) abolished the insulin-induced rise in TNF-α secretion from adipocytes compared with control.

**DISCUSSION**

The increased serum NEFA levels in type 2 diabetic patients have previously been attributed to insulin resistance within adipose tissue due to the inability of insulin to suppress lipolysis (20,24). Our data indicate, however, that chronic hyperinsulinemia may induce increased rates of lipolysis in contrast to its acute effects. This is the first report to address the long-term effects of insulin on human adipocyte lipid metabolism, and our findings are consistent with clinical observations of the association of hyperinsulinemia with increased lipolytic rate and fat accumulation in type 2 diabetes (4,21). Our findings are also consistent with previous studies investigating the effect of both chronic high insulin and glucose on mature rat adipocytes and differentiated 3T3-L1 adipocytes (7,25). These studies demonstrate that the combination of chronic high glucose and insulin stimulates lipolysis, which is in contrast to previous studies that have examined the acute effects by insulin (4,21–23). In the present study, adipocytes treated with insulin for 48 h showed a dose-dependent increase in lipolysis, as assessed by glycerol release assay. Furthermore, data from the experiment that examined adipocytes pretreated for 47 h showed a

**TABLE 1**

The chronic effect of treatments on glycerol release

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Glycerol release over 48 h (μmol/500,000 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>244.3 ± 21</td>
</tr>
<tr>
<td>Insulin (nmol/l)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>311.1 ± 44</td>
</tr>
<tr>
<td>10</td>
<td>302.0 ± 58</td>
</tr>
<tr>
<td>100</td>
<td>364.3 ± 52*</td>
</tr>
<tr>
<td>500</td>
<td>417.2 ± 71*</td>
</tr>
<tr>
<td>1,000</td>
<td>493.7 ± 80†</td>
</tr>
<tr>
<td>Insulin (nmol/l) and 10⁻⁸ mol/l RSG</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>263.5 ± 9</td>
</tr>
<tr>
<td>10</td>
<td>280.7 ± 48</td>
</tr>
<tr>
<td>100</td>
<td>274.9 ± 18</td>
</tr>
<tr>
<td>500</td>
<td>367.7 ± 22*</td>
</tr>
<tr>
<td>1,000</td>
<td>402.4 ± 35*</td>
</tr>
<tr>
<td>RSG (mol/l)</td>
<td></td>
</tr>
<tr>
<td>10⁻¹⁰</td>
<td>248.2 ± 24</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>249.7 ± 16</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>232.8 ± 19</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>225.5 ± 29</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>191.6 ± 16</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>193.2 ± 14</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05; †P < 0.01.
similar pattern of response with norepinephrine stimulation for 1 h, which has been previously reported (7), indicating that accumulation of glycerol during the 48-h incubation was not a factor affecting the results.

Hydrolysis of triacylglycerol by HSL is the rate-limiting step in the process of lipolysis, but our finding of relatively modest effects on HSL protein expression with low doses of insulin with or without rosiglitazone were not consistent with the dose-dependent increase in glycerol release shown during chronic treatment. Indeed, insulin treatment at the 100-nmol/l dose significantly reduced HSL protein expression while increasing lipolytic activity. The observed inconsistency between lipolytic activity and HSL protein expression is not surprising. HSL protein expression is less important than its activity, which is dynamically regulated by both its intracellular localization and its phosphorylation (26). A further confounding factor may be the release of TNF-α, which may increase lipolysis although it reduces the expression of HSL (27). Thus, TNF-α secretion during the experiment may have increased lipolysis while not increasing HSL protein expression as previous studies have shown (28,29). TNF-α secretion was increased by insulin but not RSG, and this may be one mechanism by which RSG negates the rise in lipolytic activity (13). At higher insulin concentrations, however, the addition of RSG appeared to stimulate lipolysis in this study, perhaps reflecting the inability of RSG, at this dose, to counteract the effect of insulin at these doses. Furthermore, at high insulin levels, there is also the possibility that insulin interacts with other receptors such as IGF-1. Finally, lipolytic response may also be regulated by another (as yet undefined) lipase, as has been suggested previously (30). Thus, the regulation of lipolysis by insulin or RSG may require more than changes in HSL expression (31). RSG has been shown to promote the differentiation of preadipocytes in vitro and thereby increase adipogenesis (32).

Insulin alone and in combination with RSG regulated LPL protein expression in a dose-dependent manner, comparable with short-term effects on adipose tissue (21,23). In addition, insulin cotreated with RSG enhanced both HSL and LPL protein expression more than insulin alone, shifting the insulin dose response to the left. This suggests that RSG may enhance the ability of insulin to modulate adipocyte lipid metabolism. Although caution should be exercised in extrapolating these in vitro data to the in vivo situation, these findings are consistent with in vivo observations of weight gain due to increase in adipose tissue mass with insulin therapy and also during RSG treatment (31). RSG has been shown to promote the differentiation of preadipocytes in vitro and thereby increase adipogenesis (32). The present study indicates that the increase in fat mass with RSG is also due to RSG increasing LPL protein expression markedly over the dose range compared with control. This is consistent with the observation that despite reduction in circulating insulin levels, weight gain is observed during treatment with RSG (32). This study on the long-term effects of RSG in adipocytes is the first in vitro study determining the effects of RSG in lipid accumulation through analysis of LPL protein expression. The results suggest that RSG may alter mature adipocyte size as well as enhancing adipogenesis to increase adipose tissue mass. It is not clear if there are depot-specific

### TABLE 2

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Glycerol release from chronically treated cells between 47 and 48 h (μmol/500,000 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No norepinephrine</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.4 ± 0.13</td>
</tr>
<tr>
<td>Insulin (nmol/l)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.7 ± 0.12</td>
</tr>
<tr>
<td>10</td>
<td>6.8 ± 1.00</td>
</tr>
<tr>
<td>100</td>
<td>7.9 ± 0.13</td>
</tr>
<tr>
<td>500</td>
<td>10.1 ± 0.22*</td>
</tr>
<tr>
<td>1,000</td>
<td>10.4 ± 0.32*</td>
</tr>
<tr>
<td>10−8 mol/l RSG</td>
<td>6.5 ± 0.31</td>
</tr>
<tr>
<td>Insulin (nmol/l) and 10−8 mol/l RSG</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.5 ± 0.12</td>
</tr>
<tr>
<td>10</td>
<td>7.1 ± 0.2</td>
</tr>
<tr>
<td>100</td>
<td>7.0 ± 0.13</td>
</tr>
<tr>
<td>500</td>
<td>9.8 ± 0.26*</td>
</tr>
<tr>
<td>1,000</td>
<td>9.7 ± 0.24†</td>
</tr>
<tr>
<td>With 1 μmol/l norepinephrine</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.6 ± 0.19</td>
</tr>
<tr>
<td>Insulin (nmol/l)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.4 ± 0.30</td>
</tr>
<tr>
<td>10</td>
<td>7.7 ± 0.26</td>
</tr>
<tr>
<td>100</td>
<td>9.9 ± 0.18†</td>
</tr>
<tr>
<td>500</td>
<td>12.2 ± 0.34†</td>
</tr>
<tr>
<td>1,000</td>
<td>12.7 ± 0.50†</td>
</tr>
<tr>
<td>10−8 nmol/l RSG</td>
<td>8.4 ± 0.28</td>
</tr>
<tr>
<td>Insulin (nmol/l) and 10−8 mol/l RSG</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8.3 ± 0.34</td>
</tr>
<tr>
<td>10</td>
<td>8.6 ± 0.35</td>
</tr>
<tr>
<td>100</td>
<td>8.6 ± 0.38</td>
</tr>
<tr>
<td>500</td>
<td>11.4 ± 0.61†</td>
</tr>
<tr>
<td>1,000</td>
<td>12.3 ± 0.60†</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05; †P < 0.01.

### TABLE 3

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Secreted TNF-α over 48 h (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>Insulin (nmol/l)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>100</td>
<td>20.0 ± 0.5*</td>
</tr>
<tr>
<td>500</td>
<td>27.0 ± 0.5*</td>
</tr>
<tr>
<td>1,000</td>
<td>32.0 ± 0.7*</td>
</tr>
<tr>
<td>Insulin (nmol/l) and 10−8 mol/l RSG</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.0 ± 0.8</td>
</tr>
<tr>
<td>10</td>
<td>0.9 ± 0.8</td>
</tr>
<tr>
<td>100</td>
<td>0.9 ± 0.7</td>
</tr>
<tr>
<td>500</td>
<td>8.5 ± 0.9</td>
</tr>
<tr>
<td>1,000</td>
<td>8.6 ± 1.8</td>
</tr>
<tr>
<td>RSG (mol/l)</td>
<td></td>
</tr>
<tr>
<td>10−10</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>10−9</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>10−8</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>10−7</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>10−6</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>10−5</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.001.
changes in fat distribution during insulin therapy, but RSG therapy appears to be associated with a preferential increase in subcutaneous rather than omental adipose tissue (33). Further studies are needed to examine whether this is due to depot-specific changes in LPL expression induced by RSG.

In conclusion, chronic insulin treatment of human adipocytes stimulates lipolysis in a dose-dependent manner in association with increased TNF-α secretion, but this does not appear to be related to changes in HSL protein expression. An increase in LPL expression was also demonstrated, during both insulin and RSG treatment. These findings may suggest, therefore, that in hyperinsulinemic states, both lipolysis and lipogenesis may increase, with a resultant increase in NEFA production as well as weight gain. Cotreatment with RSG reduced the insulin-mediated effects on lipolysis. We postulate therefore that these in vitro data may provide a potential explanation for the paradox of hyperinsulinemia coexisting with elevated serum NEFAs in type 2 diabetic patients.

ACKNOWLEDGMENTS
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