Elevated non-esterified fatty acids, triglyceride, diacglycerol, and ceramide have all been associated with insulin resistance in muscle. We set out to investigate the role of intramyocellular lipid metabolites in the induction of insulin resistance in human primary myoblast cultures. Muscle cells were subjected to adenovirus-mediated expression of perilipin or incubated with fatty acids for 18 h, prior to insulin stimulation and measurement of lipid metabolites and rates of glycogen synthesis. Adenovirus-driven perilipin expression lead to significant accumulation of triacylglycerol in myoblasts, without any detectable effect on insulin sensitivity, as judged by the ability of insulin to stimulate glycogen synthesis. Similarly, incubation of cells with the monounsaturated fatty acid oleate resulted in triacylglycerol accumulation without inhibiting insulin action. By contrast, the saturated fatty acid palmitate induced insulin resistance. Palmitate treatment caused less accumulation of triacylglycerol than did oleate but also induced significant accumulation of both diacylglycerol and ceramide. Insulin resistance was also caused by cell-permeable analogues of ceramide, and palmitate-induced resistance was blocked in the presence of inhibitors of de novo ceramide synthesis. Oleate co-incubation completely prevented the insulin resistance induced by palmitate. Our data are consistent with ceramide being the agent responsible for insulin resistance caused by palmitate exposure. Furthermore, the triacylglycerol derived from oleate was able to exert a protective role in sequestering palmitate, thus preventing its conversion to ceramide.

Obesity exerts a major impact on insulin resistance and is closely associated with type 2 diabetes (1). Two-thirds of individuals diagnosed with type 2 diabetes are overweight, and almost half are clinically obese (2), furthermore the risk of developing diabetes increases dramatically with the degree of obesity. The relationship between obesity and insulin resistance has led to the hypothesis that ectopic storage of fat in non-adipose tissues such as skeletal muscle under conditions of lipid oversupply is causal in the development of obesity-associated insulin resistance, and elevated intramyocellular triacylglycerol (IMTG) levels have been identified as a potential link between increased non-esterified fatty acid (NEFA) availability and insulin resistance. The molecular mechanism behind the coupling of IMTG content and insulin resistance is unclear, although it appears unlikely that a neutral lipid metabolite such as triacylglycerol (TG) directly impairs insulin action. Indeed, there is evidence that IMTG stores are elevated not only in insulin resistance but also in insulin-sensitive athletes (3). Hence it is generally believed that IMTG represents a surrogate for other lipid metabolites in muscle. This includes an increasing body of evidence that lipid intermediates such as diacylglycerol (DAG) and ceramides have direct effects on insulin signaling and glucose utilization.

DAG has been identified as a potential mediator of lipid-induced insulin resistance. DAG has been shown to accumulate, together with triacylglycerol, in peripheral tissues from insulin-resistant rodents (4), and to inhibit insulin action in various isolated tissues or cultured cells (5–8), including skeletal muscle (9, 10). However, other studies have reported that DAG is dispensable to the development of insulin resistance. Specifically, in one study in C2C12 myotubes, the saturated fatty acid (FA), myristate induced significant DAG synthesis while having no effect on insulin sensitivity (10).

The sphingomyelinase, ceramide, is another signaling molecule that has been implicated in several physiological events. Ceramide levels are elevated in muscle of insulin-resistant animals (4) and in skeletal muscle from obese, insulin-resistant humans (11). Ceramide has also been shown to induce insulin resistance in a variety of cultured cells (6, 12–14). Ceramide can become elevated in muscle both by activation of sphingomyelinase, which produces ceramide from the hydrolysis of sphingomyelin (15), and by de novo synthesis from palmitate (16). In C2C12 myotubes, palmitate increases ceramide content and inhibits downstream insulin-stimulated protein kinase B (PKB) phosphorylation (14). Incubation of these cells with short-chain ceramide analogues (C2-ceramide) mimics the effects of palmitate on insulin signaling, suggesting that

Key Role for Ceramides in Mediating Insulin Resistance in Human Muscle Cells*

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3 The abbreviations used are: IMTG, intramyocellular triacylglycerol; DAG, diacylglycerol; FA, fatty acid; NEFA, non-esterified fatty acid; m.o.i., multiplicity of infection; PKB, protein kinase B; TG, triacylglycerol; BSA, bovine serum albumin.
ceramide generation from saturated fatty acids is sufficient to account for inhibition of insulin signaling. Furthermore, blocking de novo ceramide synthesis in palmitate-treated C2C12 myotubes negates the inhibitory effect of saturated NEFA toward PKB activation by insulin, while inducing ceramide accumulation augments the inhibitory effect of saturated NEFA (17). Similar inhibition of PKB phosphorylation has been observed in other cell types exposed to C2-ceramide, resulting in diminished insulin-stimulated glucose uptake in muscle and fat cells (6, 7, 10).

To further investigate the roles of the various lipid metabolites in inducing insulin resistance, we have used a variety of methods to manipulate their levels in cultured human myoblasts. The effects of these manipulations on insulin sensitivity were investigated in an attempt to further define the relationship between IMTG and the development of insulin resistance.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture plates were purchased from Greiner (Gloucestershire, UK) and pipettes from Starstedt (Leicester, UK). Culture media, penicillin/streptomycin, trypsin-EDTA, and fetal calf serum were from Invitrogen (Paisley, UK). Chick embryo extract was from Sera Laboratories International (Salisbury, UK). Oleic acid, palmitic acid, fatty acid-free bovine BSA, Oil Red O, myriocin, fumonisin-B1, ceramide standards, and chemicals not specified were from Sigma (Poole, UK). Pepstatin, antipain, and leupeptin were from Peptide Institute (Osaka, Japan). Actrapid® insulin was from Novo Nordisk (Copenhagen, Denmark). Cell-permeable C2-ceramide analogues were from Tocris (Bristol, UK). Coomassie Blue G-250 and Coomassie Plus protein assay reagents were from Pierce (Chester, UK). Scintillation fluid was from National Diagnostics (Atlanta, GA). Polyvinylidine difluoride membrane was from Pall Europe Ltd. (Portsmouth, UK). Enhanced chemiluminescence (ECL) reagent was from Amersham Biosciences (Buckinghamshire, UK). Silica gel 60 thin-layer chromatography plates were from Whatman (Maidstone, UK). Anti-perilipin antibody and adenovirus-perilipin A were as described previously (18, 19). Recombinant β-galactosidase adenovirus (Ad-β-gal) (a kind gift from Dr. Calum Sutherland, University of Dundee) was generated as described previously (20). Anti-desmin antibodies were from Dako (High Wycombe, UK). Infinity™ Triglyceride reagent was from Sigma and 1,2-sn-diacglyceride reagent kit was from GE Healthcare (Buckinghamshire, UK). [U-14C]-d-Glucose (10 GBq/mmole) and [γ-32P]ATP (≈0.11 TBq/mmole) were from GE Healthcare.

Cell Culture—Human myoblasts were grown as described previously (21) from needle biopsies taken from the vastus lateralis muscle of healthy subjects with no family history of Type 2 diabetes and normal glucose tolerance and insulin sensitivity. All subjects gave informed consent and the study was approved by the Newcastle and North Tyneside Joint Ethics Committee. Myoblasts were maintained in Ham’s F-10 supplemented with 20% (v/v) fetal calf serum, 1% (v/v) chick embryo extract, 100 units/ml penicillin, and 100 μg/ml streptomycin. All experiments were performed using cells between the 5th and 15th passage at ~90% confluency. Immunocytochemical staining with anti-desmin antibody confirmed that the cultures were predominantly myoblasts.

Adenoviral Infection of Human Myoblasts—Myoblasts were infected at ~70% confluence. Cells were incubated in 500 μl serum-free Ham’s F-10 (containing 100 units/ml penicillin and 100 μg/ml streptomycin) at multiplicity of infection (m.o.i.) of 20–100 virus particles/cell for 2 h. Medium was replaced with growth medium and assays were performed, or cell extracts prepared, after 48 h.

Fatty Acid Preparation and Treatment—Stock solutions (10 mM) of oleate and palmitate, complexed to 12% (w/v) BSA, were prepared. Fatty acids were dissolved in 4 ml of ethanol supplemented with 100 μl of 5 M NaCl and dried under nitrogen prior to resuspension in 150 mM NaCl, 50 mM Tris-HCl, pH 7.9. An optically clear solution was obtained upon heating to 60 °C with continuous agitation and an equal volume of ice-cold 24% FA-free BSA was immediately added. Samples were stored at −20 °C until required. Fatty acid stocks were diluted to appropriate concentrations in serum-free Ham’s F-10 medium. Cells were washed three times with PBS prior to the addition of fatty acid-containing medium. Control cells were incubated in serum-free Ham’s F-10 medium containing an equivalent concentration of BSA.

Preparation of Cell Extracts for Immunoblotting—Cells were washed with ice-cold PBS and scraped into extraction buffer (100 mM Tris-HCl, pH 7.4, 100 mM KCl, 25 mM EDTA, 25 mM KF, 0.1% (v/v) Triton X-100, 1 mM benzamidine, 0.1 mM NaVO₄, 1 μg/ml antipain, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). Extracts were sonicated for 5 s at 3 μm amplitude (Soniprep 150, Sanyo). Protein concentration was determined using a dye binding method (22). Samples (10 μg of protein) were mixed with 4× sample buffer (0.25 M Tris-HCl pH 6.8, 8% w/v SDS, 40% (v/v) glycerol, 20% β-mercaptoethanol, 0.004% bromphenol blue), boiled for 5 min, and dispersed by sonication for 5 min (Sonibath, Dawe). Extracts were resolved on 10% (w/v) acrylamide gels by SDS-PAGE and immunoblotted as described previously (21).

Oil Red O Staining—Lipids in myoblasts were visualized by histochemical staining with Oil Red O. Cells were rinsed and fixed in 60% isopropyl alcohol for 10 min then stained in 2% (w/v) Oil Red O for 5 min at room temperature. Excess stain was removed by washing in 60% isopropyl alcohol, followed by several changes of distilled water. Stained cells were visualized under a light microscope.

Measurement of Glycogen Synthesis—The rate of glycogen synthesis was determined by measuring the incorporation of [U-14C]glucose into glycogen as described previously (21). FA-treated cells were incubated in serum-free Ham’s F-10 supplemented with 400 μM of fatty acid, 0.5% (w/v) BSA for 18 h.

Lipid Quantification—TG content was determined in cell extracts in a buffer of 50 mM Tris, 100 mM KCl, 20 mM KF, 0.5 mM EDTA, and 0.05% (v/v) Lubrol PX, pH 7.9, sonicated three times for 5 s (Soniprep 150). Homogenates were centrifuged at 11,000 × g for 15 min and supernatants collected. Total TG was measured enzymatically with Infinity™ Triglyceride kit, using trioleoylglycerol as standard. For measurement of DAG and ceramide concentrations, myoblasts were scraped into ice-cold PBS and standardized for protein concentration. Samples (100
μg protein) were transferred to polypropylene tubes and lipids extracted by adding 3 ml of ice-cold chloroform:methanol (1:2 v/v) and vortexing vigorously. NaCl (1 M) was added to bring the aqueous volume to 0.8 ml. Chloroform (1 ml) and 1 M NaCl (1 ml) were added to separate the phases. Following centrifugation at 5000 × g for 2 min, the lower chloroform phase was assayed for DAG and ceramide content using a radiometric DAG assay kit (Amersham Biosciences). Samples were applied to silica gel thin-layer plates, along with DAG and ceramide standards. Plates were developed in a tank presaturated with developing solvent (chloroform:acetic acid:acetone:methanol: water; 50:40:20:20:5). Plates were dried, and the zone corresponding to [32P] phosphatidic acid and [32P]ceramide 1-phosphate was visualized by autoradiography. Lipids were stained in iodine vapor, and spots corresponding to phosphatidic acid and ceramide 1-phosphate were scraped into scintillation vials and radioactivity determined.

Statistical Analyses—Data were expressed as means ± S.E. Significance was assessed using a two-tailed unpaired Student’s t test following analysis of variance. Sample sizes and p values for particular experiments are given in figure legends.

RESULTS

Perilipin, originally described as an adipocyte-specific protein, has now been detected in a number of tissues, located primarily on the surface of intracellular lipid droplets (23, 24). It has been demonstrated that perilipin promotes lipid accumulation by protecting nascent lipid from hydrolysis by endogenous lipases (18, 25). In view of this, recombinant adenovirus encoding mouse perilipin A was used to infect myoblasts, to promote lipid accumulation. Perilipin A expression was detected 48 h after infection, with the level of protein expression increasing with increasing m.o.i. (Fig. 1A). Using an m.o.i. of 50 virus particles/cell, the effect of ectopic perilipin expression on lipid accumulation was studied. As can be seen in Fig. 1B, expression of perilipin A led to significant accumulation of intracellular lipid, as visualized by staining with Oil Red O.

Enzymatic analysis of lipid extracted from these cells showed that they contained ~3 times more triacylglycerol than cells infected with a control adenovirus encoding β-galactosidase (Fig. 1C). There was no detectable increase in the content of diacylglycerol or ceramide (Fig. 1C).

The effect of insulin on the rate of glycogen synthesis in these cells was then examined, since this is the key physiological end point response for glucose disposal in muscle (Fig. 2). In untreated cells, insulin stimulated glycogen synthesis ~4-fold. Following infection with adenovirus encoding β-galactosidase, insulin-stimulated glycogen synthesis was essentially unchanged. In the cells expressing perilipin A and with increased triacylglycerol content, again the effect of insulin on rate of glycogen synthesis was undiminished, with both basal and insulin-stimulated rates being unaltered. These data indicate clearly that intracellular triacylglycerol accumulation per se does not cause insulin resistance in this experimental system.

Incubation of myoblasts with individual fatty acids also lead to significant accumulation of intracellular lipid, as visualized by histochemical staining (data not shown) and by enzymatic analysis for triacylglycerol (Fig. 3A). Both oleate and palmitate (respectively the major monounsaturated and saturated fatty acids in serum) caused significant accumulation of triacylglycerol. However, the levels in response to oleate were ~4-fold
higher than those formed in response to palmitate. The insulin sensitivity of these lipid-loaded cells was then investigated (Fig. 3B). Insulin stimulated glycogen synthesis ~4-fold in control cells, and this effect was maintained in cells pretreated with oleate. Neither basal nor insulin-stimulated rates of glycogen synthesis were altered, despite the high levels of intracellular triacylglycerol. In contrast, pretreatment of the cells with palmitate caused a significant diminution in the response to insulin (the basal rate of glycogen synthesis was unaltered), despite the fact that the levels of triacylglycerol were lower than these formed in response to oleate. Taken together, these findings again indicate that intracellular triacylglycerol does not induce insulin resistance. However, incubation of cells with palmitate did render cells insulin-resistant, likely via the action of a metabolite derived from this fatty acid.

Both oleate and palmitate can be esterified to DAG in addition to TG. Furthermore, palmitate is a precursor of ceramides. As both DAG and ceramides have known signaling functions, activating for example different isoforms of protein kinase C (26–28), their levels were investigated in cells preincubated with oleate and palmitate. As seen in Fig. 3, C and D, oleate treatment caused little, if any, alteration in the levels of DAG and ceramide, consistent with a preference for triacylglycerol synthesis from this fatty acid. In contrast, palmitate caused marked elevation in the amounts of DAG and ceramide, increasing them 6- and 3.5-fold, respectively. The observation that oleate, which causes mainly TG accumulation, does not induce insulin resistance is again consistent with TG not being responsible for insulin resistance. In contrast, the finding that palmitate causes insulin resistance indicates that this may be due to one or both of its major lipid metabolites, namely DAG and ceramide.

De novo ceramide synthesis occurs via palmitoyl-CoA and requires the activity of two enzymes, i.e. serine palmitoyltrans-
ferase and ceramide synthase (16), which can be specifically inhibited by myriocin and fumonisin B1, respectively. To investigate whether DAG or ceramide was the active agent in inducing insulin resistance, cells were incubated with palmitate in the presence or absence of each inhibitor. As can be seen in Fig. 4A, palmitate again induced insulin resistance, causing a dramatic decrease in the stimulation of glycogen synthesis by the hormone. The stimulation by insulin was unaffected by either inhibitor alone. However, when either inhibitor was present during palmitate treatment, insulin resistance was prevented. As expected, each inhibitor also prevented palmitate-induced ceramide accumulation (Fig. 4B) but did not prevent DAG accumulation caused by the fatty acid (Fig. 4C). These data indicate that ceramide was the agent responsible for inducing insulin resistance. This conclusion was further supported by use of C2-ceramide, a short-chain cell-permeable ceramide analogue. As seen in Fig. 5, treatment of myoblasts with this analogue completely abolished the stimulatory effect of insulin on the rate of glycogen synthesis. Use of the corresponding biologically inert analogue, C2-dihydroceramide, had no effect on the stimulation of glycogen synthesis by insulin.

The observation that oleate caused significant accumulation of TG and yet did not induce insulin resistance raised the possibility that the presence of oleate might protect the cells against the effects of palmitate by directing fatty acids into TG rather than ceramide synthesis. Indeed, co-incubation of muscle cells with oleate and palmitate for 18 h caused a significant increase in TG accumulation (843 ± 64 μg/mg protein) compared with palmitate (620 ± 35 μg/mg protein) treatment alone (p < 0.05).

Co-incubation of cells with both fatty acids virtually abolished accumulations of both DAG and ceramide that were observed after incubating cells with palmitate alone (Figs. 6, A and B).

Fig. 6C shows that co-incubation with oleate prevented the inhibitory effects of palmitate on insulin-stimulated glycogen synthesis.

**DISCUSSION**

We have presented several lines of evidence to suggest that intracellular accumulation of ceramides can invoke insulin resistance in the key end point response of glycogen synthesis in cultured human myoblasts. Furthermore, we present evidence that intracellular accumulation of TG or DAG does not in itself lead to insulin resistance and indeed that intracellular TG may
exert a protective role against insulin resistance induced by saturated fatty acids.

Although numerous studies have associated increased IMTG with insulin resistance, the evidence reported herein indicate that the IMTG itself is not responsible. Perhaps the clearest demonstration of this is the effect of adenovirus-driven perilipin expression. Although perilipin is not normally present in muscle (29), when perilipin is expressed, the balance between TG synthesis and lipolysis is disturbed, leading to lipid accumulation. This occurred in the presence of levels of endogenous lipids in the serum used for cell culture, showing that the transduced myoblasts had the ability to accumulate lipids without any necessity for elevated extracellular lipid concentrations. Furthermore, these experiments demonstrate a novel mechanism for elevation of IMTG without significant changes in DAG or ceramide.

Since both DAG and ceramide were elevated by palmitate treatment, it was necessary to ascertain which of these metabolites were responsible for the insulin resistance observed. To this end, we used myriocin and fumonisin B1 to specifically inhibit distinct enzymes involved in the biosynthesis of ceramide from palmitate. Each inhibitor prevented palmitate-induced insulin resistance. Furthermore, the addition of exogenous, cell-permeable ceramide replicated the deleterious effect of palmitate on insulin-sensitive glycogen synthesis. Together, these data provide strong evidence that the production of ceramide from palmitate is necessary for the induction of insulin resistance, consistent with recent studies performed in rodent cell lines (17, 30, 31).

Ceramide is known to have a significant impact upon a number of different intracellular signaling events important in mediating insulin action, in addition to the effects on PKB described above. Increased ceramide is also associated with processes such as oxidative stress, inflammation, and apoptosis (32). However, it is not yet established which of these are important in the mechanism by which ceramides induce insulin resistance in muscle cells. Future studies will aim to identify the molecular events that are instrumental in the mechanism of insulin resistance in this model system.

A crucial question is of course whether ceramides mediate insulin resistance in vivo. Several lines of evidence indicate that this is indeed the case. Insulin-resistant Zucker rats (4) contain elevated skeletal muscle ceramide levels, and recent findings have demonstrated elevated ceramide content in skeletal muscle from insulin-resistant humans (11, 33). Other workers have reported fatty acid-induced insulin resistance in the absence of increases in ceramide (34, 35). Such studies were performed using lipid emulsions such as Liposyn II, made from mainly unsaturated fats that are not substrates for ceramide synthesis. A number of alternative mechanisms have been proposed for insulin resistance induced by unsaturated fats, including roles for protein kinase C and tumor necrosis factor α, as discussed by Summers (32).

**FIGURE 6.** Protective effect of oleate co-incubation on the effect of palmitate on glycogen synthesis. Myoblasts were incubated for 18 h in serum-free medium containing either 0.5% (w/v) BSA, 400 μM palmitate, or a combination of 400 μM palmitate and 400 μM oleate. DAG (A) and ceramide (B) content were determined as described above. Results are expressed as fold over control and are means ± S.E. of four experiments in cells from three subjects. Statistical significance (p < 0.01) compared with respective control values is indicated by *. C, glycogen synthesis was measured in the absence (open bars) or presence (solid bars) of 100 nM insulin over 1 h. The results represent the mean ± S.E. of four experiments in cells from at least three subjects. Statistical significance (p < 0.01) compared with control (BSA alone) in the presence of insulin is indicated by *.
A key aspect of the current work is the observation that accumulated intracellular triacylglycerol may exert a protective role against the insulin resistance induced by palmitate. A similar effect was reported by Montell and colleagues (9) who showed that palmitate antagonized insulin stimulation of glucose uptake in human myotubes but that the insulin response was restored in cells treated with a mixture of oleate and palmitate. Unlike in the present study, these authors concluded that DAG played a role in saturated fatty acid-induced insulin resistance, and oleate prevented this insulin resistance due to its ability to redirect saturated FA away from the DAG pool into TG. However, this study did not consider ceramide accumulation. In addition, Chavez et al. (10) suggested that the resistance of adipocytes to fatty acid-induced insulin resistance is attributable to their increased capacity to sequester fatty acids into TG, while Listenberger et al. (36) demonstrated that TG accumulation protects against saturated fatty acid-induced lipotoxicity in pancreatic β cells. These observations are consistent with the view that the stored TG “buffers” excess fatty acids arriving at the peripheral tissues such as muscle. Once the capacity of that buffer has been exceeded, the deleterious effects of the fatty acids and their lipid derivatives such as ceramides can be manifested.

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