Arachidonic Acid Stimulates Glucose Uptake in 3T3-L1 Adipocytes by Increasing GLUT1 and GLUT4 Levels at the Plasma Membrane

EVIDENCE FOR INVOLVEMENT OF LIPOXYGENASE METABOLITES AND PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ*

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Exposure of insulin-sensitive tissues to free fatty acids can impair glucose disposal through inhibition of carbohydrate oxidation and glucose transport. However, certain fatty acids and their derivatives can also act as endogenous ligands for peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor that positively modulates insulin sensitivity. To clarify the effects of externally delivered fatty acids on glucose uptake in an insulin-responsive cell type, we systematically examined the effects of a range of fatty acids on glucose uptake in 3T3-L1 adipocytes. Of the fatty acids examined, arachidonic acid (AA) had the greatest positive effects, significantly increasing basal and insulin-stimulated glucose uptake by 1.8- and 2-fold, respectively, with effects being maximal at 4 h at which time membrane phospholipid content of AA was markedly increased. The effects of AA were sensitive to the inhibition of protein synthesis but were unrelated to changes in membrane fluidity. AA had no effect on total cellular levels of glucose transporters, but significantly increased levels of GLUT1 and GLUT4 at the plasma membrane. While the effects of AA were insensitive to cyclooxygenase inhibition, the lipoxygenase inhibitor, nordihydroguaiaretic acid, substantially blocked the AA effect on basal glucose uptake. Furthermore, adenoviral expression of a dominant-negative PPARγ mutant attenuated the AA potentiation of basal glucose uptake. Thus, AA potentiates basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes by a cyclooxygenase-independent mechanism that increases the levels of both GLUT1 and GLUT4 at the plasma membrane. These effects are at least partly dependent on *de novo* protein synthesis, an intact lipoxygenase pathway and the activation of PPARγ with these pathways having a greater role in the absence than in the presence of insulin.

A full understanding of the control of glucose homeostasis and its dysregulation in diabetes mellitus will require a better comprehension of the integration of carbohydrate and lipid metabolism (1). Free fatty acids are considered to play a pivotal role in the pathogenesis of diabetes and may be involved in the early events leading to insulin resistance in man (2). The classic studies of Randle and colleagues (3) demonstrated that oxidation of fatty acids could inhibit glucose oxidation through the effects of acetyl-CoA on pyruvate dehydrogenase activity. More recently it has been shown that increased delivery of free fatty acids *in vivo* can impair insulin-mediated glucose disposal through inhibition of glucose uptake (4–7). Furthermore, several long chain fatty acids, both saturated and unsaturated, have been demonstrated to decrease mRNA levels of the insulin-responsive glucose transporter GLUT4 *in vitro* (8).

Insulin sensitivity in whole animals can also be influenced by the manipulation of the dietary fatty acid profile. A diet high in saturated, monounsaturated (*n*-9) or polyunsaturated (*n*-6) fatty acids (PUFA)[1] leads to insulin resistance in rodents (9, 10). Substituting a small percentage of fatty acids in the *n*-6 PUFA diet with *n*-3 PUFA normalizes insulin action (9, 10). Similar dietary intervention studies in humans have been inconclusive (11). However, in both humans and rodents, PUFA (particularly *n*-3) levels in skeletal muscle membrane phospholipids are positively correlated with insulin sensitivity (10, 12, 13).

In addition to their roles as metabolic fuel, certain fatty acids can act as precursors for signaling molecules which may influence insulin action and glucose metabolism. Interest in these activities has been greatly enhanced by the recent recognition of the importance of the nuclear hormone receptor PPARγ in the control of insulin sensitivity (14, 15). Thus, pharmacological agonists for PPARγ can enhance glucose disposal both *in vitro* (16–18) and *in vivo* (19) and loss of function mutations in human PPARγ result in extreme insulin resistance and diabetes mellitus (20). While the true endogenous ligand(s) for PPARγ have not been established with certainty, the receptor can be activated *in vitro* by a variety of lipophilic ligands including certain polyunsaturated fatty acids (21–25) and their metabolites (21, 24, 26–29).

Grunfeld *et al.* (30) originally reported that exposure of 3T3-L1 adipocytes to certain unsaturated fatty acids during differentiation could enhance basal glucose uptake. These

[1] The abbreviations used are: PUFA, polyunsaturated fatty acid; PPARγ, peroxisome proliferator-activated receptor γ; AA, arachidonic acid; NBCS, newborn calf serum; 6-MNA, 6-methoxy-2-napthylacetic acid; NDGA, nordihydroguaiaretic acid; GFP, green fluorescent protein; aP2, adipocyte P2; PGE$_2$, prostaglandin E$_2$; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; COX, cyclooxygenase; LOX, lipoxygenase.

workers suggested that relatively nonspecific effects on membrane fluidity might underlie these effects. More recently, a similar effect on basal glucose uptake has been observed in fully differentiated 3T3-L1 adipocytes supplemented with the *n*-6 PUFA, arachidonic acid (AA) (31, 32). In light of recent advances in the understanding of the possible roles of lipid mediators in the control of glucose homeostasis we have re-examined the effects of a range of fatty acids on glucose uptake in 3T3-L1 adipocytes. Having established that AA had the most marked effects on enhancement of glucose disposal we have attempted to dissect the molecular mechanisms underlying these effects.

<div align="center">EXPERIMENTAL PROCEDURES</div>

Materials—2-Deoxy-D-[2,6-³H]glucose, [1-¹⁴C]AA, and the enhanced chemiluminescence (ECL) kit were purchased from Amersham Pharmacia Biotech. Analytical grade solvents were obtained from BDH Biochemicals (Dorset, United Kingdom). Rabbit anti-GLUT4 antibody was a gift from Prof. G. Gould (University of Glasgow), rabbit anti-GLUT1 was a gift from Prof. S. Baldwin (University of Leeds), and rabbit anti-PPARγ was a gift from Dr. M. Lazar (University of Pennsylvania School of Medicine). The PGE_2 monoclonal enzyme immunoassay kit was purchased from Cayman Chemical. Ibuprofen, 6-methoxy-2-napthylacetic acid (6-MNA), and piroxicam were purchased from Biomol Research Laboratories. The RNeasy total RNA kit was from Qiagen. Rosiglitazone was provided by Dr. S. Smith (SmithKline Beecham Pharmaceuticals). Reverse transcription reagents were obtained from Promega, UK, and TaqMan reagents were from PE Biosystems. All other reagents were from Sigma.

Tissue Culture—3T3-L1 fibroblasts (ATCC) were maintained at no higher than 70% confluency in DMEM containing 10% newborn calf serum, 25 mM glucose, 2 mM glutamine, and antibiotics (DMEM/NBCS). For differentiation they were grown 2 days post-confluence in DMEM/NBCS and then for 2 days in medium containing fetal bovine serum instead of newborn calf serum (DMEM/FBS) supplemented with 0.83 μM insulin, 0.25 μM dexamethasone, and 0.5 mM isobutylmethylxanthine. The medium was then changed to DMEM/FBS supplemented only with 0.83 μM insulin for 2 days and then to DMEM/FBS alone for a further 3–5 days. Differentiated cells were only used when at least 95% of the cells showed an adipocyte phenotype by accumulation of lipid droplets.

Fatty Acid Solutions—Fatty acid-supplemented media were prepared as described previously (30). In short, a 0.2 M stock solution of the fatty acid in ethanol was diluted 1:25 into 20% fatty acid-free bovine serum albumin in Dulbecco's phosphate-buffered saline (PBS) at 60 °C with shaking. This solution was diluted 1:10 into serum-free DMEM (containing 25 mM glucose and 2 mM glutamine) for studies with up to 4-h incubation times, or into DMEM/FBS for studies with >4-h incubation times. This gives a final concentration of 8×10^{-4} M fatty acid and 0.4% ethanol.

Glucose Uptake—Adipocytes (at day 9 after initiation of differentiation) in 6- (or 24-) well plates were incubated for 4 h in fatty acid-supplemented DMEM. In the time course experiment, incubations of 48 and 24 h were commenced on days 7 and 8 of differentiation, respectively, and on day 9 cells were serum-starved in DMEM (containing 25 mM glucose and 2 mM glutamine) for 2 h prior to the assay. For the 1-h time point, cells were starved for 1 h prior to incubation in the AA medium. Glucose uptake assays were performed as described previously (33).

Western Blotting—Treated cells (adipocytes/pre-adipocytes) were solubilized by scraping and passing 10 times through a 25-gauge needle in lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 30 mM NaF, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM $Na_2P_2O_7$, 2.5 mM benzamidine, 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). The lysate was clarified by centrifugation at 13,500 × g for 10 min at 4 °C. Crude cell extracts were resolved by SDS-PAGE before electroblotting to polyvinylidene difluoride membranes (Millipore). Membranes were blocked in 1% bovine serum albumin and specific proteins were detected by incubation with appropriate primary and secondary (horseradish peroxidase-conjugated) antibodies in TBST (150 mM NaCl, 50 mM Tris, 0.1% Tween 20). Proteins were visualized using an ECL kit.

Plasma Membrane Lawn Assay—3T3-L1 adipocytes (day 9), grown on collagen-coated glass coverslips, were treated for 4 h with AA medium. Cells were incubated ± 10 nM insulin for 30 min and a modified version of the plasma membrane lawn assay (33) was performed. Cells were washed twice in ice-cold buffer A (50 mM Hepes, 10 mM NaCl, pH

7.2), twice in ice-cold buffer B (20 mM Hepes, 10 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.2), and sonicated using a probe sonicator (Kontes, Vineland, NJ) to generate a lawn of plasma membrane fragments attached to the coverslip. The membranes were washed twice again in ice-cold buffer B and fixed to the coverslips for 15 min using freshly prepared 3% paraformaldehyde. Membranes were then serially washed: 3 times in PBS, 3 times in 50 mM NH_4Cl in PBS over 10 min, 3 times in PBS, 3 times in PBS-gelatin (PBS containing 0.2% gelatin and 1 μl/ml goat serum) over 5 min, and finally 3 times in PBS. Membranes were incubated in either anti-GLUT4 or anti-GLUT1 antibody (1:100 dilution in PBS-gelatin) for 1 h at room temperature. After washing 3 times in PBS-gelatin and 3 times in PBS, the coverslips were incubated with the secondary antibody, fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG for 1 h at room temperature, washed 3 times in PBS-gelatin, and 3 times in PBS and mounted on glass slides. Coverslips were viewed using a ×60 objective lens on a Nikon Optiphot-2/Bio-Rad MRC-1000 microscope operated in laser scanning confocal mode. Samples were illuminated at 488 nm, and images were collected at 510 nm. Duplicate coverslips were prepared at each experimental condition and eight random images of plasma membrane lawn were collected from each. The images were quantified using Bio-Rad MRC-1000 confocal microscope operating software (CoMOS, version 6.05.8), on an AST premmia SE P/60 personal computer.

[1-¹⁴C]Arachidonic Acid Uptake—0.2 M AA, containing a trace of [1-¹⁴C]AA, was diluted into 20% bovine serum albumin and serum-free DMEM to a final concentration of 8×10^{-4} M as described above. 3T3-L1 adipocytes (day 9) in six-well plates were supplemented with 2 ml of medium containing 0.05 μCi of [1-¹⁴C]AA per well for 2, 4, 6, 8, and 10 h. At these time points, media was removed and cells were washed twice with PBS. Cells were solubilized in 1 ml of 0.1 M NaOH per well and quenched using 50 μl of concentrated HCl. Radioactivity in media and cell samples was determined by liquid scintillation counting.

Membrane Preparation, Phospholipid Extraction, and Fatty Acid Analysis—3T3-L1 adipocytes (day 9) in 3 × T175 tissue culture flasks were treated with 8×10^{-4} M AA for 4 h. Cells were washed twice with PBS and solubilized by scraping in 4 ml of PBS/flask and passing 10 times through a 25-gauge needle. Cell lysates were spun at 10,000 × g for 10 min at 4 °C to remove nuclei and mitochondria and at 100,000 × g for 1 h at 4 °C to pellet membranes. The supernatant, including the floating triglyceride layer, was removed and the pellet resuspended in 1 ml of PBS by passing 10 times through a 27-gauge needle. Total lipids were extracted and phospholipids separated and transmethylated as described previously (34). The resulting fatty acid methyl esters were separated and measured on a Unicam 610 gas chromatograph with flame ionization detection and a 30 m × 0.53-mm Supelco SP2380 megabore column. Helium was used as the carrier gas at a flow rate of 19.4 ml/min. A temperature gradient program was used with an initial temperature of 70 °C increasing at 1 °C/min to 180 °C, then 5 °C/min to 200 °C and remaining at 200 °C for a further 6 min. Identification of fatty acid methyl esters was made by comparison with retention times of standard mixtures.

Membrane Fluidity Assay—3T3-L1 adipocytes (day 9) in 2 × 6-well plates were treated with 8×10^{-4} M AA for 4 h. Cells were washed and solubilized in PBS and membranes were prepared as described above. Fluidity of the membranes was assessed by measurement of steady state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene incorporated into the hydrophobic core of the membrane bilayer. A modified version of the previously described assay (35) was performed. Briefly, a stock solution of 250 μM 1,6-diphenyl-1,3,5-hexatriene in tetrahydrofuran was prepared. 0.9 mg of membrane in 3 ml of PBS was incubated for 30 min in the dark with 0.25 μM 1,6-diphenyl-1,3,5-hexatriene at 37 °C. Fluorescence anisotropy measurements were performed in a PerkinElmer Life Sciences (LS-5B) luminescence spectrometer at 37 °C, with excitation and emission wavelengths of 360 and 430 nm, respectively, and slit widths of 10 nm. Intensities were corrected for intrinsic membrane fluorescence by using the tetrahydrofuran vehicle. Fluorescence anisotropy is inversely proportional to membrane fluidity.

Inhibitor Studies—Fresh stock solutions of inhibitors were prepared in absolute ethanol (ibuprofen, nordihydroguaiaretic acid, and cycloheximide) or dimethyl sulfoxide (piroxicam and 6-MNA). 3T3-L1 adipocytes (day 9) in 6-well plates were given 1.8 ml of serum-free DMEM and 20 μl of inhibitor solution for 30 min. 0.2 M AA in ethanol was diluted 1:25 into 20% fatty acid-free bovine serum albumin in PBS as before and 200 μl of this solution was added per well for 4 h. This gives a final 1:100 dilution of the original inhibitor stock solution. The glucose uptake assay was performed as above.

Enzyme Immunoassay for PGE_2—Cells were treated ±AA/inhibitor for 4 h. 1-ml aliquots of medium were taken immediately prior to the

glucose uptake assay and analyzed for PGE_2 content using an enzyme immunoassay kit for PGE_2 (Cayman Chemical) based on the enzyme-linked immunosorbent assay method.

Adenovirus Expression—Recombinant adenoviruses were generated as described previously (36), expressing GFP (Ad-GFP) or GFP and full-length L468A/E471A human $PPAR\gamma1$ ($Ad\gamma_m$). 3T3-L1 preadipocyte (2 days post-confluence) or day 7 adipocyte cultures in 24-well plates were infected with recombinant virus by addition of 1×10^9 plaque-forming units/well. 12 h later medium containing free virus was removed and appropriate experimental medium was added. Comparable viral infection efficiency was verified by microscopy using a Zeiss axiovert 135 inverted fluorescence microscope. Only cells with >70% infectivity were used in experiments.
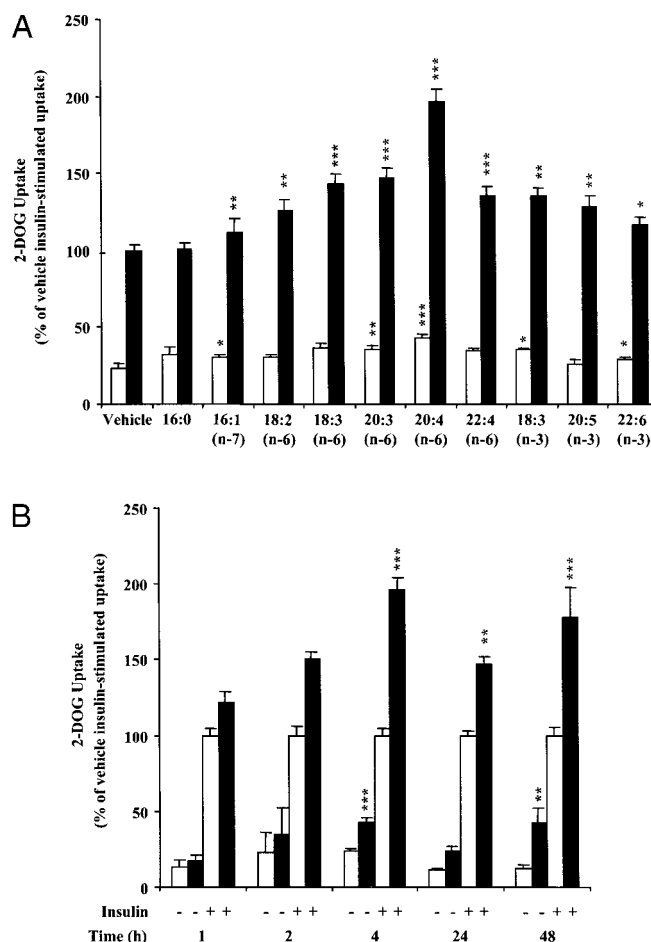
RNA Extraction/Quantitative Reverse Transcriptase-PCR—Virally infected pre-adipocytes/day 2 adipocytes were scraped and total RNA was extracted using the RNeasy mini kit from Qiagen. Adipocyte P2 (aP2) gene expression was quantified using real time quantitative reverse transcriptase-PCR. Briefly, cDNA was prepared from 100 ng of RNA using 200 units of Moloney murine leukemia virus reverse transcriptase (Promega). Real time quantitative PCR was performed using an ABI-PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Inc., Foster City, CA) as described (37). The primers and probes for aP2 were: forward, CACCGCAGACGACA-GGAAG; reverse, GCACCTGCACCAGGG; probe, TGAAGAGCATCAA-ACCCTAGATGGCGG (all 5′-3′). Results were normalized to the endogenous control, glyceraldehyde-3-phosphate dehydrogenase.

Statistical Analysis—Data are presented as mean ± S.E. Statistical significance of treatments was determined using the paired Student's t test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

RESULTS

Effect of Fatty Acids on Glucose Uptake in 3T3-L1 Adipocytes—Fully differentiated 3T3-L1 adipocytes were incubated with 8×10^{-4} M free fatty acids for 4 h and basal and insulin-stimulated glucose uptake was compared with cells incubated in vehicle alone. Ten different fatty acids were studied, including several members of the n-3 and n-6 series.[2] Basal glucose uptake was significantly increased with 16:1(n-7), 20:3(n-6), 20:4(n-6), 18:3(n-3), and 22:6(n-3) with all others showing a nonsignificant trend in the same direction (Fig. 1A). All fatty acids, except 16:0, significantly increased insulin-stimulated glucose uptake. The largest effect on glucose uptake was observed with 20:4(n-6) (AA). This increased basal glucose uptake by 1.8-fold and insulin-stimulated glucose uptake 2-fold. To establish the time course of these effects, cells were incubated with AA for 1, 2, 4, 24, and 48 h. Significant enhancement of glucose uptake, both basal and insulin stimulated, was seen from 4 h onward, at which time point maximum effects were seen (Fig. 1B). In repeat experiments, occasional cellular toxicity was noted at 24- and 48-h exposures. Concentrations of AA lower than 5×10^{-4} M had no discernible effect (data not shown). All further experiments were undertaken using 8×10^{-4} M AA at the 4-h time point.

Effect of Arachidonic Acid on the Expression and Cellular Localization of Glucose Transporters—Arachidonic acid had no effect on total cellular levels of GLUT1 and GLUT4 as assessed by Western blotting (Fig. 2A). To examine whether AA might have an effect on levels of GLUT1 or GLUT4 at the plasma membrane the plasma membrane lawn assay was used. Insulin treatment of 3T3-L1 cells produced an ~2-fold increase in GLUT1 and a 5-fold increase in GLUT4 plasma membrane expression, results which are consistent with previous published studies (33). AA increased levels of GLUT1 at the plasma membrane in the absence and presence of insulin by 2- ($p < 0.05$) and 1.3-fold ($p < 0.001$), respectively, and increased levels

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[2] The following fatty acid nomenclature was used: 16:0, palmitic acid; 16:1(n-7), palmitoleic acid; 18:2(n-6), linoleic acid; 18:3(n-6), γ-linolenic acid; 20:3(n-6), dihomo-γ-linolenic acid (DGLA); 20:4(n-6), arachidonic acid; 22:4(n-6), adrenic acid; 18:3(n-3), α-linolenic acid; 20:5(n-3), EPA; and 22:6(n-3), DHA.

FIG. 1. **Glucose uptake.** *A*, effect of fatty acids. Day 9 adipocytes in six-well plates were supplemented with 8×10^{-4} M fatty acid in serum-free DMEM for 4 h and stimulated for 30 min ± 10 nM insulin (*open columns*, unstimulated; *filled columns*, stimulated). 2-Deoxyglucose uptake was measured over 5 min as described under "Experimental Procedures." Data are mean uptakes ± S.E. from four or more independent experiments performed in triplicate, normalized to vehicle insulin-stimulated uptake (mean insulin responses, 21,630 dpm/well). *B*, time course of AA supplementation. Days 7–9 adipocytes in six-well plates were supplemented with 8×10^{-4} M AA in serum-free DMEM (1, 2, and 4 h) or in DMEM/FBS followed by 2 h in serum-free DMEM (24 and 48 h) as described under "Experimental Procedures." 2-Deoxyglucose (*DOG*) uptake was measured over 5 min following stimulation ± 10 nM insulin for 30 min. Data are mean uptakes ± S.E. from four or more independent experiments performed in triplicate, normalized to vehicle insulin-stimulated uptake at each time point (mean insulin-responses, 17,000 dpm/well). *Open columns*, vehicle; *filled columns*, AA.

of GLUT4 at the plasma membrane in the absence and presence of insulin by 1.5-fold ($p < 0.01$) and 1.4-fold ($p < 0.05$), respectively (Fig. 2B).

Effect of Arachidonic Acid on Membrane Phospholipid Composition and Fluidity—The rapidity of the effects of AA on glucose uptake raised the question of whether the uptake of AA into the cell and its incorporation into membrane phospholipid could be accomplished significantly within 4 h. 3T3-L1 adipocytes were incubated with $[1-^{14}C]AA$. Within 4 h, the cells had taken up approximately half of the labeled AA (Fig. 3A). 4 h incubation in 8×10^{-4} M AA also had a highly significant effect on the fatty acid composition of cellular membranes with a 4.5-fold increase in the AA content of membrane phospholipid ($p < 0.001$) (Fig. 3B). The positive effects of AA on glucose uptake did not appear to be mediated by nonspecific effects on membrane fluidity which was unchanged after 4 h of incubation with AA (Fig. 3C).

Effects of the Inhibition of Protein Synthesis—The protein
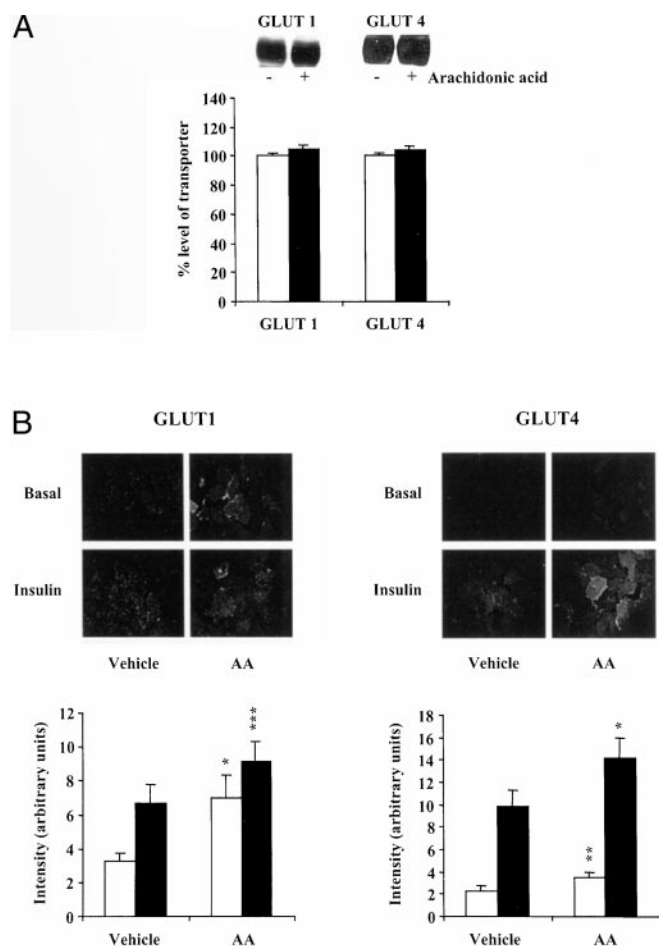
FIG. 2. **Effect of arachidonic acid on glucose transporters.** Day 9 adipocytes were supplemented with 8×10^{-4} M AA in serum-free DMEM for 4 h. *A*, Western blotting. Cells were lysed as described under "Experimental Procedures." 40 μg of total protein was resolved by SDS-PAGE and immunoblotted with anti-GLUT1 or GLUT4 antibody. A representative gel is shown for each data set. Numerical data are percentage mean ± S.E. obtained by quantitation of gels from four independent experiments, normalized to transporter levels in vehicle-supplemented cells. *Open columns*, vehicle; *filled columns*, AA. *B*, plasma membrane lawn assay. Cells were stimulated ± 10 nM insulin for 30 min before preparation of plasma membrane lawns and assay of glucose transporter translocation. Representative images from a typical experiment are shown. Data from each experiment, utilizing 16 fields for each condition, were quantified as described under "Experimental Procedures" and overall results are shown as mean ± S.E. from five independent experiments. *Open columns*, unstimulated; *filled columns*, 10 nM insulin.

synthesis inhibitor, cycloheximide, has previously been shown to influence glucose uptake in 3T3-L1 adipocytes (38, 39) and it was therefore important to choose a concentration which had no effect on glucose uptake in the absence of AA. While 2.5 μM cycloheximide had no effect on glucose uptake in control cells (Fig. 4*A*), it completely inhibited the AA potentiation of basal glucose uptake ($p < 0.001$) and partially inhibited the potentiation of insulin-stimulated glucose uptake ($p < 0.05$) (Fig. 4*B*). Thus, despite the fact that the effects of AA on glucose uptake are rapid (4 h), these appear to be highly dependent on *de novo* protein synthesis.

Effect of Inhibitors of Cyclooxygenase and Lipoxygenase on the Arachidonic Acid Potentiation of Glucose Uptake—Arachidonic acid is rapidly converted to a number of eicosanoids. To identify involvement of either cyclooxygenase (COX) or lipoxygenase (LOX) in the AA potentiation of glucose uptake, inhibitors of the two enzyme systems were used. Cyclooxygenase, or prostaglandin H synthase, exists as two isozymes, COX1,

which is constitutively expressed, and COX2, which is inducible (40). Ibuprofen inhibits both isozymes nonselectively with an IC_{50} of 10 μM (41). Piroxicam and 6-MNA selectively inhibit COX1 and COX2 with IC_{50} values of 18 and 15–55 μM, respectively (42, 41). None of these COX inhibitors significantly inhibited either the AA potentiation of basal or insulin-stimulated glucose uptake (Fig. 5*A*). To confirm that the COX enzyme activity had indeed been inhibited in these experiments, samples of media were taken prior to the studies of glucose uptake and tested for the presence of prostaglandin E_2, a major metabolite of AA in the adipocyte (43). All three compounds significantly inhibited the production of PGE_2 from the AA-supplemented cells (Fig. 5*B*).

Nordihydroguaiaretic acid (NDGA) is a selective inhibitor of the lipoxygenases ($IC_{50} = 0.2$ μM for 5-LOX and 30 μM for 12- and 15-LOX (44)). NDGA significantly inhibited the AA potentiation of basal glucose uptake at both 30 μM ($p < 0.05$) and 60 μM ($p < 0.01$) and showed a similar trend to inhibit the potentiation of insulin-stimulated uptake ($p = 0.08$) (Fig. 5*A*).

Effect of Dominant-negative PPARγ on the Arachidonic Acid Potentiation of Glucose Uptake—The L468A/E471A human PPARγ1 mutant is a powerful dominant-negative inhibitor of PPARγ action (36). A recombinant adenovirus (Adγ_m) which coexpresses this mutant PPARγ receptor and green fluorescent protein (GFP) has previously been demonstrated to markedly inhibit thiazolidinedione-induced differentiation of human pre-adipocytes (36). We first established that this virus could infect both 3T3-L1 pre-adipocytes and differentiated 3T3-L1 adipocytes with high efficiency, resulting in an increase in levels of PPARγ1 expression (Fig. 6*A* and Fig. 7, *A* and *B*). Adenoviral infection with a control virus expressing only GFP (Ad-GFP) had no effect on endogenous PPARγ levels (Figs. 6*A* and 7*B*). Expression of the dominant-negative PPARγ in 3T3-L1 pre-adipocytes significantly inhibited the induction of the differentiation marker aP2 by rosiglitazone ($p < 0.05$) (Fig. 6*B*) and also inhibited adipocyte differentiation in response to a standard differentiation mixture ($p < 0.05$) (Fig. 6*C*). No such effects were seen with Ad-GFP (data not shown). Thus, these studies confirmed that the human PPARγ mutant receptor could inhibit the function of endogenous PPARγ activity in a murine pre-adipocyte line.

To investigate the effect of the PPARγ mutant on the AA potentiation of glucose uptake, 3T3-L1 adipocytes were infected on day 7 of differentiation and the glucose uptake studies were undertaken 2 days later. Transduction efficiencies were ascertained by fluorescence microscopy (Fig. 7*A*) and plates with <70% infectivity were discarded. The high-level expression of mutant PPARγ1 in Adγ_m-infected adipocytes was confirmed by Western blotting (Fig. 7*B*). In these cells Adγ_m significantly inhibited the AA potentiation of basal, but not insulin-stimulated, glucose uptake compared with cells infected with the control virus ($p < 0.01$) (Fig. 7*D*). It is also noteworthy that there was a trend for Adγ_m to reduce basal and insulin-stimulated glucose uptake in the absence of AA (Fig. 7*C*).

DISCUSSION

A role for fatty acids in the modulation of insulin sensitivity *in vivo* and *in vitro* is well established (11), although the precise nature of the mechanisms underlying these effects is unknown. The recently established fact that certain fatty acids and their metabolites can act as endogenous ligands for the nuclear hormone receptor PPARγ provides a potential mechanism (15). With this in mind, we investigated the effect of a range of fatty acids on glucose uptake in 3T3-L1 adipocytes. Having established that AA had the most marked effect on the enhancement of glucose uptake, we have attempted to dissect the molecular
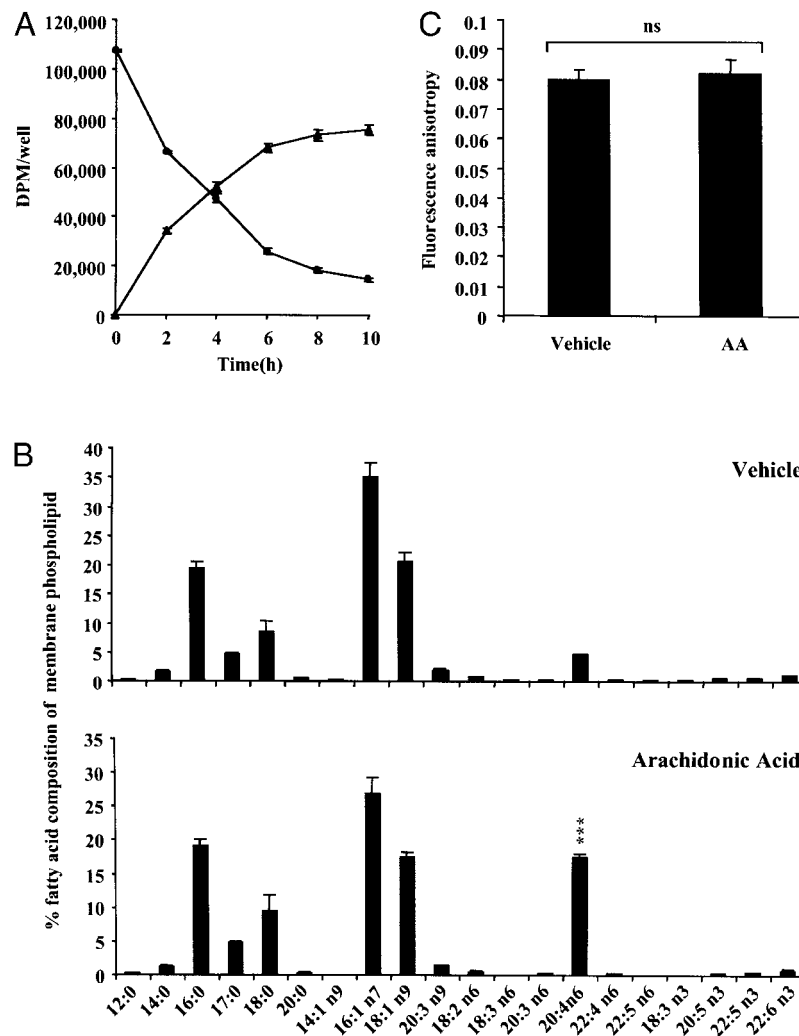
FIG. 3. **Effect of arachidonic acid on membrane phospholipid composition and fluidity.** *A*, [1-^{14}C]AA uptake. Day 9 adipocytes in six-well plates were supplemented with 8×10^{-4} M AA, including a trace of [1-^{14}C]AA, in serum-free DMEM for 2, 4, 6, 8, and 10 h. Radioactivity in media and cell lysates was measured. Data are mean dpm/well $\pm$ S.E. from six wells in one representative experiment. *Circles*, medium; *triangles*, cell lysate. *B*, membrane phospholipid fatty acid composition. Day 9 adipocytes in $3 \times$ T175 flasks were supplemented with 8×10^{-4} M AA in serum-free DMEM for 4 h. Membranes were prepared, phospholipids extracted, and their fatty acid composition analyzed as described under "Experimental Procedures." Data are mean percentage fatty acid composition of membrane phospholipids $\pm$ S.E. from four independent experiments. *C*, membrane fluidity. Day 9 adipocytes in six-well plates were supplemented with 8×10^{-4} M AA in serum-free DMEM for 4 h. Membranes were prepared and the fluidity assay performed as described under "Experimental Procedures." Data are mean fluorescence anisotropy "r" $\pm$ S.E. from eight independent experiments performed in duplicate.

events underlying this effect. Our studies have found that 4 h of exposure to AA potentiates basal and insulin-stimulated glucose uptake by increasing levels of GLUT1 and GLUT4 at the plasma membrane. The potentiation of glucose uptake is independent of cyclooxygenase but at least partly dependent on an intact lipoxygenase pathway, *de novo* protein synthesis, and PPARγ activation. Our results also suggest that the molecular mechanisms whereby AA potentiates basal *versus* insulin-stimulated glucose uptake may differ slightly.

The systematic study of the effects of various fatty acids on glucose uptake in adipocytes was first reported by Grunfeld *et al.* (30). These workers showed that incubation of 3T3-L1 cells, during the process of differentiation, with a range of saturated and monounsaturated fatty acids increased basal glucose uptake. Saturated fatty acids decreased insulin-stimulated uptake, whereas monounsaturated fatty acids had no effect. Our studies investigated the effect of 10 mainly polyunsaturated, fatty acids on glucose uptake in fully differentiated 3T3-L1 adipocytes. Differentiated cells were used to eliminate confounding effects of fatty acids on the adipogenic process itself. In agreement with Grunfeld *et al.* (30) there appeared to be a

relatively nonspecific effect of all fatty acids to increase basal glucose uptake. However, all fatty acids studied, except the saturated palmitic acid (16:0), increased insulin-stimulated glucose uptake and this effect did not appear to be specific to fatty acid series. The most marked effects on glucose uptake were observed with AA, which increased basal and insulin-stimulated glucose uptake at all time points studied. Two previous studies have shown a similar effect of AA on basal glucose uptake in 3T3-L1 adipocytes. Fong *et al.* (32) observed an increased basal glucose uptake with 0.2 mM AA at all time points from 1 to 8 h (32). Similarly, Tebbey *et al.* (31) reported such an effect with 50 μM AA following 24 and 72 h incubation. Neither study, however, observed a significant effect of AA on insulin-stimulated glucose uptake. Consistent with the observations of Fong *et al.* (32) who demonstrated that the potentiating effects of 8 h exposure to AA on glucose uptake were partially inhibited by cycloheximide, we found that the effects of 4 h of AA were also sensitive to the inhibition of protein synthesis suggesting the involvement of a rapidly synthesized protein intermediate in the mediation of this response.
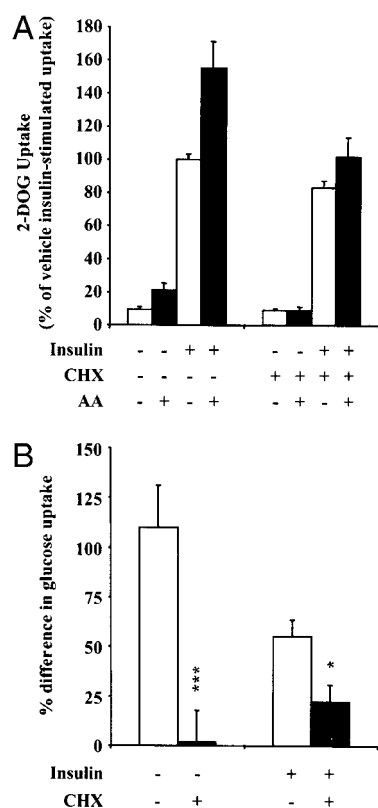
Having established the effect of AA on glucose uptake, we

FIG. 4. **Effect of cycloheximide on the arachidonic acid potentiation of glucose uptake.** Day 9 adipocytes were supplemented with 2.5 μM cycloheximide for 30 min and with 8×10^{-4} M AA for a further 4 h as described under "Experimental Procedures." Cells were stimulated for 30 min $\pm$ 10 nM insulin and 2-deoxyglucose uptake was measured over 5 min. *A*, data are mean 2-deoxyglucose (*DOG*) uptake $\pm$ S.E. from seven independent experiments performed in triplicate, normalized to nil cycloheximide (*CHX*), vehicle insulin-stimulated uptake (mean insulin responses 19,800 dpm/well). *Open columns*, vehicle; *filled columns*, AA. *B*, data from *A* is expressed as mean percentage difference in glucose uptake (AA, *cf.* fatty acid vehicle) $\pm$ S.E.

FIG. 5. **Effect of metabolic inhibitors on the arachidonic acid potentiation of glucose uptake.** *A*, glucose uptake. Day 9 adipocytes were supplemented with the indicated concentrations of inhibitors for 30 min and with 8×10^{-4} M AA for a further 4 h as described under "Experimental Procedures." Cells were stimulated for 30 min $\pm$ 10 nM insulin and 2-deoxyglucose uptake was measured over 5 min. Data are mean percentage difference in 2-deoxyglucose uptake (AA, *cf.* fatty acid vehicle) $\pm$ S.E. from four or more independent experiments performed in triplicate, normalized to inhibitor control (mean control values, basal, 57%; insulin, 128%). *B*, PGE$_2$ enzyme immunoassay. Aliquots of medium were removed immediately prior to the glucose uptake assay and tested for the presence of PGE$_2$. Data are mean picomole of PGE$_2$/ml $\pm$ S.E. for AA-treated cells $\pm$ inhibitor from four independent experiments performed in triplicate. Fatty acid vehicle-treated cells gave values below the level of detection of the EIA kit (4.25 pmol/ml).

examined whether this was mediated through increased expression or altered cellular location of glucose transporters. Previous studies have demonstrated a decrease in total cellular and plasma membrane GLUT4 in 3T3-L1 adipocytes supplemented with AA for 24 and 48 h (31, 8). Conversely, 3T3-L1 adipocytes supplemented with AA for 8 and 48 h exhibited increased total cellular and plasma membrane levels of GLUT1 (32, 31). This effect was not, however, observed with 2 h AA incubation (32). In our experiments, 4 h AA incubation had no effect on total cellular levels of GLUT1 or GLUT4. However, a highly significant increase in the plasma membrane levels of both transporters, in the absence and presence of insulin was observed. Thus AA appears to enhance either the translocation of both major adipocyte glucose transporters to the plasma membrane or reduce their rate of internalization.

It has been suggested that fatty acids may have nonspecific effects on cellular membranes which could secondarily alter the intrinsic activity of transmembrane glucose transporters (12, 30). In particular, alterations in the fatty acid composition of membrane phospholipids can affect membrane fluidity (45). It was unclear, however, whether as short an exposure as 4 h would be sufficient to lead to such changes in plasma membrane composition. We observed that, within 4 h, at least half of the available AA had entered the cells and that there was a highly significant increase in the AA content of membrane phospholipids. However, this did not have any discernible effect on membrane fluidity. Therefore, although we have not excluded an effect of AA on the intrinsic activity of membrane
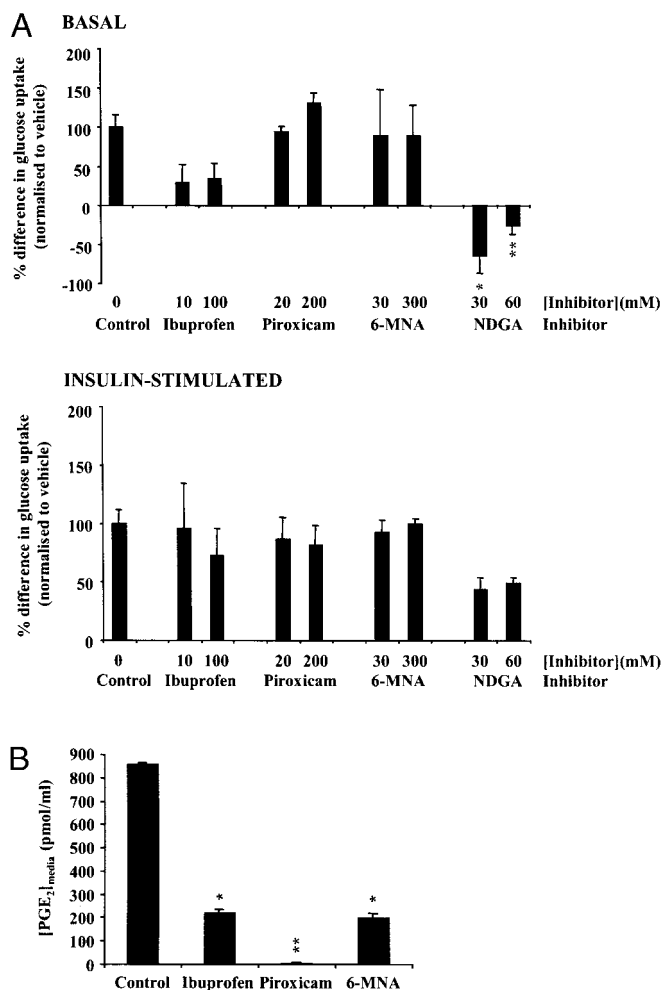
glucose transporters any such effect is unlikely to be mediated by changes in membrane fluidity.

Arachidonic acid is capable of being metabolized by both the cyclooxygenase and the lipoxygenase enzyme systems. The resulting metabolites have been implicated in the control of a wide range of physiological and pathological processes. Inhibitors of the individual enzyme systems were employed to examine which, if either, of these systems was involved in the generation of an AA metabolite that might mediate the effects on glucose uptake. Cyclooxygenase, or prostaglandin H synthase, catalyzes the conversion of AA to the prostanoids and the thromboxanes. The enzyme exists as two isozymes, COX1, which is constitutively expressed, and COX2, which is inducible (40). We inhibited the COX enzyme activity in 3T3-L1 adipocytes for 30 min prior to addition of AA using ibuprofen (nonselective), piroxicam (COX1), or 6-MNA (COX2). None of these compounds inhibited the AA potentiation of either basal or insulin-stimulated glucose uptake in our system, despite the
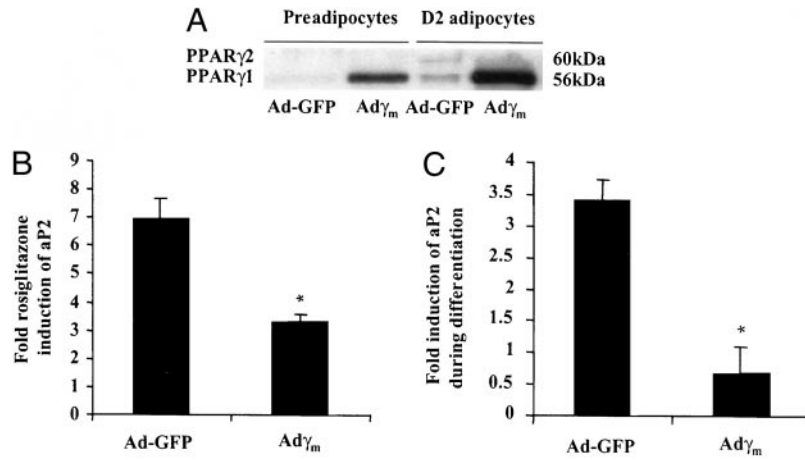
FIG. 6. **Transduction of 3T3-L1 pre-adipocytes with dominant-negative PPARγ.** Confluent pre-adipocytes in 24-well plates were infected for 12 h with 1×10^9 pfu/well Ad-GFP or Adγ$_m$. Medium was changed to DMEM/FBS with differentiation mixture or DMEM/NBCS $\pm 1 \times 10^{-7}$ M ROSIGLITAZONE FOR 48 H. *A*, Western blotting. Infected pre-adipocytes (minus rosiglitazone) and day 2 adipocytes were scraped and lysed as described under "Experimental Procedures." 10 μg of total protein was resolved by SDS-PAGE and immunoblotted with anti-PPARγ antibody. *B*, aP2 induction by rosiglitazone in pre-adipocytes. Infected pre-adipocytes $\pm 1 \times 10^{-7}$ M rosiglitazone were lysed and RNA extracted and reverse transcribed. aP2 gene expression was quantified using real time quantitative PCR as described under "Experimental Procedures." Data are mean fold rosiglitazone induction of aP2 $\pm$ S.E. *C*, aP2 induction during differentiation. Infected pre-adipocytes (minus rosiglitazone) and day 2 adipocytes were lysed and RNA extracted and reverse transcribed. aP2 gene expression was quantified using real time quantitative PCR. Data are mean fold induction of aP2 during differentiation $\pm$ S.E. For experiments B and C, data are from four independent experiments performed in triplicate, normalized to the endogenous control, glyceraldehyde-3-phosphate dehydrogenase. Ad-GFP data are representative of data from uninfected cells.
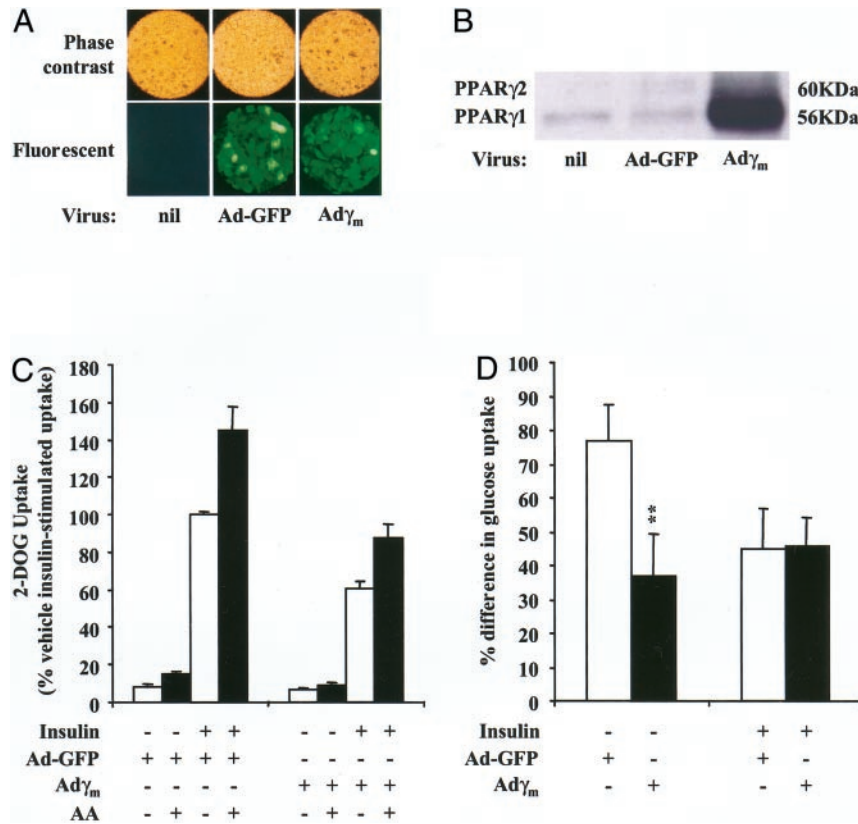
FIG. 7. **Transduction of 3T3-L1 adipocytes with dominant-negative PPARγ.** Day 7 adipocytes in 24-well plates were infected for 12 h with 1×10^9 pfu/well Ad-GFP or Adγ$_m$. Medium was changed to DMEM/FBS for 48 h. *A*, microscopy. Infection efficiency was estimated using fluorescence microscopy. Representative images at $\times320$ magnification from a typical experiment are shown. *B*, Western blotting. Infected adipocytes were scraped and lysed as described under "Experimental Procedures." 10 μg of total protein was resolved by SDS-PAGE and immunoblotted with anti-PPARγ antibody. *C* and *D*, glucose uptake. Infected adipocytes were supplemented with 8×10^{-4} M AA IN SERUM-FREE DMEM FOR 4 H AND STIMULATED FOR 30 MIN $\pm$ 10 nM insulin. 2-Deoxyglucose uptake was measured over 5 min. *C*, data are mean 2-deoxyglucose uptake $\pm$ S.E. from four independent experiments performed in triplicate, normalized to Ad-GFP, vehicle insulin-stimulated uptake (mean insulin responses 11,310 dpm/well). *Open columns*, vehicle; *filled columns*, AA. *D*, data from *C* is expressed as mean percentage difference in glucose uptake (AA, *cf*. fatty acid vehicle) $\pm$ S.E.

demonstration that the COX enzyme activity had indeed been inhibited. These results contrast with those of Fong *et al.* (46) who reported that indomethacin (a nonselective COX inhibitor) inhibited the 8-h AA potentiation of basal glucose uptake. The reasons for this discrepancy are unclear but may be explained by the differing experimental conditions of the two studies,

specifically the time points and particular COX inhibitors studied. Several groups have demonstrated that certain COX metabolites of AA, particularly the J series of prostaglandins, can activate PPARγ *in vitro* and could therefore act as endogenous ligands (21, 26, 27). Our results would suggest that the AA potentiation of glucose uptake is not mediated via the action of a COX metabolite on PPARγ. However, the involvement of PPARγ in mediating the AA effect on glucose uptake is not excluded. It is possible that other metabolites, or AA itself, could act as PPARγ activators in this system. It should also be noted that COX inhibitors themselves have also been demonstrated to act as ligands at PPARγ (47). Ibuprofen was shown to induce PPARγ activity 20-fold at 10^{-4} M but piroxicam had a negligible effect. The effects of 6-MNA have not been reported.

Lipoxygenase enzymes catalyze the conversion of AA to the leukotrienes and the hydroxyeicosatetraenoic acid. The enzyme exists as three isozymes, 5-LOX, 12-LOX, and 15-LOX. We inhibited the LOX enzyme activity in 3T3-L1 adipocytes for 30 min prior to addition of AA using NDGA, a nonselective LOX inhibitor. We observed a significant inhibition of the AA potentiation of basal glucose uptake with NDGA. Furthermore, there was a trend for NDGA to inhibit the AA potentiation of insulin-stimulated uptake. These results suggest that a LOX metabolite(s) of AA may be involved in its effect on glucose uptake. Indeed, it has been shown that the 15-LOX metabolites of both AA and linoleic acid, 15-hydroxyeicosatetraenoic acid and 9- and 13-hydroxyoctadecadienoic acid, respectively, can function as micromolar PPARγ agonists (29).

The nuclear hormone receptor, PPARγ, has recently been shown to have an important role in the control of insulin sensitivity (14, 15). Loss of function mutations in human PPARγ result in extreme insulin resistance and diabetes mellitus (20). As previously mentioned, several metabolites of AA have been recognized as potential endogenous ligands for PPARγ. Such metabolites include 15d-PGJ$_2$ (26, 27), Δ12-PGJ$_2$ (28), and PGD$_2$ (21). It has also been demonstrated that AA itself can act as a PPARγ ligand (22–25). To investigate the role of PPARγ in our system, we inhibited endogenous activity by transducing fully differentiated adipocytes with a dominant-negative hPPARγ mutant receptor. This mutant form of hP-PARγ has previously been shown to inhibit co-transfected wild-type receptor action and to inhibit the thiazolidinedione-induced differentiation of human pre-adipocytes (36). We demonstrated that the transduced hPPARγ mutant receptor was highly expressed in murine 3T3-L1 pre-adipocytes and adipocytes and inhibited endogenous wild-type activity. We then showed that the AA potentiation of basal glucose uptake was partially, but significantly, inhibited in cells transduced with the mutant receptor. There was, however, no significant effect of the mutant receptor on the AA potentiation of insulin-stimulated glucose uptake. These results suggest that PPARγ activation is at least partly required for the AA effect on glucose uptake. While the dominant-negative mutant is clearly capable of specifically inhibiting PPARγ function some caveats of this system should be mentioned. First, although high levels of infectivity were observed, 3T3-L1 adipocytes are notoriously difficult to transduce with high efficiency using adenoviruses and full inhibition of PPARγ function in all cells may not have been achieved. It is also theoretically possible that this dominant-negative mutant might, at high concentrations, occupy and inhibit transcription through response elements other than the true native peroxisome proliferator response elements. Such effects, however, have not actually been demonstrated.

In summary, we have undertaken the most comprehensive study to date of the effects of free fatty acids on basal and insulin-stimulated glucose uptake in an insulin-responsive cell type. The potentiating effects of even short-term exposure to AA are at least partially dependent on new protein synthesis and involve enhanced expression of both GLUT1 and GLUT4 at the plasma membrane. While activation of PPARγ appears to be necessary, particularly in the absence of insulin, this does not appear to involve cyclooxygenase metabolites of AA but, somewhat surprisingly, our results suggest a greater role for lipoxygenase metabolites in the mediation of this important metabolic effect.

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Arachidonic Acid Stimulates Glucose Uptake in 3T3-L1 Adipocytes by Increasing GLUT1 and GLUT4 Levels at the Plasma Membrane: EVIDENCE FOR INVOLVEMENT OF LIPOXYGENASE METABOLITES AND PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ

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