# Resistin expression in 3T3-L1 adipocytes is reduced by arachidonic acid

**Fred Haugen, Naeem Zahid, Knut T. Dalen, Kristin Hollung, Hilde I. Nebb, and Christian A. Drevon**[1]

Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway

**Abstract** **The resistin gene is expressed in adipocytes and encodes a protein proposed to link obesity and type 2 diabetes. Increased plasma FFA is associated with insulin resistance. We examined the effect of separate FFAs on the expression of resistin mRNA in cultured murine 3T3-L1 adipocytes. The FFAs tested did not increase resistin expression, whereas both arachidonic acid (AA) and eicosapentaenoic acid (EPA) reduced resistin mRNA levels. AA was by far the most potent FFA, reducing resistin mRNA levels to ~20% of control at 60–250 μM concentration. Selective inhibitors of cyclooxygenase-1 and of mitogen-activated protein kinase kinase counteracted AA-induced reduction in resistin mRNA levels. Transient overexpression of sterol-regulatory element binding protein-1a (SREBP-1a) activated the resistin promoter, but there was no reduction in the abundance of ~65 kDa mature SREBP-1 after AA exposure. Actinomycin D as well as cycloheximide abolished the AA-induced reduction of resistin mRNA levels, indicating dependence on de novo transcription and translation. Our data suggest that reductions in resistin mRNA levels involve a destabilization of the resistin mRNA molecule. An inhibitory effect of AA and EPA on resistin expression may explain the beneficial effect of ingesting PUFAs on insulin sensitivity.**—Haugen, F., N. Zahid, K. T. Dalen, K. Hollung, H. I. Nebb, and C. A. Drevon. **Resistin expression in 3T3-L1 adipocytes is reduced by arachidonic acid.** ***J. Lipid Res.*** **2005.** 46: **143–153.**

**Supplementary key words** fatty acid • insulin resistance • peroxisome proliferator-activated receptor γ • liver X receptor α • sterol-regulatory element binding protein-1 • polyunsaturated fatty acids • prostaglandin • E-prostanoid 1 • E-prostanoid 4 • F-prostanoid

An essential function of the adipose tissue is to secrete adipokines, a group of hormones with effects on whole body metabolism. Adipokines are potentially important for the development of obesity-related diseases such as diabetes, cardiovascular diseases, and cancer (1). Insulin resistance is a hallmark of diabetes, and its development may involve several adipokines, including leptin (2), tumor necrosis factor-α (3), interleukin-6 (4), adiponectin (5), and resistin (6).

The resistin gene belongs to the resistin-like molecule (RELM) gene family (6–8). In mice, resistin is preferentially expressed in adipose tissue (6, 7), whereas RELMα is found mostly in adipose tissue and lung (7, 8), RELMβ is in the intestine (8), and RELMγ is expressed in hematopoietic tissues (9). In humans, resistin and RELMβ homologous genes have been identified, with highest expression in macrophages (10, 11) and the intestine (8), respectively. RELMs encode peptides (105–117 amino acids) with 10 cysteine residues in the C terminus having identical spacing (7, 12). Resistin peptides are linked in pairs with disulfide bonds via the conserved cysteine residues and secreted as homodimers (13).

Administration of recombinant resistin reduced insulin-mediated glucose uptake in 3T3-L1 adipocytes (6) as well as L6 rat skeletal muscle cells (14). Resistin may also inhibit glucose oxidation and insulin-stimulated glycogenesis in isolated muscle from rats (15). Normally, insulin inhibits hepatic glucose production when plasma glucose levels increase in mice, but resistin attenuates this effect of insulin, promoting increased plasma glucose concentration (16). Mice lacking resistin exhibit low blood glucose levels after fasting as a result of reduced hepatic glucose production (17). Transgenic expression of the mouse resistin gene in adipose tissue impaired oxidative and nonoxidative glucose disposal in skeletal muscle and promoted glucose intolerance (15).

The resistin mRNA level was reduced by insulin-sensitiz-

Abbreviations: AA, arachidonic acid; BADGE, bisphenol A diglycidyl ether; C/EBP, CCAAT/enhancer binding protein; COX, cyclooxygenase; DHA, docosahexaenoic acid; EP, E-prostanoid; EPA, eicosapentaenoic acid; FP, F-prostanoid; LA, linoleic acid; LDH, lactate dehydrogenase; LN, α-linolenic acid; LUC, luciferase; LXR, liver X receptor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; OA, oleic acid; PA, palmitic acid; PG, prostaglandin; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; RELM, resistin-like molecule; RXR, retinoic acid X receptor; SA, stearic acid; SREBP, sterol-regulatory element binding protein; TTA, tetradecylthioacetic acid.

[1] To whom correspondence should be addressed.
e-mail: c.a.drevon@basalmed.uio.no

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ing thiazolidinedione ligands of the transcription factor peroxisome proliferator-activated receptor γ (PPARγ) in 3T3-L1 adipocytes (6, 18, 19), although the regulation in vivo has been disputed (6, 20, 21). Both suppressors (insulin) and activators (β-adrenoceptor agonists) of lipolysis have been reported to reduce resistin mRNA levels in 3T3-L1 adipocytes (18, 22, 23). Furthermore, intracellular levels of cAMP (forskolin) involving protein kinase A may modulate the level of resistin mRNA (18, 23).

Increased plasma FFA is associated with insulin resistance (24). However, dietary intake of PUFAs may alter the development of human diseases such as cardiovascular diseases, diabetes, and cancer by affecting enzyme activities, cellular signal transduction, and composition of membrane lipids (25). In addition, PUFAs may influence gene expression by direct interaction with the transcription machinery (26). Fatty acids may modulate the activity of the adipogenic transcription factors PPARγ, liver X receptor α (LXRα), and sterol-regulatory element binding protein (both SREBP-1a and SREBP-1c) (27–30).

Our aim in the present work was to study the effects of selected dietary FFAs on resistin expression in murine 3T3-L1 adipocytes to determine if resistin could represent a link between increased FFA plasma levels and insulin resistance. FFAs in general did not affect resistin mRNA levels. Only stearic acid (SA) promoted a small increase in resistin expression, but we observed a marked suppression of resistin mRNA levels by arachidonic acid (AA) and to a smaller extent by eicosapentaenoic acid (EPA). AA promoted its effects dependent on cyclooxygenase-1 (COX-1) and mitogen-activated protein kinase kinase (MEK) activity. In transfection experiments, SREBP-1a transactivated the resistin promoter, but we found no evidence that AA regulated SREBP-1 activity in 3T3-L1 adipocytes. Our data also suggested an active degradation of resistin mRNA dependent on the de novo synthesis of mRNA and protein.

## MATERIALS AND METHODS

### Cell culture and supplements

3T3-L1 and COS-1 cells from the American Type Culture Collection (Rockville, MD) were cultured in DMEM supplemented with 10% FBS (Integro, Dieren, Holland), 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37°C in 5% $CO_2$ (31). 3T3-L1 fibroblasts were seeded on 12-well plates, and differentiation was initiated 2 days after confluence (18). After 6 days of differentiation, adipocytes were given fresh growth medium daily for up to 4 days and every other day thereafter. The cells were maintained for up to 8 days or 15 days after induction of differentiation, at which time specific treatments were initiated. Cells incubated under serum-free conditions were pretreated in DMEM containing 2 mM L-glutamine, streptomycin (50 μg/ml), penicillin (50 U/ml), and 1% FFA-free BSA (Sigma) for 24 h. The sodium salts of the fatty acids palmitic acid (PA; 16:0), SA (18:0), oleic acid (OA; 18:1, n-9), linoleic acid (LA; 18:2, n-6), α-linolenic acid (LN; 18:3, n-3), AA (20:4, n-6), EPA (20:5, n-3), or docosahexaenoic acid (DHA; 22:6, n-3) (Nu-Chek-Prep, Elysian, MN) were dissolved at 50°C to make 6 mM stock solutions in culture medium containing 2.4 mM FFA-free BSA (Sigma, St. Louis, MO) and stored for up to 1 month at −20°C under argon (32). Fatty acid-containing media were dilutions of stock solutions, and the molar ratio of BSA to FFA was 1:2.5 for all cell treatments. Cytotoxicity was controlled by determining lactate dehydrogenase (LDH) activity in the medium with the Cytoxicity Detection Kit (Boehringer Mannheim, Indianapolis, IN) after incubation with the different fatty acids. We used COX inhibitors dissolved in vehicle liquids: indomethacin (ethanol), flurbiprofen (DMSO), and meloxicam (DMSO), as well as the isoform-specific COX inhibitors SC-506 (DMSO) for COX-1 and NS-398 (DMSO) for COX-2 (Calbiochem, La Jolla, CA). MEK and phosphatidylinositol 3-kinase (PI3K) signaling was inhibited with DMSO-dissolved PD98059 and LY294002 (Calbiochem), respectively. Transcription and translation were inhibited with actinomycin D (DMSO) and cycloheximide (DMSO), respectively (Sigma). Other chemicals and the vehicles we used were prostaglandins (PGs; Calbiochem; ethanol), insulin (Sigma; water), 25-OH-cholesterol (Sigma; ethanol), darglitazone (AstraZeneca R&D, Molndal, Sweden; DMSO), T0901317 (Alexis, Lausen, Switzerland; DMSO), bisphenol A diglycidyl ether (BADGE; Fluka, Buchs, Switzerland; DMSO), and tetradecylthioacetic acid (TTA; Rolf K. Berge, University of Bergen, Norway; water). The cells were harvested in TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA), and samples were stored at −70°C until RNA isolation.

### RNA preparation and Northern blot/RT-PCR analysis

Total RNA was prepared using the TRIzol method, and RNA quality and quantity were determined spectrophotometrically. For Northern blots, RNA (10 μg) was electrophoresed on a 1.0% agarose gel containing 6.7% formaldehyde, transferred onto Hybond-N nylon membranes (Amersham, Little Chalfont, UK) by capillary action, and UV cross-linked (33). Primer sequences for amplifying cDNA from the mouse resistin gene (approved name, Retn) (AGCGGATGAAGAACCTTTCA and GGAGGAGACTGTCCAGCAAT) were designed based on the available mRNA sequence (gi:12228705) using Primer3 software (34). Using 3T3-L1 mRNA as a template, the 185 bp region of resistin cDNA was amplified on a LightCycler with the RNA Amplification Kit (Roche Diagnostics GmbH, Mannheim, Germany). The RT-PCR product was cloned and sequenced before hybridization of membranes (33). Membranes were probed with [α-$^{32}$P]dCTP (Amersham)-radiolabeled cDNAs synthesized using a multiple DNA labeling system (Amersham) and purified on ProbeQuant G50 Micro columns (Amersham) (33). The tissue specificity of resistin mRNA detection using the probe described in this paper was the same as that reported previously (18). Blots were stripped and reprobed for other mRNAs: SREBP-1, PPARγ (33), LXRα (31), L27 (RPL27; American Type Culture Collection catalog no. 107385), and 36B4 (acidic ribosomal phosphoprotein P0; used here as an internal control). Hybridization signals were detected by the PhosphorImager and quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For RT-PCR analysis of PG receptor mRNA, exon-spanning primer pairs for E-prostanoid 1 (EP1; ptger1; ACGCACACGATGTGGAAAT and CGCTGCAGGGAGTTAGAGTT; 127 bp), EP2 (ptger2; ATCACCTTCGCCATATGCTC and AGTATGGCAAAGACCCAAGG; 143 bp), EP3 (ptger3; GGATCATGTGTGTGCTGTCC and CCCATCTGTGTCTTGCATTG; 103 bp), EP4 (ptger4; ACCTCAGTTCCGGAAAGTTG and GCTCACGGTTCGATCTAGGA; 132 bp), and F-prostanoid (FP; ptgfr; AAGGCAGATCTCACCACCTG and TTCACAGGTCACTGGGGAAT; 137 bp) were designed with Primer3 (34) and mRNA input sequences (gi:7363446, gi:31560647, gi:6755217, gi:6679530, and gi:31560648, respectively). The one-step RT-PCR amplification of PG receptors was performed for each primer pair with Thermoscript Plus reverse transcriptase and Platinum *Taq* DNA polymerase (Invitrogen) using 1 μg of to-



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tal RNA from 3T3-L1 adipocytes 15 days after induction of differentiation (24 h BSA-treated, serum-free conditions) or water only (negative control) as a template (annealing temperature, 60°C), and after 40 cycles the entire 25 μl reaction volumes were loaded onto 3% agarose gels and evaluated visually.

### Whole cell protein extracts and Western blot analysis

After treatment, 3T3-L1 adipocytes were washed twice with PBS and added 100 μl/well of lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, and 0.2 mM PMSF) with Complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Plates were shaken at 4°C for 30 min, and the resulting lysate was collected in a microtube and centrifuged at 13,000 rpm in a table centrifuge. The protein concentrations in the cleared lysates were assayed using the bicinchoninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, IL). A total of 100 μg of protein from each lysate was boiled for 1 min in SDS buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 50 mM DTT) before separation by SDS-PAGE on a Criterion precast 7.5% Tris-glycine gel (Bio-Rad, Hercules, CA) together with a prestained Precision Plus Dual Color protein standard (Bio-Rad) and finally blotted onto a 0.2 μm nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The blots were incubated at room temperature in different TBS (pH 7.5) solutions: first, 30 min of blocking with 3% dry milk, then without washing, 2 h incubation with 1:100 dilution of SREBP-1 rabbit polyclonal antibody (K-10; Santa Cruz Biotechnology, Santa Cruz, CA) with 0.005% Tween 20 (Sigma), and then washing three times in 0.05% Tween 20, and finally, 1:4,000 dilution of goat anti-rabbit IgG(H+L)-HRP, mouse/human absorbed antibodies (Southern Biotechnology, Birmingham, AL) for 2 h followed by two washes in 0.05% Tween 20 and TBS alone. SREBP-1 was visualized on film (Eastman Kodak, Rochester, NY) using the ECL+ chemo-luminescence kit (Amersham).

### Cloning of the mouse resistin promoter

The available resistin mRNA (gi:12228705) was used as a bait in a Basic Local Alignment Search Tool search against nonredundant and high-throughput genomic sequence databases to identify the mouse resistin promoter (annealed to gi: 9964856). Primers (TACTCGAGTTTTTGGTGTTTGGAGACAGGGTTT and TACTCGAGCACATACACAGACATGGACAGCACA) were designed with Primer3 software (34) to amplify a long fragment of the resistin promoter (5,358 bp; −4638 to +720). The PCR amplification was performed with *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) as described (31) from mouse genomic DNA (Clontech; BALB/c, #6650-1). The amplified promoter fragment was subsequently cloned into the pPCR-Script vector (Stratagene). A truncated version of this fragment, obtained by restriction cutting with *Nhe*I and *Xho*I generating a 3.3 kb fragment (−2610 to +720 of the resistin gene), was subcloned into the *Nhe*I/*Xho*I sites in the pGL3-basic firefly luciferase (LUC) reporter vector (Promega, Madison, WI) to generate the resistin reporter vector. A more truncated version of the resistin reporter (−393 to +720 of the resistin gene) was obtained by restriction digestion with *Kpn*I followed by religation of the reporter vector.

### Transfection and LUC assay

COS-1 cells were transiently transfected on six-well plates with firefly LUC reporter (5 μg) and cotransfected with pSV-β-galactosidase (pCMV-βgal; 3 μg; for use as an internal standard) and CCAAT/enhancer binding protein-α (C/EBPα) expression vector (1 μg) (35) with calcium phosphate precipitation (36). The mouse C/EBPα expression vector (pCDNA-3.1-C/EBPα) was described previously (37) and was a gift from O. A. MacDougald (University of Michigan, Ann Arbor, MI). Total DNA concentration was adjusted to 12 μg with empty expression vector and pGL3-basic vector. Undifferentiated 3T3-L1 fibroblasts were plated on 24-well plates and were semiconfluent on the day of transfection. Wells were added to a transfection solution containing 1 μl of LipofectAMINE reagent, 4 μl of LipofectAMINE Plus reagent (Invitrogen Life Technologies), and ~500 ng of DNA (same amount for all wells in separate experiments), including firefly LUC reporter (200 ng) and pTK Renilla LUC expression vector (20 ng; Promega) and different transcription factor expression vectors (10 ng of each if not stated otherwise). The retinoic acid X receptor (RXRα; pCMX-RXR) and LXRα (pCMX-LXR) expression vectors have been described (38) and were provided by D. J. Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). The PPARγ (pSG5 hPPARγ2) expression vector was a gift from J. Auwerx (CNRS/INSERM/Université Louis Pasteur, Illkirch, France) (31, 39). The SREBP-1a (pCMV-CSA10) expression vector was provided by T. F. Osborne (University of California, Los Angeles, CA) (40). The C/EBPα expression vector is described above. Total DNA concentration was adjusted using empty reporter and expression vectors. After 3 h of transfection, medium containing supplements of AA or PGs was added and cells were incubated for 24 h. Cells were harvested in 200 μl of lysis buffer, and LUC activities were measured using the dual LUC assay kit (Promega) in a Luminometer (TD-20/20 Luminometer; Turner Designs, Sunnyvale, CA).

### Presentation of data and statistical analysis

Relative resistin mRNA levels were calculated as the ratio of resistin and 36B4 signal intensities. The effects of different incubations were expressed relative to the control incubation, which was assigned a value of 100%, and are presented in bars representing means ± SEM of three to five experiments. The statistical significance of differences between incubations was assessed with a two-tailed Student's *t*-test (* $P < 0.05$, ** $P < 0.01$).

## RESULTS

To test whether fatty acids affect the transcriptional level of resistin expression, we differentiated 3T3-L1 adipocytes until day 8 or day 15 and incubated with vehicle (BSA) or with 250 μM OA, LN, AA, or EPA. Initially, we observed that resistin mRNA levels were consistently reduced by incubation with AA in 3T3-L1 adipocytes (**Fig. 1A**). Judging by the size of the intracellular lipid droplets, cells differentiated for 15 days had more lipids than after 8 days of differentiation (data not shown). The PUFAs we tested (LN, AA, and EPA) appeared to reduce resistin expression at day 8, whereas AA reduced resistin mRNA at day 15 (Fig. 1A). No effect was observed with OA (Fig. 1A). Leakage of LDH into the media after incubation with the different FFAs was 5–6% (day 8) and 2–4% (day 15) of total LDH released after complete cell lysis and did not indicate toxic side effects of the different FFAs. In the subsequent experiments with FFAs, we omitted serum and used the more lipid-loaded and fully differentiated (day 15) 3T3-L1 adipocytes. The level of resistin mRNA was significantly reduced at concentrations of ≥30 μM in adipocytes incubated with AA, and expression levels at ~20% of the BSA control were observed at concentrations of 60–250 μM (Fig. 1B). Incubation with 250 μM EPA reduced the level of resistin mRNA to 78 ± 11%, whereas 30 μM SA



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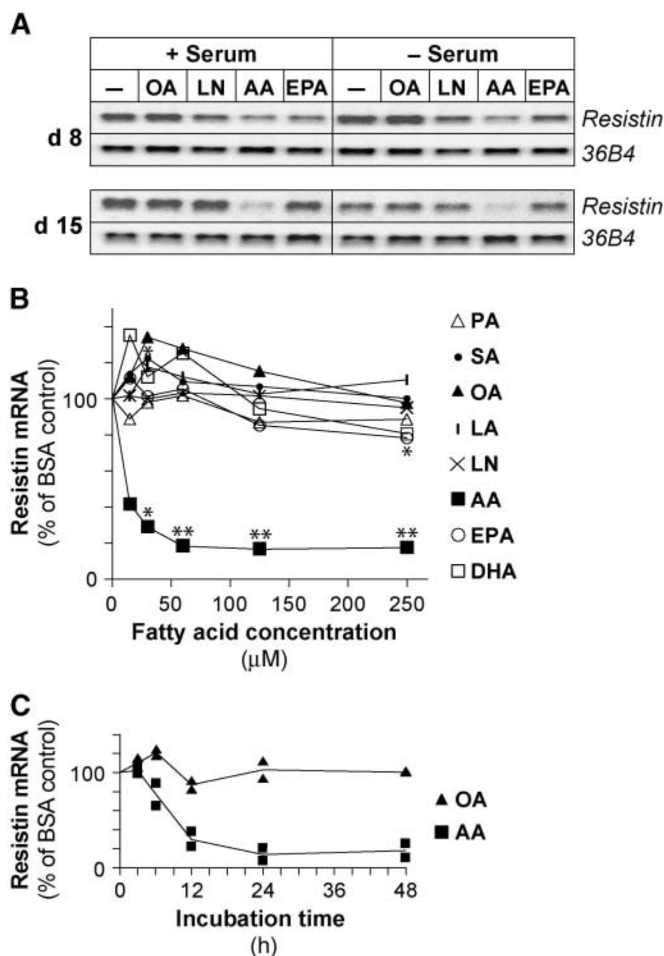


**Fig. 1.** Reduction of resistin mRNA by arachidonic acid (AA). Resistin mRNA was monitored in 3T3-L1 adipocytes by Northern blotting using 36B4 as an internal control. A: After 8 days (d 8) or 15 days (d 15) of differentiation, the adipocytes were incubated for 48 h with fatty acid-free BSA vehicle (−) or 250 μM BSA-bound oleic acid (OA), α-linolenic acid (LN), AA, or eicosapentaenoic acid (EPA) in medium with (+ Serum) and without (− Serum) 10% FBS. Further FFA incubations of adipocytes were performed with 15 day differentiated adipocytes incubated without FBS. B: Resistin mRNA levels in adipocytes incubated with BSA-bound palmitic acid (PA), stearic acid (SA), OA, linoleic acid (LA), LN, AA, EPA, or docosahexaenoic acid (DHA) at 0, 15, 30, 60, 125, and 250 μM for 48 h. Relative resistin mRNA levels were calculated as the ratio of resistin and 36B4 signal intensities related as percentage to the respective negative control stimulated with BSA only. Values shown are mean percentages of three to four experiments. * $P < 0.05$, ** $P < 0.01$. C: Relative resistin mRNA levels in adipocytes incubated for 0, 3, 6, 12, 24, or 48 h with 250 μM OA or AA. The plots represent means of two determinations.

promoted a significant increase in resistin expression to 122 ± 8% of the control (Fig. 1B). There was no significant effect on resistin expression of PA, OA, LA, LN, and DHA in the range 15–250 μM (Fig. 1B). To determine the time course of the AA effect on resistin expression, we harvested adipocytes at different time points after incubation with 250 μM OA or AA. There was a reduction of resistin mRNA to ~30% of the control after 12 h of incubation with AA and to ~20% after 24 and 48 h, whereas no effect was observed with OA (Fig. 1C).

PGs of the 2-series are synthesized from AA, and we tested if PG synthesis was involved in regulating resistin expression. Unspecific COX inhibitors (indomethacin, flurbiprofen, and meloxicam) neutralized the AA-induced reduction in resistin mRNA (**Fig. 2A**). Experiments with specific inhibitors of COX-1 (SC-506) and COX-2 (NS-398) revealed that SC-506, but not NS-398, could inhibit the AA effect on resistin mRNA (Fig. 2B). Incubations with AA alone reduced resistin mRNA levels to 6 ± 1% of control, whereas this was reversed to 71 ± 17% of control by SC-506 (Fig. 2B), implying a mechanism involving COX-1. After incubation with AA and NS-398, resistin mRNA was 30 ± 16% of the control, but the AA effect with this inhibitor was not significantly different from the AA effect without the inhibitor added (Fig. 2B). As a control, OA was included in the experiments and had no effect on resistin expression (Fig. 2B). We tested if the effect of AA on resistin expression was mimicked by direct exposure of adipocytes to different concentrations of PGs. At concentrations of 1 and 10 μM, $PGE_2$ downregulated resistin mRNA levels to 53 ± 8% and 19 ± 6% of control, respectively (Fig. 2C), and no effect was seen using 0.001, 0.01, or 0.1 μM. In addition, 0.1, 1, and 10 μM $PGF_{2\alpha}$ reduced resistin mRNA levels to 37 ± 9%, 24 ± 4%, and 19 ± 3% of control, respectively (Fig. 2C). The COX-1 selective inhibitor SC-506 did not affect the inhibitory effect of $PGE_2$ and $PGF_{2\alpha}$ on resistin expression (Fig. 2D). The suggested PPARγ agonist 15deoxy-δ12,14-$PGJ_2$ (10 μM) did not have any significant effect on resistin expression (data not shown). Physiological effects of PGs are mediated via interactions with G-protein-coupled receptors at the cell surface (41). By the use of known prostanoid receptor agonists, we examined the involvement of EP and FP receptors in the regulation of resistin mRNA. We incubated the differentiated 3T3-L1 cells with agonists of the different prostanoid receptors in the range $10^{-14}$ to $10^{-4}$ M and measured resistin mRNA levels by Northern blotting (n = 3–4). The EP1/EP3 agonist sulprostone and the EP2/EP4 agonist misoprostol had an $EC_{50} > 1$ μM, whereas the EP2 agonist butaprost did not decrease resistin mRNA levels dose-dependently (data not shown). The FP agonist fluprostenol $EC_{50}$ was in the range of 0.1–10 nM (data not shown). By RT-PCR analysis (see Materials and Methods), we could detect mRNA encoding EP1, EP4, and FP in 3T3-L1 adipocytes, but not EP2 and EP3 (Fig. 2E). These results indicate that reduced resistin mRNA expression by AA depends on its conversion to PGs and possibly on the subsequent autocrine activation of EP1, EP4, and FP.

We examined if the AA-induced reduction of resistin mRNA expression depends on activation of the mitogen-activated protein kinase (MAPK) and PI3K pathways, both of which are important for signal transduction (e.g., leading to transcription factor activation). The inhibitory effect of AA on resistin expression was reversed by an inhibitor of MEK (PD98059) in the MAPK pathway; resistin mRNA levels were reduced to 84 ± 7% of control by AA together with PD98059, as opposed to 6 ± 1% with AA alone (**Fig. 3A**). Incubation with a PI3K inhibitor



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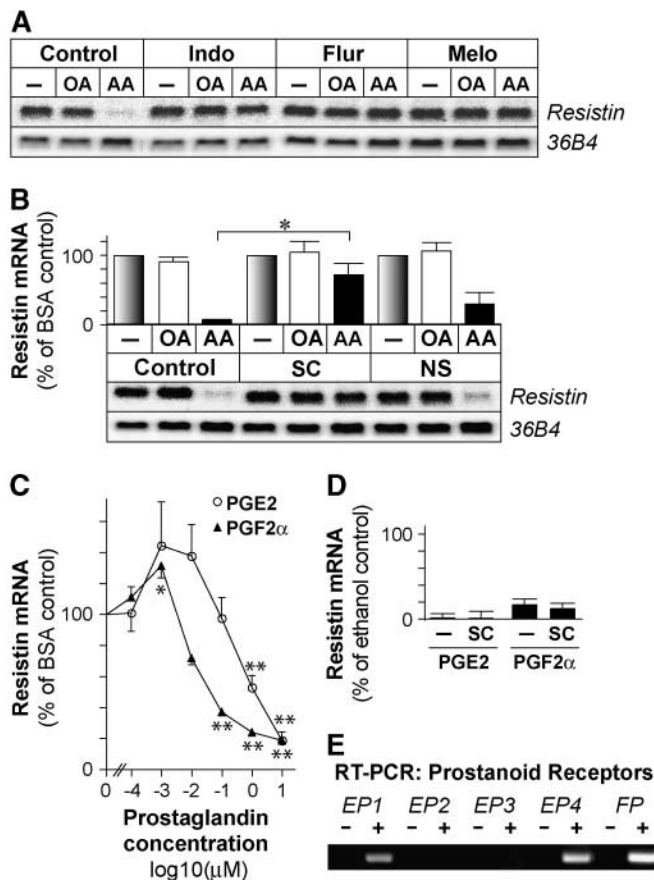


**Fig. 2.** AA-induced reduction in resistin mRNA depends on cyclooxygenase-1 (COX-1) activity and is mimicked by prostaglandins (PGs). Northern blots show resistin and reference 36B4 mRNA in adipocytes incubated with BSA vehicle (−) or 250 μM OA or AA for 24 h. A: Adipocytes were preincubated at 1 h and throughout the fatty acid stimulation with vehicle (Control) or with inhibitors of COX-1 and COX-2: 10 μM indomethacin (Indo), 100 μM flurbiprofen (Flur), and 10 μM meloxicam (Melo). Results shown are representative of two experiments. B: Similarly, cells were treated with vehicle (Control) or isoform-specific inhibitors: 0.3 μM SC-506 (SC; COX-1 inhibitor) and 30 μM NS-398 (NS; COX-2 inhibitor). Columns represent means ± SEM of the relative resistin levels from three to five experiments with the BSA incubations set to 100% (* $P < 0.05$). C: Relative resistin mRNA levels in adipocytes incubated for 24 h with ethanol vehicle alone or different concentrations of $PGE_2$ and $PGF_{2\alpha}$ ($10^{-4}$, $10^{-3}$, $10^{-2}$, $10^{-1}$, 1, or 10 μM). The plots represent means ± SEM of the relative resistin levels from five experiments with the ethanol incubations set to 100% (* $P < 0.05$, ** $P < 0.01$). D: Relative levels of resistin mRNA determined by Northern blotting in serum-starved adipocytes incubated in media with 0.1% fatty acid-free BSA supplements. Cells were first treated for 1 h with DMSO vehicle (−) or 0.3 μM SC-506 (SC; COX-1 inhibitor). Incubations with inhibitor continued for another 24 h after the addition of 10 μM $PGE_2$, 10 μM $PGF_{2\alpha}$ or ethanol vehicle only (set to 100%). Values shown are means ± SEM of three experiments. E: RT-PCR analysis of the E-prostanoid receptors EP1, EP2, EP3, and EP4 and F-prostanoid (FP) mRNA expression in 3T3-L1 adipocytes 15 days after induction of differentiation. One-step RT-PCR was performed using water only (−) or 1 μg of total RNA (+) as a template.

(LY294002) partly inhibited the AA effect (33 ± 4% of control; Fig. 3A). At concentrations >50 μM, LY294002 alone had an inhibitory effect on resistin expression (data not shown), and side effects of the inhibitor in combina-

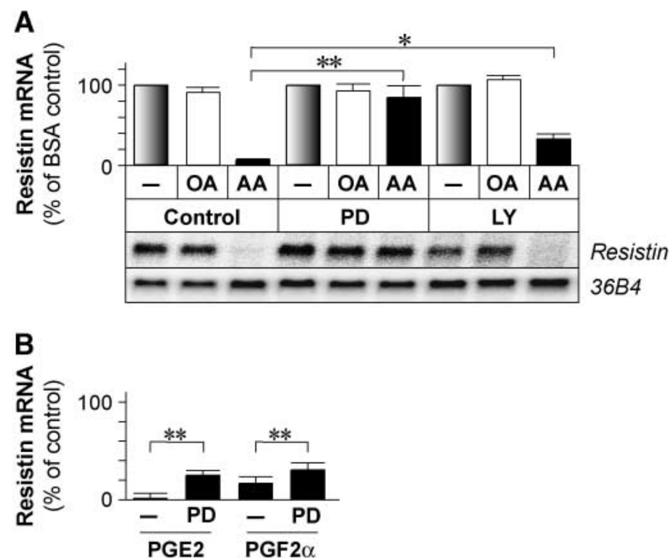


**Fig. 3.** AA- and PG-induced reduction in resistin mRNA involves mitogen-activated protein kinase kinase (MEK) activity. A: Northern blots of resistin and reference 36B4 mRNA in adipocytes incubated with BSA vehicle (−) or 250 μM OA or AA for 24 h in the presence of DMSO vehicle (Control), 50 μM PD98059 (PD; MEK inhibitor), or 10 μM LY294002 (LY; phosphatidylinositol 3-kinase inhibitor). Columns represent means ± SEM of relative resistin mRNA levels from three to four experiments with the BSA controls set to 100% (* $P < 0.05$, ** $P < 0.01$). B: Relative resistin mRNA levels were determined by Northern blot analysis in adipocytes incubated in media with 0.1% FFA-free BSA. Cells were treated for 1 h in advance with DMSO vehicle (−) or 50 μM PD98059 (PD; MEK inhibitor) and for another 24 h after the addition of 10 μM $PGE_2$, 10 μM $PGF_{2\alpha}$ or ethanol vehicle only (set to 100%). Values shown are means ± SEM of three experiments (** $P < 0.01$).

tion with fatty acids cannot be excluded. Control incubations with OA in combination with the inhibitors did not affect resistin expression (Fig. 3A). Furthermore, the inhibitory effects of $PGE_2$ and $PGF_{2\alpha}$ on resistin expression were partially antagonized by the MEK inhibitor PD98059 (Fig. 3B). This finding indicates that activation via the MAPK pathway is involved in AA-induced reduction of resistin mRNA expression, whereas signaling through the PI3K pathway seems less important.

As potential mediators of AA-regulated resistin expression, we investigated if the insulin signaling pathway and activity of the nuclear receptors PPARγ and LXRα affect resistin expression. In the absence of serum, adipocytes differentiated for 15 days responded to insulin with a dose-dependent reduction in resistin mRNA (**Fig. 4A**, upper panels). PD98059 reduced the effect of insulin on resistin expression (Fig. 4A, lower panels). Contrary to our earlier experiments performed in the presence of serum (18), serum-starved cells did not respond to darglitazone with decreased resistin expression even at high doses (Fig. 4A, upper panels). In contrast, the LXR agonist T0901317 dose-dependently reduced the expression of resistin mRNA in the absence of serum (Fig. 4A, upper panels). Adding the MEK inhibitor PD98059 did not affect the LXR-induced reduction of resistin mRNA expression (Fig.



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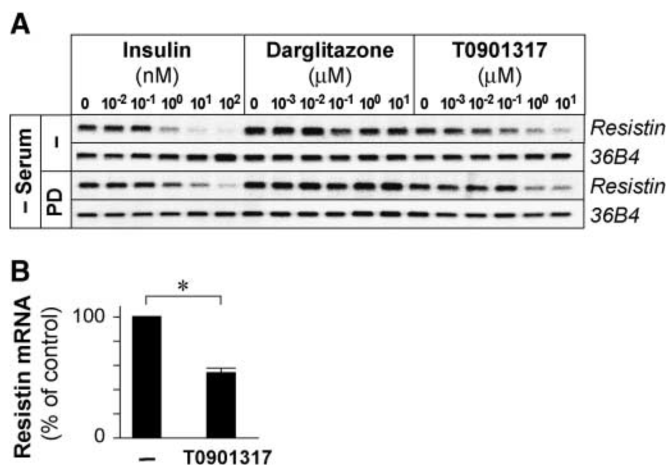


**Fig. 4.** The liver X receptor α (LXRα) agonist T0901317 down-regulated resistin mRNA levels. A: Northern blots of resistin and reference 36B4 mRNA from serum-starved adipocytes incubated for 24 h with insulin (0, 0.01, 0.1, 1, 10, and 100 nM), the peroxisome proliferator-activated receptor γ (PPARγ) agonist darglitazone, or the LXRα agonist T0901317 (both 0, 0.001 0.01, 0.1, 1, and 10 μM) in the absence (−) or presence (PD) of 50 μM PD98059 dissolved in vehicle. B: Adipocytes were incubated for 24 h with vehicle (−) or 1 μM T0901317. Relative resistin mRNA levels as percentages of the DMSO controls of three experiments are presented as mean percentages ± SEM (* $P < 0.05$).

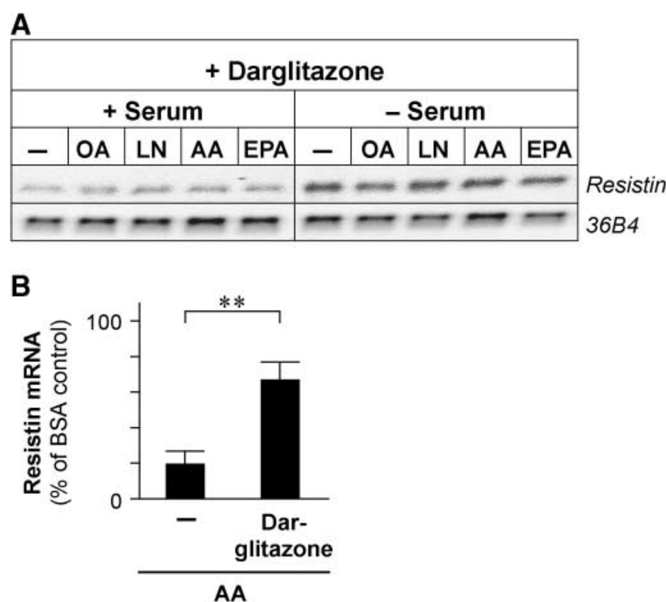


**Fig. 5.** The PPARγ agonist darglitazone interfered with the inhibitory effect of AA on resistin mRNA levels. A: Northern blots from adipocytes were incubated for 48 h with BSA vehicle (−) or 250 μM OA, LN, AA, or EPA in medium with (+ Serum) and without (− Serum) 10% FBS in the presence of 0.1 μM darglitazone. B: Relative resistin mRNA levels in serum-starved adipocytes after 24–48 h of incubation with BSA or 250 μM AA together with vehicle (−) or 0.1 μM darglitazone. Columns represent percentages of the respective BSA controls given as means ± SEM of four experiments (** $P < 0.01$).

4A, lower panels), suggesting that the LXR activator executes its effect independently of the MAPK pathway. In separate experiments, we observed that the LXR agonist T0901317 reduced the resistin mRNA levels to 54 ± 4% of the DMSO-treated control (Fig. 4B). This shows for the first time that LXRα may regulate resistin expression.

To further clarify the role of PPARγ in resistin expression, we tested the combination of AA and darglitazone. The presence of the PPARγ agonist darglitazone interfered with the inhibitory effect of AA on resistin expression (**Fig. 5A**). Without darglitazone, AA reduced resistin mRNA to 19 ± 8% of the fatty acid-free control, but when darglitazone was included in the media this effect was significantly changed to 67 ± 10% (Fig. 5B). The PPARγ antagonist BADGE (42) did not affect the AA-induced reduction in resistin mRNA (data not shown).

To test if AA-inhibited resistin expression is dependent on the direct interactions of transcription factors with the resistin promoter, we constructed a firefly LUC reporter containing the −2610 to +720 bp and the −393 to +720 bp fragments of the mouse resistin promoter (**Fig. 6A**). C/EBPα has been shown to be a positive regulator of the resistin promoter (43, 44), and we tested if the promoter region included in our constructs was able to convey activation by a C/EBPα expression vector. Transiently overexpressing C/EBPα in COS-1 cells induced a 60-fold increase in relative LUC activity conferred by the −2610 to +720 bp resistin promoter (Fig. 6B). With this reporter in COS-1 cells, PPARγ2 overexpression alone did not affect LUC activity, whereas together with darglitazone it induced a 2-fold increase (Fig. 6C). In the same assay, cotransfection with the LXRα expression plasmid alone or with T0901317 increased LUC activity 2-fold and 3-fold, respectively (Fig. 6C). We tested the capacity of several adipocyte-enriched transcription factors to activate the resistin promoter in undifferentiated 3T3-L1 fibroblasts. Although to a much lesser extent than in COS-1, C/EBPα overexpression also activated the −2610 to +720 bp resistin reporter construct in 3T3-L1 fibroblasts, resulting in a 3-fold increase in relative LUC activity (Fig. 6D). When we overexpressed mature SREBP-1a in 3T3-L1 fibroblasts, we observed an >12-fold increase in relative LUC activity with the −2610 to +720 bp resistin promoter construct (Fig. 6D). Transfection with RXRα induced a small (3-fold) increase in relative LUC activity, and the effect of PPARγ2 or LXRα in combination with RXRα was only slightly inhibitory (Fig. 6D). Incubation of fibroblasts with AA or $PGE_2$ did not significantly affect the ability of C/EBPα or SREBP-1a to induce the resistin reporter (Fig. 6D). However, relative LUC activity induced by SREBP-1a was reduced by ∼50% after incubation of the transfected fibroblasts with $PGF_{2\alpha}$ (Fig. 6D). In undifferentiated 3T3-L1 fibroblasts, SREBP-1a activated the −2610 to +720 and the −393 to +720 resistin constructs in a concentration-dependent manner, inducing up to a 12-fold increase in relative LUC activity for both constructs (Fig. 6E).

We also examined other potential transcriptional and posttranscriptional processes in the AA-induced reductions in resistin mRNA. Incubation of adipocytes with AA did not change mRNA levels of LXRα, SREBP-1, and



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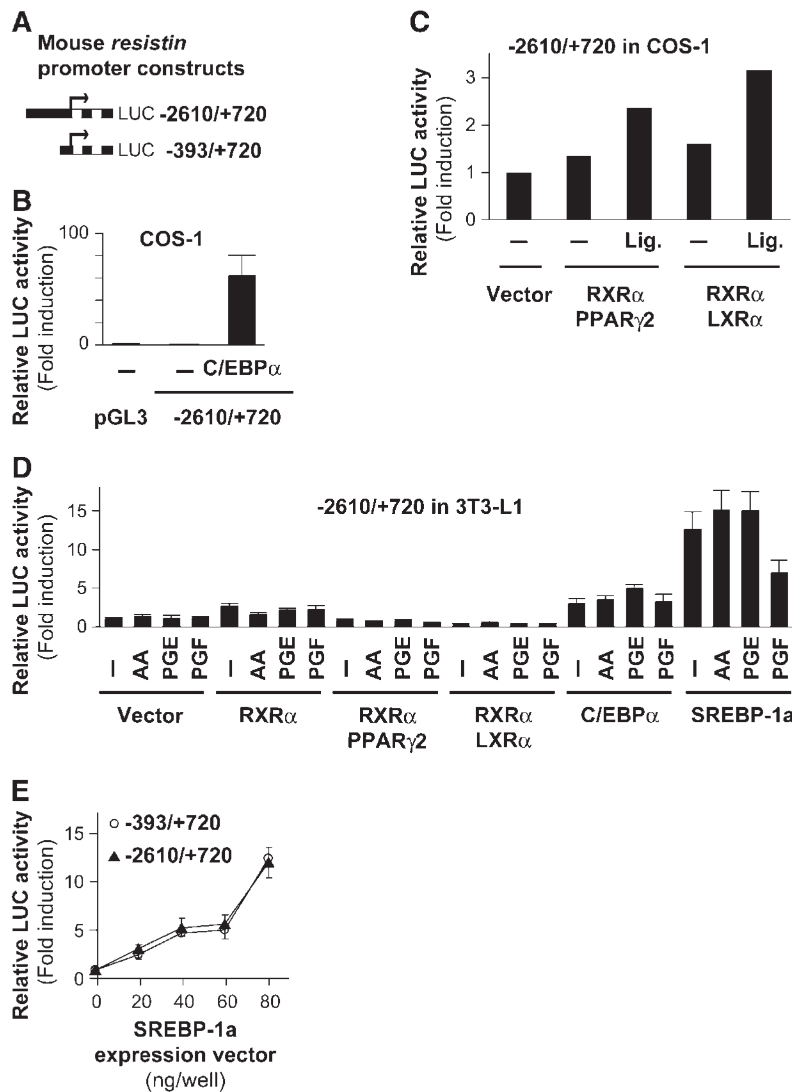


**Fig. 6.** Resistin promoter activity is enhanced with overexpression of CCAAT/enhancer binding protein-α (C/EBPα) and sterol-regulatory element binding protein-1a (SREBP-1a). A: The −2610 to +720 bp and the −393 to +720 bp fragments of the mouse resistin promoter were cloned into firefly luciferase (LUC) reporter plasmid. B: The reporter vector alone (pGL3) or the −2610/+720 resistin-firefly LUC construct was cotransfected with the C/EBPα expression vector or the empty vector (−) together with the β-galactosidase expression vector (as an internal control) into COS-1 cells. Relative LUC activity was calculated as the ratio of firefly LUC activity to β-galactosidase activity, and fold induction is relative to the −2610/+720 resistin-firefly LUC construct in the absence of C/EBPα expression vector. Values shown are means ± SEM of three experiments. C: The −2610/+720 resistin-firefly LUC construct was cotransfected into COS-1 cells with the PPARγ or LXRα expression vector. After transfection, the activity of PPARγ and LXRα was amplified by incubating cells with vehicle (−) or 1 μM of the appropriate ligand (Lig.), darglitazone or T0901317. Relative LUC activity was calculated as the ratio of firefly LUC activity to β-galactosidase activity, and fold induction is relative to the −2610/+720 resistin-firefly LUC construct cotransfected with empty expression vector. Bars represent means of two experiments performed in triplicate. D: The −2610/+720 resistin-firefly LUC construct was cotransfected with empty expression vector (Vector) or combinations of transcription factor expression vectors [retinoic acid X receptor (RXRα), PPARγ2, LXRα, C/EBPα, and SREBP-1a] into 3T3-L1 preadipocytes. Renilla LUC expression vector was included in the transfections as an internal control. Cells were incubated with vehicle (−), 100 μM AA, 10 μM $PGE_2$ (PGE), or 10 μM $PGF_{2\alpha}$ (PGF). Relative LUC activity was calculated as the ratio of firefly LUC activity to Renilla LUC activity, and fold induction is relative to BSA incubation in the absence of transcription factor expression vectors. Values shown are means ± SEM from a representative experiment of two experiments performed in triplicate. E: The −2610/+720 and −393/+720 resistin-firefly LUC constructs were cotransfected with different amounts (ng/well) of SREBP-1a expression vector in 3T3-L1 preadipocytes as above. Values shown are means ± SEM from a representative experiment of two experiments performed in triplicate.

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PPARγ (**Fig. 7A**), suggesting that transcriptional regulation of these transcription factor genes is of minor importance for resistin expression. SREBP-1 activity is also regulated at the posttranslational level, and cleavage of SREBP-1 is required to release its mature and active form (45). However, in adipocytes, the level of the mature SREBP-1 protein was unchanged after incubation with AA, EPA, or 25-hydroxycholesterol (Fig. 7B). This does not show regulated SREBP-1 maturation in 3T3-L1 adipocytes, and it does not support SREBP-1 maturation as an important factor in AA-regulated resistin expression. The effect of AA on resistin mRNA levels in adipocytes was abolished when the cells were incubated with cycloheximide or actinomycin D for 24 h (Fig. 7C). Surprisingly, there was no reduction in the amount of resistin mRNA in the controls after incubation with actinomycin D for 24 h (Fig. 7C), whereas extending the incubation to 48 h resulted in a marked reduction of resistin mRNA in the controls (Fig. 7D). The suppressive effect on resistin mRNA levels of both AA and T0901317 was more rapid than the effect of actinomycin D alone (Fig. 7D).

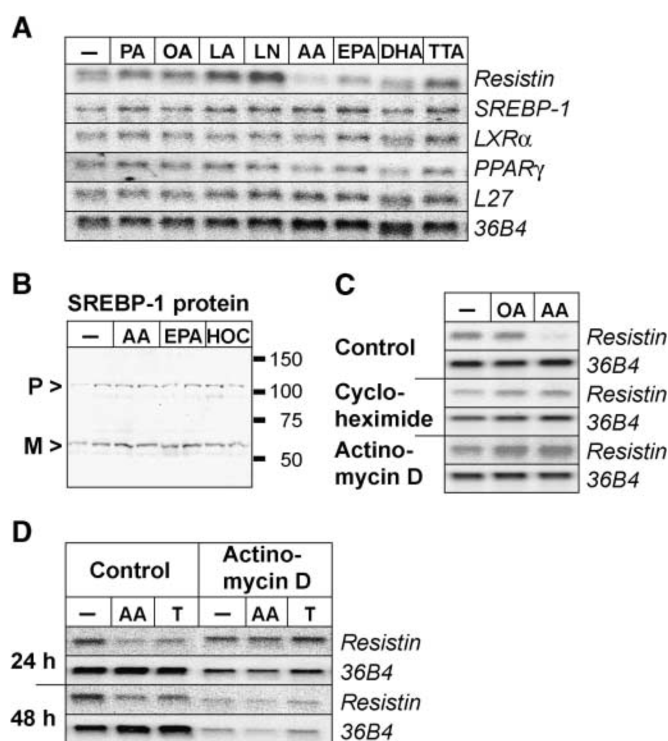


**Fig. 7.** Reduced levels of resistin mRNA are not associated with reduced expression of adipogenic transcription factors but require de novo protein and mRNA synthesis. A: Northern blots showing mRNA encoding resistin, SREBP-1, LXRα, and PPARγ after 48 h of incubation of adipocytes with vehicle (−) or PA, OA, LA, LN, AA, EPA, DHA, or tetradecylthioacetic acid (TTA). Bands of internal controls (L27 and 36B4) were included to demonstrate similar loading of wells. B: Immunoblot analysis showing the precursor (P) and mature (M) forms of SREBP-1 extracted from adipocytes incubated for 24 h with vehicle (−) or 250 μM AA, 250 μM EPA, or 5 μg/ml 25-hydroxycholesterol (HOC). The presented blot is representative of three experiments. C: Northern blot analysis of resistin mRNA in adipocytes incubated for 24 h with BSA vehicle (−), 250 μM OA or AA in the absence (Control) or presence of 10 μM cycloheximide (inhibitor of protein synthesis) or 1 μg/ml actinomycin D (inhibitor of RNA synthesis). Data are representative of two experiments. D: Northern blot showing adipocytes incubated with vehicle (−), 250 μM AA, or 1 μM T0901317 (T) in the absence (Control) or presence of 1 μg/ml actinomycin D for 24 or 48 h.

## DISCUSSION

In this study, we have searched for a link between the availability of fatty acids and resistin gene expression, with possible implications for the development of insulin resistance. We incubated 3T3-L1 adipocytes with different FFAs, and only SA induced a marginal increase in resistin mRNA. We observed that AA at ⩾30 μM promoted a marked reduction in resistin mRNA in 3T3-L1 adipocytes independent of serum constituents and the degree of intracellular lipid loading. The strong effect indicates that AA might be a physiological regulator of resistin expression in adipocytes. A much smaller reduction in resistin mRNA was also induced by EPA at 250 μM. In contrast to our data, an earlier study of cultured primary rat adipocytes indicated the inhibition of resistin mRNA expression by OA (46). Our present study reveals a strong inhibition of resistin mRNA expression specifically by AA and does not indicate a regulation by FFAs in general.

Our experiments with isoform-specific COX inhibitors indicate that COX-1 and not COX-2 activity is required for the inhibitory effect of AA on resistin expression. This is in accordance with findings demonstrating that AA inhibits fatty acid synthase and S14 gene expression in 3T3-L1 adipocytes via COX (47). Also, COX-1 is the most abundant isoform in differentiated 3T3-L1 cells, with COX-2 being expressed at a much lower level (48). $PGE_2$ and $PGF_{2\alpha}$ are secreted from 3T3-L1 adipocytes (49, 50), and we observed that high doses (10 μM) of both of these PGs reduced resistin expression to the same extent as AA, in agreement with the fact that AA is the precursor of PGs. Earlier studies have reported detectable expression of the FP receptor gene (ptgfr) by RT-PCR in 3T3-L1 adipocytes (51). Quite recently, mRNA expression of the four EP receptor genes ptger1, ptger2, ptger3, and ptger4, expressing the EP1, EP2, EP3, and EP4 receptors, respectively, was investigated in 3T3-L1 adipocytes (52). In agreement with those findings, we were able to detect mRNAs encoding FP, EP1, and EP4 but no expression of EP2 and EP3 by RT-PCR analysis of 3T3-L1 adipocytes. Earlier studies show that the $K_d$ values of $PGE_2$ and $PGF_{2\alpha}$ to their respective murine prostanoid receptors are in the 1–20 nM range (53). The dose-response curve of PG-regulated resistin expression we present here shows an effect of $PGF_{2\alpha}$ in the physiological range (>10 nM), whereas the effect of $PGE_2$ depends on its superphysiological concentrations (>100 nM). Furthermore, the FP receptor agonist fluprostenol effectively downregulated resistin mRNA at nanomolar concentrations ($EC_{50} \sim 0.1$–10 nM). High doses of the EP1/EP3 agonist sulprostone and the EP2/EP4 agonist misoprostol ($EC_{50} > 1$ μM) were required, whereas the EP2 agonist butaprost had no effect on resistin mRNA lev-



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els. The high doses of $PGE_2$, sulprostone, and misoprostol required to obtain an effect suggest that EP receptors are of small importance for the regulation of resistin mRNA levels, and the effect may be indirect. However, the potent effects of $PGF_{2\alpha}$ and flurprostenol suggest that FP receptor is a major mediator of PG-regulated resistin gene expression. The alleged biological PPARγ ligand 15deoxy-δ12,14-$PGJ_2$ (54) did not have any effect and therefore seems not to play a role in regulating resistin expression. Furthermore, pharmacological COX-1 inhibitors did not block the effect of PGs on resistin expression, supporting the notion that enzymatic transformation of AA into PGs is required to downregulate resistin mRNA.

Of the prostanoid receptors present in 3T3-L1 adipocytes, EP1 acts via the phospholipase C/inositol trisphosphate pathway, EP4 receptors activate the cAMP/protein kinase A pathway, and FP receptors signal through the heterotrimeric G-protein Gq (55). We also found that a specific MEK inhibitor (PD98059) nearly abolished the AA-induced reduction of resistin expression. This inhibitor also partially blocked the downregulation of resistin mRNA when PG was included in the incubation media. This indicates that MEK activity is required downstream of COX-1 activity for the full AA-mediated downregulation of resistin mRNA. This suggests a mechanism involving the secretion of PGs and subsequent activation of G-protein-coupled receptors at the cell surface and further signaling via the MAPK pathway. Moreover, an inhibitor of PI3K (LY294002) did not block the AA-induced reduction of resistin mRNA. This is in contrast to observations by Song et al. (44) demonstrating that overexpression of PI3K suppressed resistin expression.

The transcription factors PPARγ, LXRα, and SREBP-1 are induced during adipogenesis, and their transcriptional activities may be regulated by PUFAs. Previously, we have shown that the PPARγ agonist darglitazone reduced resistin mRNA in the presence of serum (18). In the present study, we observed that the downregulatory effect of darglitazone was dependent on serum in the culture medium. Under these conditions, we also observed that darglitazone neutralized the inhibiting effect of AA, suggesting an interaction between the two. We first suspected that AA could execute its effect on resistin via an association with the PPARγ ligand binding pocket and that darglitazone under these conditions functioned as a PPARγ antagonist. Incubation with BADGE (42), a ligand for PPARγ that can antagonize thiazolidinedione stimulation of PPARγ transcriptional activity, did not affect resistin mRNA levels. However, in contrast to darglitazone, BADGE did not counteract the reduction of resistin mRNA induced by AA. Our data do not support the idea that the interference between darglitazone and AA is the result of competition for the PPARγ ligand binding pocket. PPARγ ligands exert PPARγ-independent effects in part through activation of the MAPK pathway (56). On the other hand, MAPK-mediated phosphorylation may reduce PPARγ transcriptional activity (57), and it is possible that the AA-induced reduction in resistin mRNA is modulated by darglitazone via MAPK signaling affecting PPARγ transcriptional activity.

In the present study, we also found that the LXRα agonist T0901317 is a negative regulator of resistin mRNA levels in 3T3-L1 adipocytes, which is in accordance with LXRα agonists promoting increased expression of mRNA for the glucose transporter GLUT4 and increasing insulin sensitivity (31). This made us speculate that the downregulation of resistin expression by AA was via the LXRα nuclear receptor. With transient cell transfections, we attempted to narrow the list of candidate transcription factors involved in the AA-induced inhibition of resistin expression. A firefly LUC reporter gene controlled by the resistin proximal promoter was used to test the promoter's responsiveness to various adipocyte-enriched transcription factors. We found that transfection of a C/EBPα expression vector in COS-1 cells caused more than 60-fold induction of the resistin promoter activity. It has been shown that C/EBPα can transactivate the resistin promoter (43, 44). In undifferentiated 3T3-L1 fibroblasts, the effect of C/EBPα was less prominent, possibly because of mechanisms repressing differentiation at the preadipocyte stage. We further tested if the reduction in resistin mRNA levels by PPARγ and LXRα ligands could be paralleled in transfection studies. Both PPARγ and LXRα overexpression reduced RXRα-induced resistin promoter activity, but these effects were so small that their biological significance is uncertain. In addition, we did not see any effect of AA treatment on mRNA levels of PPARγ and LXRα. In accordance with similar experiments with the human resistin promoter (58), we found that SREBP-1a is a dose-responsive positive regulator of resistin promoter activity in 3T3-L1 fibroblasts. This is particularly interesting because the SREBP-1 transcription factors are involved in PUFA-inhibited gene expression in the liver (59). However, the mRNA levels of SREBP-1 (transcripts of both the -a and -c isoforms) were unaffected by incubation with AA, suggesting that AA does not reduce resistin mRNA levels by inhibiting SREBP-1 transcription. Because SREBP-1 activity requires proteolysis of the membrane-bound precursor and its subsequent translocation to the nucleus (45), we investigated if AA affected the amount of mature SREBP-1 (both the -a and -c isoforms), but our experiments did not support the involvement of a regulated SREBP-1 maturation.

Applying inhibitors of transcription (actinomycin D) and translation (cycloheximide) indicated that both de novo transcription and translation are necessary for the AA-induced reduction of resistin mRNA. Furthermore, inhibiting transcription with actinomycin D per se had no apparent effect on resistin mRNA levels after 24 h, and a reduction was only seen after 48 h. This indicates that the resistin transcript is stable, with a half-life of >24 h. It should also be noted that the reduction in resistin mRNA observed after incubation with AA occurred faster than by blocking transcription with actinomycin D. This suggests that resistin mRNA is actively degraded through a process induced by AA and that AA is not likely to exert its effect by regulating resistin transcription via the gene's promoter. Our results also indicate that resistin mRNA is reduced by the LXR agonist (T0901317) in a similar man-



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ner as AA. A mechanism involving the degradation of mRNA is likely to reduce resistin mRNA levels in 3T3-L1 adipocytes not only after AA and LXR agonist incubation but also after exposure to insulin (60).

In conclusion, we observe that AA reduces resistin mRNA levels in 3T3-L1 adipocytes involving COX-1 and MEK activity, and possibly a destabilization of the resistin transcript.

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## REFERENCES

1. Trayhurn, P., and J. H. Beattie. 2001. Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc. Nutr. Soc.* **60:** 329–339.
2. Segal, K. R., M. Landt, and S. Klein. 1996. Relationship between insulin sensitivity and plasma leptin concentration in lean and obese men. *Diabetes.* **45:** 988–991.
3. Hotamisligil, G. S., N. S. Shargill, and B. M. Spiegelman. 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science.* **259:** 87–91.
4. Kern, P. A., S. Ranganathan, C. Li, L. Wood, and G. Ranganathan. 2001. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am. J. Physiol. Endocrinol. Metab.* **280:** E745–E751.
5. Weyer, C., T. Funahashi, S. Tanaka, K. Hotta, Y. Matsuzawa, R. E. Pratley, and P. A. Tataranni. 2001. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J. Clin. Endocrinol. Metab.* **86:** 1930–1935.
6. Steppan, C. M., S. T. Bailey, S. Bhat, E. J. Brown, R. R. Banerjee, C. M. Wright, H. R. Patel, R. S. Ahima, and M. A. Lazar. 2001. The hormone resistin links obesity to diabetes. *Nature.* **409:** 307–312.
7. Holcomb, I. N., R. C. Kabakoff, B. Chan, T. W. Baker, A. Gurney, W. Henzel, C. Nelson, H. B. Lowman, B. D. Wright, N. J. Skelton, G. D. Frantz, D. B. Tumas, F. V. Peale, Jr., D. L. Shelton, and C. C. Hebert. 2000. FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. *EMBO J.* **19:** 4046–4055.
8. Steppan, C. M., E. J. Brown, C. M. Wright, S. Bhat, R. R. Banerjee, C. Y. Dai, G. H. Enders, D. G. Silberg, X. Wen, G. D. Wu, and M. A. Lazar. 2001. A family of tissue-specific resistin-like molecules. *Proc. Natl. Acad. Sci. USA.* **98:** 502–506.
9. Gerstmayer, B., D. Kusters, S. Gebel, T. Muller, E. Van Miert, K. Hofmann, and A. Bosio. 2003. Identification of RELMgamma, a novel resistin-like molecule with a distinct expression pattern. *Genomics.* **81:** 588–595.
10. Nagaev, I., and U. Smith. 2001. Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle. *Biochem. Biophys. Res. Commun.* **285:** 561–564.
11. Savage, D. B., C. P. Sewter, E. S. Klenk, D. G. Segal, A. Vidal-Puig, R. V. Considine, and S. O'Rahilly. 2001. Resistin/Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. *Diabetes.* **50:** 2199–2202.
12. Schinke, T., M. Haberland, A. Jamshidi, P. Nollaub, J. M. Rueger, and M. Amling. 2004. Cloning and functional characterization of resistin-like molecule-gamma. *Biochem. Biophys. Res. Commun.* **314:** 356–362.
13. Banerjee, R. R., and M. A. Lazar. 2001. Dimerization of resistin and resistin-like molecules is determined by a single cysteine. *J. Biol. Chem.* **276:** 25970–25973.
14. Moon, B., J. J. Kwan, N. Duddy, G. Sweeney, and N. Begum. 2003. Resistin inhibits glucose uptake in L6 cells independently of changes in insulin signaling and GLUT4 translocation. *Am. J. Physiol. Endocrinol. Metab.* **285:** E106–E115.
15. Pravenec, M., L. Kazdova, V. Landa, V. Zidek, P. Mlejnek, P. Jansa, J. Wang, N. Qi, and T. W. Kurtz. 2003. Transgenic and recombinant resistin impair skeletal muscle glucose metabolism in the spontaneously hypertensive rat. *J. Biol. Chem.* **278:** 45209–45215.
16. Rajala, M. W., S. Obici, P. E. Scherer, and L. Rossetti. 2003. Adipose-derived resistin and gut-derived resistin-like molecule-beta selectively impair insulin action on glucose production. *J. Clin. Invest.* **111:** 225–230.
17. Banerjee, R. R., S. M. Rangwala, J. S. Shapiro, A. S. Rich, B. Rhoades, Y. Qi, J. Wang, M. W. Rajala, A. Pocai, P. E. Scherer, C. M. Steppan, R. S. Ahima, S. Obici, L. Rossetti, and M. A. Lazar. 2004. Regulation of fasted blood glucose by resistin. *Science.* **303:** 1195–1198.
18. Haugen, F., A. Jorgensen, C. A. Drevon, and P. Trayhurn. 2001. Inhibition by insulin of resistin gene expression in 3T3-L1 adipocytes. *FEBS Lett.* **507:** 105–108.
19. Lehmann, J. M., L. B. Moore, T. A. Smith-Oliver, W. O. Wilkison, T. M. Willson, and S. A. Kliewer. 1995. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J. Biol. Chem.* **270:** 12953–12956.
20. Moore, G. B., H. Chapman, J. C. Holder, C. A. Lister, V. Piercy, S. A. Smith, and J. C. Clapham. 2001. Differential regulation of adipocytokine mRNAs by rosiglitazone in db/db mice. *Biochem. Biophys. Res. Commun.* **286:** 735–741.
21. Way, J. M., C. Z. Gorgun, Q. Tong, K. T. Uysal, K. K. Brown, W. W. Harrington, W. R. Oliver, Jr., T. M. Willson, S. A. Kliewer, and G. S. Hotamisligil. 2001. Adipose tissue resistin expression is severely suppressed in obesity and stimulated by peroxisome proliferator-activated receptor gamma agonists. *J. Biol. Chem.* **276:** 25651–25653.
22. Shojima, N., H. Sakoda, T. Ogihara, M. Fujishiro, H. Katagiri, M. Anai, Y. Onishi, H. Ono, K. Inukai, M. Abe, Y. Fukushima, M. Kikuchi, Y. Oka, and T. Asano. 2002. Humoral regulation of resistin expression in 3T3-L1 and mouse adipose cells. *Diabetes.* **51:** 1737–1744.
23. Fasshauer, M., J. Klein, S. Neumann, M. Eszlinger, and R. Paschke. 2001. Isoproterenol inhibits resistin gene expression through a G(S)-protein-coupled pathway in 3T3-L1 adipocytes. *FEBS Lett.* **500:** 60–63.
24. Lam, T. K., A. Carpentier, G. F. Lewis, G. van de Werve, I. G. Fantus, and A. Giacca. 2003. Mechanisms of the free fatty acid-induced increase in hepatic glucose production. *Am. J. Physiol. Endocrinol. Metab.* **284:** E863–E873.
25. Drevon, C. A. 1993. Sources, chemistry and biochemistry of dietary lipids. *In* Omega-3 Fatty Acids: Metabolism and Biological Effects. I. Baksaas, H. E. Krokan, and C. A. Drevon, eds. Birkhäuser, Basel, Switzerland. 1–10.
26. Jump, D. B. 2002. Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr. Opin. Lipidol.* **13:** 155–164.
27. Kliewer, S. A., S. S. Sundseth, S. A. Jones, P. J. Brown, G. B. Wisely, C. S. Koble, P. Devchand, W. Wahli, T. M. Willson, J. M. Lenhard, and J. M. Lehmann. 1997. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc. Natl. Acad. Sci. USA.* **94:** 4318–4323.
28. Worgall, T. S., S. L. Sturley, T. Seo, T. F. Osborne, and R. J. Deckelbaum. 1998. Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J. Biol. Chem.* **273:** 25537–25540.
29. Ou, J., H. Tu, B. Shan, A. Luk, R. A. DeBose-Boyd, Y. Bashmakov, J. L. Goldstein, and M. S. Brown. 2001. Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. *Proc. Natl. Acad. Sci. USA.* **98:** 6027–6032.

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30. Yoshikawa, T., H. Shimano, M. Amemiya-Kudo, N. Yahagi, A. H. Hasty, T. Matsuzaka, H. Okazaki, Y. Tamura, Y. Iizuka, K. Ohashi, J. Osuga, K. Harada, T. Gotoda, S. Kimura, S. Ishibashi, and N. Yamada. 2001. Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c gene promoter. *Mol. Cell. Biol.* **21:** 2991–3000.
31. Dalen, K. T., S. M. Ulven, K. Bamberg, J. A. Gustafsson, and H. I. Nebb. 2003. Expression of the insulin-responsive glucose transporter GLUT4 in adipocytes is dependent on liver X receptor alpha. *J. Biol. Chem.* **278:** 48283–48291.
32. Nossen, J. O., A. C. Rustan, S. H. Gloppestad, S. Malbakken, and C. A. Drevon. 1986. Eicosapentaenoic acid inhibits synthesis and secretion of triacylglycerols by cultured rat hepatocytes. *Biochim. Biophys. Acta.* **879:** 56–65.
33. Reseland, J. E., F. Haugen, K. Hollung, K. Solvoll, B. Halvorsen, I. R. Brude, M. S. Nenseter, E. N. Christiansen, and C. A. Drevon. 2001. Reduction of leptin gene expression by dietary polyunsaturated fatty acids. *J. Lipid Res.* **42:** 743–750.
34. Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **132:** 365–386.
35. Hwang, C. S., S. Mandrup, O. A. MacDougald, D. E. Geiman, and M. D. Lane. 1996. Transcriptional activation of the mouse obese (ob) gene by CCAAT/enhancer binding protein alpha. *Proc. Natl. Acad. Sci. USA.* **93:** 873–877.
36. Tobin, K. A., H. H. Steineger, S. Alberti, O. Spydevold, J. Auwerx, J. A. Gustafsson, and H. I. Nebb. 2000. Cross-talk between fatty acid and cholesterol metabolism mediated by liver X receptor-alpha. *Mol. Endocrinol.* **14:** 741–752.
37. Ross, S. E., R. L. Erickson, N. Hemati, and O. A. MacDougald. 1999. Glycogen synthase kinase 3 is an insulin-regulated C/EBPalpha kinase. *Mol. Cell. Biol.* **19:** 8433–8441.
38. Willy, P. J., K. Umesono, E. S. Ong, R. M. Evans, R. A. Heyman, and D. J. Mangelsdorf. 1995. LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev.* **9:** 1033–1045.
39. Fajas, L., D. Auboeuf, E. Raspe, K. Schoonjans, A. M. Lefebvre, R. Saladin, J. Najib, M. Laville, J. C. Fruchart, S. Deeb, A. Vidal-Puig, J. Flier, M. R. Briggs, B. Staels, H. Vidal, and J. Auwerx. 1997. The organization, promoter analysis, and expression of the human PPARgamma gene. *J. Biol. Chem.* **272:** 18779–18789.
40. Jackson, S. M., J. Ericsson, T. F. Osborne, and P. A. Edwards. 1995. NF-Y has a novel role in sterol-dependent transcription of two cholesterogenic genes. *J. Biol. Chem.* **270:** 21445–21448.
41. Bos, C. L., D. J. Richel, T. Ritsema, M. P. Peppelenbosch, and H. H. Versteeg. 2004. Prostanoids and prostanoid receptors in signal transduction. *Int. J. Biochem. Cell Biol.* **36:** 1187–1205.
42. Wright, H. M., C. B. Clish, T. Mikami, S. Hauser, K. Yanagi, R. Hiramatsu, C. N. Serhan, and B. M. Spiegelman. 2000. A synthetic antagonist for the peroxisome proliferator-activated receptor gamma inhibits adipocyte differentiation. *J. Biol. Chem.* **275:** 1873–1877.
43. Hartman, H. B., X. Hu, K. X. Tyler, C. K. Dalal, and M. A. Lazar. 2002. Mechanisms regulating adipocyte expression of resistin. *J. Biol. Chem.* **277:** 19754–19761.
44. Song, H., N. Shojima, H. Sakoda, T. Ogihara, M. Fujishiro, H. Katagiri, M. Anai, Y. Onishi, H. Ono, K. Inukai, Y. Fukushima, M. Kikuchi, H. Shimano, N. Yamada, Y. Oka, and T. Asano. 2002. Resistin is regulated by C/EBPs, PPARs, and signal-transducing molecules. *Biochem. Biophys. Res. Commun.* **299:** 291–298.
45. Brown, M. S., and J. L. Goldstein. 1999. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc. Natl. Acad. Sci. USA.* **96:** 11041–11048.
46. Juan, C. C., L. C. Au, V. S. Fang, S. F. Kang, Y. H. Ko, S. F. Kuo, Y. P. Hsu, C. F. Kwok, and L. T. Ho. 2001. Suppressed gene expression of adipocyte resistin in an insulin-resistant rat model probably by elevated free fatty acids. *Biochem. Biophys. Res. Commun.* **289:** 1328–1333.
47. Mater, M. K., D. Pan, W. G. Bergen, and D. B. Jump. 1998. Arachidonic acid inhibits lipogenic gene expression in 3T3-L1 adipocytes through a prostanoid pathway. *J. Lipid Res.* **39:** 1327–1334.
48. Petersen, R. K., C. Jorgensen, A. C. Rustan, L. Froyland, K. Muller-Decker, G. Furstenberger, R. K. Berge, K. Kristiansen, and L. Madsen. 2003. Arachidonic acid-dependent inhibition of adipocyte differentiation requires PKA activity and is associated with sustained expression of cyclooxygenases. *J. Lipid Res.* **44:** 2320–2330.
49. Hyman, B. T., L. L. Stoll, and A. A. Spector. 1982. Prostaglandin production by 3T3-L1 cells in culture. *Biochim. Biophys. Acta.* **713:** 375–385.
50. Long, S. D., and P. H. Pekala. 1996. Regulation of GLUT4 gene expression by arachidonic acid. Evidence for multiple pathways, one of which requires oxidation to prostaglandin E2. *J. Biol. Chem.* **271:** 1138–1144.
51. Miller, C. W., D. A. Casimir, and J. M. Ntambi. 1996. The mechanism of inhibition of 3T3-L1 preadipocyte differentiation by prostaglandin F2alpha. *Endocrinology.* **137:** 5641–5650.
52. Tsuboi, H., Y. Sugimoto, T. Kainoh, and A. Ichikawa. 2004. Prostanoid EP4 receptor is involved in suppression of 3T3-L1 adipocyte differentiation. *Biochem. Biophys. Res. Commun.* **322:** 1066–1072.
53. Kiriyama, M., F. Ushikubi, T. Kobayashi, M. Hirata, Y. Sugimoto, and S. Narumiya. 1997. Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. *Br. J. Pharmacol.* **122:** 217–224.
54. Kliewer, S. A., J. M. Lenhard, T. M. Willson, I. Patel, D. C. Morris, and J. M. Lehmann. 1995. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell.* **83:** 813–819.
55. Ito, S., K. Sakamoto, N. Mochizuki-Oda, T. Ezashi, K. Miwa, E. Okuda-Ashitaka, V. I. Shevchenko, Y. Kiso, and O. Hayaishi. 1994. Prostaglandin F2 alpha receptor is coupled to Gq in cDNA-transfected Chinese hamster ovary cells. *Biochem. Biophys. Res. Commun.* **200:** 756–762.
56. Gardner, O. S., B. J. Dewar, H. S. Earp, J. M. Samet, and L. M. Graves. 2003. Dependence of peroxisome proliferator-activated receptor ligand-induced mitogen-activated protein kinase signaling on epidermal growth factor receptor transactivation. *J. Biol. Chem.* **278:** 46261–46269.
57. Hu, E., J. B. Kim, P. Sarraf, and B. M. Spiegelman. 1996. Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. *Science.* **274:** 2100–2103.
58. Seo, J. B., M. J. Noh, E. J. Yoo, S. Y. Park, J. Park, I. K. Lee, S. D. Park, and J. B. Kim. 2003. Functional characterization of the human resistin promoter with adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element binding protein 1c and CCAAT enhancer binding protein-alpha. *Mol. Endocrinol.* **17:** 1522–1533.
59. Xu, J., H. Cho, S. O'Malley, J. H. Park, and S. D. Clarke. 2002. Dietary polyunsaturated fats regulate rat liver sterol regulatory element binding proteins-1 and -2 in three distinct stages and by different mechanisms. *J. Nutr.* **132:** 3333–3339.
60. Kawashima, J., K. Tsuruzoe, H. Motoshima, A. Shirakami, K. Sakai, Y. Hirashima, T. Toyonaga, and E. Araki. 2003. Insulin down-regulates resistin mRNA through the synthesis of protein(s) that could accelerate the degradation of resistin mRNA in 3T3-L1 adipocytes. *Diabetologia.* **46:** 231–240.

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