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Octylphenol stimulates resistin gene expression in 3T3-L1 adipocytes via the estrogen receptor and extracellular signal-regulated kinase pathways

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Lee M-J, Lin H, Liu C-W, Wu M-H, Liao W-J, Chang H-H, Ku H-C, Chien Y-S, Ding W-H, Kao Y-H. Octylphenol stimulates resistin gene expression in 3T3-L1 adipocytes via the estrogen receptor and extracellular signal-regulated kinase pathways. *Am J Physiol Cell Physiol* 294: C1542–C1551, 2008. First published April 16, 2008; doi:10.1152/ajpcell.00403.2007.—Resistin is known as an adipocyte-specific secretory hormone that can cause insulin resistance and decrease adipocyte differentiation. It can be regulated by sexual hormones. Whether environmental estrogens regulate the production of resistin is still not clear. Using 3T3-L1 adipocytes, we found that octylphenol upregulated resistin mRNA expression in dose- and time-dependent manners. The concentration of octylphenol that increased resistin mRNA levels by 50% was ~100 nM within 6 h of treatment. The basal half-life of resistin mRNA induced by actinomycin D was lengthened by octylphenol treatment, suggesting that octylphenol decreases the rate of resistin mRNA degradation. In addition, octylphenol stimulated resistin protein expression and release. The basal half-life of resistin protein induced by cycloheximide was lengthened by octylphenol treatment, suggesting that octylphenol decreases the rate of resistin protein degradation. While octylphenol was shown to increase activities of the estrogen receptor (ER) and MEK1, signaling was demonstrated to be blocked by pretreatment with either ICI-182780 (an ERα antagonist) or U-0126 (a MEK1 inhibitor), in which both inhibitors prevented octylphenol-stimulated phosphorylation of ERK. These results imply that ERα and ERK are necessary for the octylphenol stimulation of resistin mRNA expression. Moreover, U-0126 antagonized the octylphenol-increased resistin protein expression and release. These data suggest that the way octylphenol signaling increases resistin protein levels is similar to that by which it increases resistin mRNA levels; it is likely mediated through an ERK-dependent pathway. In vivo, octylphenol increased adipose resistin mRNA expression and serum resistin and glucose levels, supporting its in vitro effect.

environmental hormone; adiponectin; leptin; nonylphenol; bisphenol A

ADIPOCYTES have been traditionally viewed as the depot site for fat energy. They are now known to express and secrete a variety of bioactive peptides that act at the autocrine, paracrine, and endocrine levels (23). Of these adipokines, resistin is a cysteine-rich hormone that was first isolated from adipose tissues and found to link obesity to Type 2 diabetes in rodents (45). In particular, administration of exogenous resistin to normal mice causes glucose intolerance and hyperinsulinemia, while an anti-resistin antibody decreases blood glucose and improves insulin sensitivity in obese mice (45). In addition, resistin suppresses insulin-stimulated glucose uptake in adipocytes (45) and muscle cells (32). Moreover, transgenic mice overexpressing a dominant negative form of resistin showed increased adipogenesis and improved insulin sensitivity (24). However, the involvement of resistin in obesity and insulin resistance in humans is still controversial. Some studies have shown no relationship of resistin gene expression with body weight or insulin resistance (25). Others found that a single-nucleotide polymorphism in the resistin gene promoter is associated with obesity (17) and diabetes (39), that plasma resistin levels are elevated in patients with obesity (14) and Type 2 diabetes (50), and that resistin mRNA expression in adipose tissues of obese humans is higher than that in normal subjects (30). One possible explanation for these disparate findings is the presence of various isoforms (1, 35) or dimers (37) of resistin. This contention may also explain the functional diversity of resistin in different species or systems. For example, resistin regulates fasting blood glucose levels, lipid metabolism, catecholamine release, inflammation, atherosclerosis, hepatic insulin resistance, vasodilation, and the proliferation and activation of endothelial cells and smooth muscle cells (3, 9, 15, 22, 25, 33, 36). Resistin can stimulate muscle cell proliferation through the activation of ERK and phosphatidylinositol 3-kinase (PI3K) (9) in additional to inhibiting insulin signaling of 3T3-L1 adipocytes through the induction of the gene expression of suppressor of cytokine signaling 3 (46).

The expression of the adipocyte resistin gene can be regulated by endocrine, nutritional, pharmacological, genetic, and developmental cues (2, 25, 44, 45). For example, 17β-estradiol (E_2) increased resistin gene expression and protein secretion of murine primary and secondary adipocytes, whereas ICI-182780, an estrogen receptor (ER) antagonist, prevented E_2-stimulated resistin expression (11). The signal elements responsible for transducing the direct action of E_2 on resistin gene expression and secretion were found in 3T3-L1 adipocytes to include ERα and ERK (11).

Octylphenols are alkylphenolic compounds that are called environmental estrogens because they strongly compete with native estrogen for binding to the ER as well as stimulating vitellogenin production in hepatocytes and cell proliferation in cultures of human MCF-7 breast cancer cells (48). Determining whether octylphenols have the same ability as the estrogen hormone in directly stimulating resistin gene expression and protein secretion by adipocytes (11) requires further investiga-

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tion. Octylphenols have been reported to stimulate cell proliferation and adipocyte formation in cultures of murine 3T3-L1 adipocytes, as indicated by an increase in the DNA content and by decreases in triglyceride content and lipoprotein lipase activity (28, 29). Whether octylphenols exert their effects through the control of resistin's production or modulation of resistin's action is unknown. The fact that ER, MAPK, and PI3K have been reported to be essential signal transducers of E_2 or xenoestrogens in regulating other genes in 3T3-L1 adipocytes (28, 34, 43) and the fact that ER and ERK proteins are necessary for E_2 to regulate the expression of the adipocyte resistin gene have created much speculation surrounding the possible involvement of ER and ERK proteins in octylphenol's regulation of resistin gene expression. Further in vitro cell lines that are free from interfering influences present in whole animals and that allow precise octylphenol concentrations to be achieved should help clarify these notions.

In this study, we used 3T3-L1 adipocytes to examine the influence and signaling of octylphenol on resistin gene expression and protein secretion. We investigated whether octylphenol-regulated resistin gene expression and protein secretion are dependent on ER, MAPK, and/or PI3K pathways.

MATERIALS AND METHODS

Chemical reagents. All materials (e.g., octylphenol, U-0126, and so forth) were purchased from Sigma (St. Louis, MO) unless otherwise stated. DMEM, penicillin-streptomycin, FBS, trypsin, agarose, the 1-kb plus DNA ladder marker, and the protein marker were purchased from GIBCO-BRL (New York, NY). Except for the resistin antibody, which was obtained from Linco Research (St. Charles, MO), all other antibodies [phosphorylated (p-)ERKs, ERK1, ERK2, ER, goat anti-guinea pig IgG-horseradish peroxidase, etc.] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The 3′-rapid amplification of cDNA ends (RACE) system, TRIzol, and *Taq* polymerase were purchased from Invitrogen Life Science Technologies (Carlsbad, CA).

Cell culture. 3T3-L1 adipocytes (American Type Culture Collection, Manassas, VA) were obtained according to a previously published method (11), in which 2-day postconfluent 3T3-L1 preadipocytes (3×10^6 cells on a 10-cm plate) were treated with DMEM containing a final concentration of 10 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10% FBS for 48 h. The medium was then changed to DMEM containing 10% FBS for an additional 6∼10 days. With this protocol, >90% adipocyte differentiation was achieved, as indicated by a phenotypical appearance and triglyceride accumulation (11). Differentiated adipocytes expressed 3.4-fold more resistin mRNA than did preadipocytes or differentiating preadipocytes.

All experiments were carried out according to the method described by Chen et al. (11) unless otherwise noted. Briefly, adipocytes were serum starved for 12 h in DMEM containing 0.1% (fatty acid free) BSA and then, unless otherwise noted, incubated with or without octylphenol at various concentrations for the indicated time periods. Based on results of previous dose-response studies described by Masuno et al. (29) and White et al. (48), concentrations of 1–1,000 nM octylphenol were used in our study. Octylphenol was dissolved in 0.1% ethanol and sterile medium for cell treatment. Actinomycin D (Acti-D; 5 μg/ml), cycloheximide (5 μg/ml), U-0126 [a MEK1 inhibitor (18), 10 μM], and ICI-182780 [an ER inhibitor (47), 1 μM] were used to inhibit transcriptional, translational, MEK1, and ER activities, respectively (12, 47). In the experiments, serum-starved 3T3-L1 adipocytes were pretreated with or without either Acti-D for 30 min or other inhibitors for 90 min. Adipocytes were then stimulated with or without octylphenol (100 nM) for the indicated time periods. After treatment, resistin mRNA and protein levels were measured. Despite the high dose of some inhibitors used in the study, no adverse effects on the cell viability of adipocytes for 24 h were noted (12). In this study, through the use of the trypan blue dye exclusion method, the cell viability of 3T3-L1 adipocytes remained at 90–100% with concentrations of octylphenol of 0.001–10 μM during the 24-h treatment. Nonylphenol (0.1 and 10 μM), bisphenol A (0.1 and 10 μM), and inhibitors (i.e., U-0126, SB-203580, ICI-182780, Acti-D, cycloheximide, and LY-294002) used in this study reduced the numbers of 3T3-L1 adipocytes by 5–10% during the 24-h treatment.

In vivo experiments. Male C57BL/6J mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mice were ∼10 wk of age and weighed 24∼26 g. Animal experimental protocols were reviewed and approved by the Laboratory Animal Ethics Committee, Academia Sinica, Taipei, Taiwan. All animals were injected daily with 50 and 100 mg octylphenol/kg body wt for 3 days according to the method described by Laws et al. (26). The octylphenol compound was dissolved in DMSO and subcutaneously given in a total volume of 0.1 ml/animal. Six hours after the last injection, epididymal adipose tissues were collected and stored in liquid N_2 for the later analysis of hormone gene expression. Blood was collected from the tip of the tail of mice after they had been anesthetized with ethyl ether. Serum was stored in a −80°C freezer for later assays of hormones and glucose. Circulating levels of resistin, adiponectin, and leptin were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN). Based on the standard value of each hormone, the amounts of the three hormones in a sample were determined and then expressed as absolute values. Serum glucose levels were directly determined by a commercially available strip for glucose (Fuji Dri-Chem Slide Glu-PIII, Fuji Photo Film). mRNA levels of tissue resistin, adiponectin, and leptin were determined by real-time PCR.

ELISA or real-time PCR for resistin mRNA and extracellular resistin protein. Resistin mRNA levels were measured using a commercial PCR ELISA kit with digoxigenin (DIG) labeling and detection (Roche Applied Science, Mannheim, Germany) (11, 12). The forward and reverse primers were 5′-GTACCCACGGGATGAAGAACC-3′ and 5′-GCAGAGCCACAGGAGCAG-3′ for mouse resistin (accession no. AF323080), 5′-CCAGGGTGTGATGGTGGGAATG-3′ and 5′-CGCACGATTTCCCTCTCAGCTG-3′ for actin (accession no. X03672), and 5′-CCTCTGGAAAGCTGTGGCGT-3′ and 5′-TTGGCAGGTTTCTCCAGGCG-3′ for mouse GAPDH (accession no. M32599), respectively. Sample resistin mRNA levels were determined in relation to a standard curve of resistin cDNAs ranging from 3 to 200 ng/well [optical density at 405 nm ($OD_{405\ nm}$) = 0.1141 + 0.0031 × ng DNA/well; $R^2 = 0.998$]. An almost linear range in the number of PCR amplifications for resistin was observed between 20 and 40 cycles compared with the β-actin standard. Thus 30 cycles of PCR amplification were subsequently used for all experiments. After normalization to β-actin or GAPDH mRNA, resistin levels were expressed as a percentage of the control. To analyze secreted resistin protein, a homologous ELISA procedure (12) was used. The interassay and intra-assay coefficients of variation in the ELISA were 7–9% and 3–4%, respectively. Reproducible results were obtained in the range of resistin from 5 to 80 ng/well ($OD_{405\ nm} = 0.1269 + 0.0044 \times$ ng/well; $R^2 = 0.979$).

In some experiments, we used real-time PCR (38) to determine mRNA levels of resistin, adiponectin, and leptin. The real-time PCR analysis was performed twice in duplicate with use of a power SYBR green PCR master mix and ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) under the following conditions: an initial denaturing cycle at 95°C for 5 min, followed by 45 cycles of amplification consisting of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s. A final cooling period at 40°C for 10 s was added after the last cycle. The forward and reverse primers were 5′-TGAGATGATTCAGTGGGTAAAGATG-3′ and 5′-TCCACCATGTAGTTTCCAGGAA-3′ for

mouse resistin (accession no. AF510100), 5′-AAGGGCTCAGGATGCTACTGTT-3′ and 5′-AGTAACGTCATCTTCGGCATG-3′ for mouse adiponectin (accession no. NM_009605), 5′-TCTCCGAGACCTCCTCCATCT-3′ and 5′-CATCCAGGCTCTCTGGCTTCT-3′ for mouse leptin (accession no. NM_008493), and 5′-GGAGCCAAACGGGTCATCATCTC-3′ and 5′-GAGGGGCCATCCACAGTCTTG-3′ for GAPDH, respectively. Normalization involved GAPDH mRNA levels as controls in parallel reactions. The relative expression ratio of resistin, adiponectin, and leptin transcripts to GAPDH transcript was calculated as previously described (38) and then expressed as a percentage of the control.

Western blot analysis. The Western blot method for analyzing p-ERK1/2 and other proteins was performed according to the method described by Chen et al. (11). Following experimental treatments, adipocytes were washed twice in PBS and then lysed in 1 ml of buffer containing 20 mM Tris·HCl (pH 7.6), 1 mM EDTA, 1 mM Na_3VO_4, 0.2% Triton X-100, and 1 mM PMSF. The lysate was agitated for 15 min at 4°C and then centrifuged for 10 min to collect the supernatant. The protein content of the lysates was determined in duplicate by the dye-binding method (6) using a Bio-Rad (Richmond, CA) microplate reader and BSA (Sigma) as the standard. Supernatant fractions of adipocytes (50 μg protein) were separated by 12.5% SDS-PAGE with loading buffer [100 mM Tris·HCl (pH 6.8), 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 10% β-mercaptoethanol] and then blotted onto an Immobilon-NC transfer membrane (Millipore, Bedford, MA). Immunoblots were blocked for 1 h at room temperature with 10 mM PBS containing 0.1% Tween 20 and 5% defatted milk. After a wash with PBS-Tween 20, immunoblot analyses with primary antibody were performed. All primary antibodies (ERK1, ERK2, p-ERKs, p-p38, p-Akt, ERα, β-actin, and resistin antisera) were used at a dilution concentration of 1:1,000 (~0.2 μg/ml). Donkey anti-rabbit IgG, donkey anti-mouse IgG, donkey anti-goat IgG, or goat anti-guinea pig conjugated with horseradish peroxidase were used as the secondary antibodies at a dilution of 1:2,000 (~0.2 μg/ml). We visualized immunoblots using the Western Lightning chemiluminescence reagent plus kit (Perkin-Elmer Life Science, Boston, MA) for 3 min; this was followed by an exposure to Fuji film for 2–3 min. We quantified blots using a Molecular Imager (Bio-Rad). After normalization to β-actin protein, levels of the intracellular resistin protein, ERα, and kinases were expressed as a percentage of the control unless otherwise noted. The amounts of p-ERK1/2 proteins indicative of ERα activation (11, 43) in the immunoprecipitates were measured by Western blot analysis using a p-ERK1/2 antibody.

Statistical analysis. Data are expressed as means ± SE. An unpaired Student's *t*-test was used to examine differences between control and E_2-treated groups. One-way ANOVA followed by the Student-Newman-Keuls multiple-range test were used to examine differences among multiple groups. Differences were considered significant at $P < 0.05$. Statistics were performed using SigmaStat (Jandel Scientific, Palo Alto, CA), and data were log transformed.

RESULTS

Effects of octylphenol on resistin mRNA expression. Octylphenol increased the steady-state levels of resistin mRNA in concentration-dependent (Fig. 1*A*) and time-dependent (Fig. 1*B*) manners. The activation concentration of octylphenol to increase the resistin mRNA levels by 50% was ~100 nM after 6 h of treatment. The possibility that the octylphenol-induced stimulation of resistin mRNA expression resulted from an alteration in resistin mRNA stability was also examined (Fig. 1*C*). 3T3-L1 adipocytes were pretreated with the transcriptional inhibitor Acti-D and then treated with or without 100 nM octylphenol. Octylphenol increased the basal half-life of resistin mRNA induced by Acti-D alone (Fig. 1*C*).

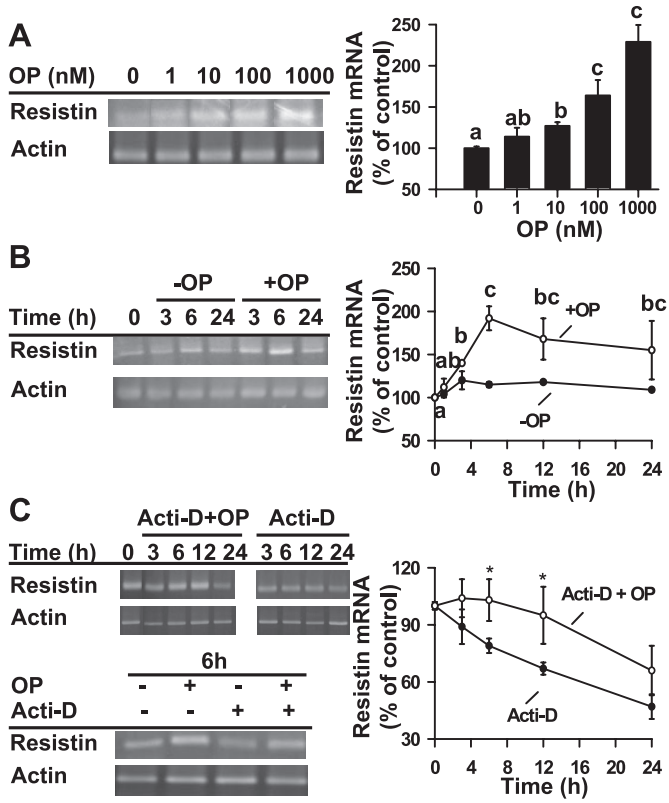


Fig. 1. Stimulatory effect of octylphenol (OP) on resistin mRNA expression by 3T3-L1 adipocytes resulted from an increase in resistin mRNA stability. *A*: dose-dependent effect of OP was observed 6 h after treatment. *B*: time-dependent effect of OP (100 nM) was observed. *C*: OP increased resistin mRNA stability induced by actinomycin D (Acti-D; 5 μg/ml) alone. Bands (*right*) show representative RT-PCR, whereas graphs (*left*) show normalized data. Data are expressed as means ± SE from triplicate experiments after quantitative digoxigenin (DIG)-PCR ELISA. SE bars are too small to be seen in *B* and *C*. In *A*, the control experiment was not treated with OP, whereas in *B* and *C* controls were set at *time 0*, when OP was added. [a,b,c]Groups with different letters are significantly different ($P < 0.05$) from each other. *$P < 0.05$, Acti-D vs. Acti-D + OP at a given time.

Effects of octylphenol on resistin protein expression. To determine whether octylphenol-stimulated resistin gene expression also occurred at the level of translation, changes in the intracellular resistin protein content were measured (Fig. 2). Intracellular and extracellular levels of resistin protein significantly increased in the presence of 1~100 nM octylphenol after 6 h of treatment (Fig. 2, *A* and *B*). The possibility that octylphenol-stimulated expression of resistin protein was related to changes in resistin protein stability was also examined (Fig. 2*C*). 3T3-L1 adipocytes were pretreated with the translational inhibitor cycloheximide and then treated with or without 100 nM octylphenol. Octylphenol increased protein stability based on the observations of significant changes in the intracellular resistin protein content when comparing two cycloheximide treatment groups in the presence and absence of octylphenol.

Effect of octylphenol on resistin gene expression is dependent on the ER pathway. To further demonstrate whether octylphenol-induced expression of the resistin gene is mediated through the ER, adipocytes were pretreated with the ER antagonist ICI-182780 (1 μM) for 90 min and then incubated with or without 100 nM octylphenol for 6 h (Fig. 3). Treatment

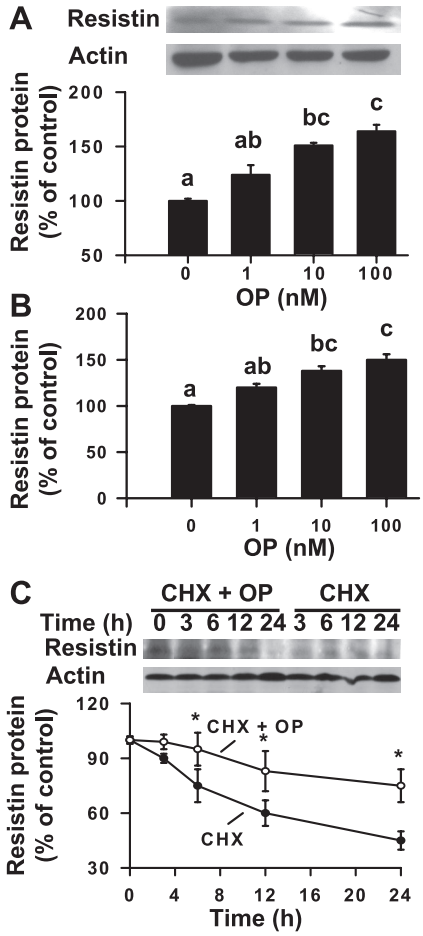
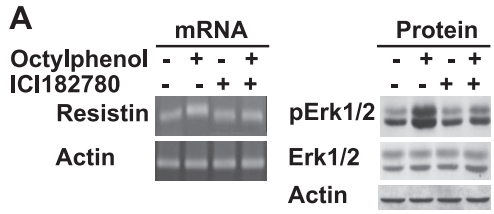


Fig. 2. OP increased intracellular (*A*) and extracellular (*B*) resistin protein levels in 3T3-L1 adipocytes in a dose-dependent manner. Also, octylphenol increased the stability of the intracellular resistin protein induced by cycloheximide (CHX; 5 μg/ml) alone (*C*). Bands (*A* and *C*, *top*) show representative Western blots, whereas graphs (*A* and *C*, *bottom*) show normalized data. Data are expressed as means ± SE from triplicate experiments. SE bars are too small to be seen in *A*–*C*. In *A* and *B*, the control experiment was not treated with OP, whereas in *C* it was set at *time 0*, when octylphenol was added. [a,b,c]Groups with different letters are significantly different ($P < 0.05$) from each other. *$P < 0.05$, CHX vs. CHX + octylphenol at a given time.

with ICI-182780 alone did not alter the steady-state level of resistin mRNA but prevented octylphenol-induced increases in resistin mRNA expression. Because ERK MAPK has been implicated as being necessary for the estrogen stimulation of adipocyte proliferation from the ER (43), the effect of octylphenol on ER activity was assessed by changes in the amount of the phosphorylated form of ERK MAPK. Indeed, treatment with ICI-182780 prevented octylphenol-induced increases in the amount of p-ERK1/2 proteins (Fig. 3). However, neither octylphenol nor ICI-182780 changed the total amounts of ERK-1 and ERK-2 proteins (Fig. 3*A*).

Effect of octylphenol on resistin gene expression is dependent on the ERK pathway. To further demonstrate whether octylphenol-induced expression of the resistin gene is mediated through ERK MAPKK, adipocytes were pretreated with the ERK MAPKK antagonist U-0126 (10 μM) for 90 min and then incubated with or without 100 nM octylphenol for 6 h (Fig. 4). Activities of ERK MAPKK were assessed by changes in the amounts of the phosphorylated forms of ERK1 and ERK2. Octylphenol alone had no effect on ERK1 and ERK2 proteins (Fig. 4*A*) but increased the amounts of p-ERK1 and p-ERK2 proteins (Fig. 4, *A* and *B*). In contrast, U-0126 alone reduced the basal activity of ERK MAPKK and, in the presence of octylphenol, suppressed octylphenol-increased levels of p-ERK proteins (Fig. 4). Compared with the octylphenol-treated group, U-0126 prevented the octylphenol-stimulated expression of resistin mRNA. Whether octylphenol-induced increases in resistin protein production are dependent on the ERK MAPKK pathway was also examined during the course of the experiment (Fig. 4). There was no significant effect of U-0126 on the basal intracellular resistin protein content or the basal release of resistin protein after 6 h of treatment. However, this ERK MAPKK inhibitor prevented octylphenol-increased levels of the intracellular resistin protein and reduced octylphenol-induced increases in resistin protein release.

Whether octylphenol-induced alterations in resistin gene expression are dependent on other MAPK or PI3K pathways was also examined when 3T3-L1 adipocytes were pretreated with the respective p38 MAPK and PI3K antagonists SB-203580 (10 μM) (16) and LY-294002 (50 μM) (7) for 90 min and then incubated with or without 100 nM octylphenol for 6 h (Fig. 5). The activity of p38 MAPK was assessed by changes in the amount of the Tyr^{182}-phosphorylated form of p38, whereas the activity of PI3K was assessed by changes in the amount of the phosphorylated form of its protein substrate, Akt. Regardless of the presence of octylphenol, SB-203580 pretreatment significantly decreased the amount of p-p38 protein in adipocytes relative to the control. SB-203580 did not alter the increases in resistin gene expression induced by octylphenol. LY-294002 significantly reduced the amount of p-Akt protein in adipocytes treated with or without octylphenol

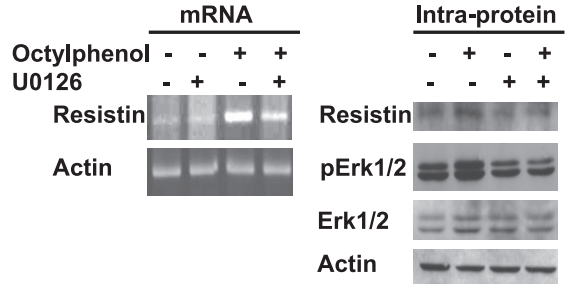


B

Treatments	Resistin mRNA	pErk1/2
Control	100±1	100±1
Octylphenol	177±10[a]	182±17[a]
ICI182780	92±4	87±12
Octylphenol + ICI182780	127±5[b]	96±5[b]

Fig. 3. Effects of ICI-182780 on the OP-induced increase in resistin mRNA of 3T3-L1 adipocytes. *A*: representative RT-PCR and Western blots are shown. *B*: data are expressed as means ± SE of 3 experiments. Serum-starved 3T3-L1 adipocytes were pretreated with or without ICI-182780 (1 μM, 90 min) and then stimulated with 100 nM OP for 6 h. Resistin mRNA expression was analyzed by DIG-PCR ELISA, and levels are expressed as a percentage of the control after normalization to actin mRNA. Amounts of phosphorylated (p-)ERK1/2 proteins were determined by Western blot analysis after 6 h of OP treatment and are expressed as a percentage of the control after normalization to actin protein. For each experiment, significant differences were assessed between the two groups after one-way ANOVA and a Student-Newman-Keuls multiple-range test. [a]$P < 0.05$, OP vs. control; [b]$P < 0.05$, OP vs. OP + ICI-182780.

A



B

Treatments	Resistin			pErk1/2
	mRNA	Intra-protein	Extra-protein	
Control	100±1	100±1	100±3	100±1
Octylphenol	171±11[a]	164±6[a]	143±3[a]	179±10[a]
U0126	94±2	83±5	98±2	52±4[a]
Octylphenol + U0126	114±9[b]	84±5[b]	110±3[b]	44±4[a, b]

Fig. 4. Effects of U-0126 on OP-induced alterations in resistin mRNA and protein of 3T3-L1 adipocytes. *A*: representative RT-PCR and Western blots are shown. *B*: data are expressed as means ± SE of 3 experiments. Serum-starved 3T3-L1 adipocytes were pretreated with or without U-0126 (10 μM) for 90 min and then stimulated with 100 nM OP for 6 h. Resistin mRNA and protein levels are expressed as a percentage of the control after normalization to actin mRNA and protein, respectively. The average level of resistin released from control adipocytes was 47 ± 5 ng/ml. Amounts of p-ERK1/2 proteins were determined by Western blot analysis and are expressed as a percentage of the control after normalization to actin protein. Significant differences were assessed between the two groups after one-way ANOVA and a Student-Newman-Keuls multiple-range test. [a]$P < 0.05$, OP vs. control or U-0126 vs. control; [b]$P < 0.05$, OP vs. OP + U-0126.

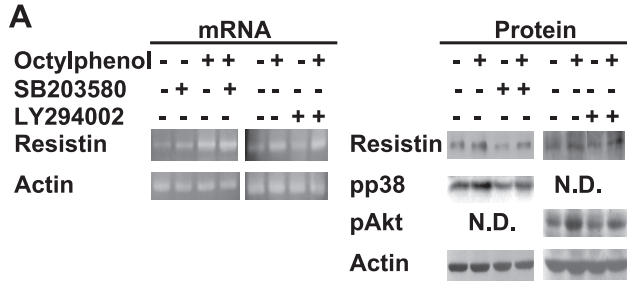
compared with the control. Similar to SB-203580, LY-294002 neither changed resistin gene expression alone nor significantly affected octylphenol-induced increases in resistin mRNA levels. In other experiments, whether octylphenol-induced alterations in resistin protein expression are dependent on p38 MAPK and PI3K pathways was also examined (Fig. 5). There was a trend for SB-203580 to decrease the basal content of intracellular resistin protein. In addition, this p38 MAPK inhibitor decreased octylphenol-increased levels of the intracellular resistin protein. The effects of LY-294002 differed from those of SB-203580. There was no significant effect of LY-294002 to alter the basal or octylphenol-increased content of the intracellular resistin protein. Neither SB-203580 nor LY-294002 significantly prevented octylphenol-stimulated resistin protein release by 3T3-L1 adipocytes (data not shown). These data indicated that U-0126, as previously described, was more significant than SB-203580 or LY-294002 in modifying octylphenol-induced changes in resistin protein contents between intracellular and extracellular compartments.

Differences among several environmental estrogens in the regulation of resistin gene expression. Differences in the regulation of resistin mRNA expression among octylphenol, nonylphenol, and bisphenol A were also assessed (Fig. 6). At a given concentration of 100 nM for 6 h of treatment, octylphenol and nonylphenol, but not bisphenol A, increased resistin mRNA levels. With 6 or 24 h of treatment, octylphenol and nonylphenol at 10 μM tended to be more effective than bisphenol A in stimulating resistin mRNA expression.

Effects of octylphenol, nonylphenol, and bisphenol on adiponectin and leptin mRNA expressions. To determine whether different adipokines in addition to resistin are regulated by octylphenol, changes in adiponectin and leptin mRNA levels were assessed (Fig. 7). At a given concentration of 100 nM, octylphenol did not significantly alter the steady-state levels of adiponectin and leptin mRNAs (Fig. 7*A*). However, octylphenol at 10 μM tended to decrease adiponectin mRNA expression after 6 or 24 h of treatment while stimulating leptin mRNA levels.

Differences in the regulation of adiponectin and leptin mRNA expressions among octylphenol, nonylphenol, and bisphenol A were also assessed by treating serum-starved 3T3-L1 adipocytes with or without each compound for 6 and 24 h (Fig. 7, *A* and *B*). Nonylphenol at 100 nM for 6 h significantly increased levels of adiponectin (Fig. 7*A*) and leptin (Fig. 7*B*) mRNAs, whereas 6 or 24 h of 10 μM nonylphenol treatment significantly decreased levels of adiponectin mRNA and tended to increase levels of leptin mRNA. Unlike octylphenol and nonylphenol, bisphenol A did not significantly alter the level of any of these hormone mRNAs after 6 h of 0.1 or 10 μM treatment but reduced the amount of adiponectin mRNA by 54% (Fig. 7*A*) and increased the amount of leptin mRNA by 122% (Fig. 7*B*) after 24 h of 10 μM treatment.

A



B

Treatments	Resistin		pp38	pAkt
	mRNA	Protein		
Control	100 ±1	100 ±1	100 ±1	100 ±1
OP	171 ±11[a]	164 ±6[a]	145 ±2[a]	152 ±5[a]
SB203580	104 ±3	65 ±5[a]	62 ±4[a]	ND
LY294002	101 ±3	90 ±5	ND	60 ±5[a]
OP+SB203580	162 ±5	92 ±5[b]	85 ±6[b]	ND
OP+LY294002	144 ±10	152 ±5	ND	99±6[b]

Fig. 5. Effects of SB-203580 and LY-294002 on OP-induced alterations in resistin mRNA and protein of 3T3-L1 adipocytes. *A*: representative RT-PCR and Western blots are shown. *B*: data are expressed as means ± SE of 3 experiments. Serum-starved 3T3-L1 adipocytes were pretreated with or without SB-203580 (10 μM, 90 min) or LY-294002 (50 μM, 90 min) and then stimulated with 100 nM OP for 6 h. Resistin mRNA and protein levels are expressed as a percentage of the control after normalization to actin mRNA and protein, respectively. Amounts of p-p38 and p-Akt proteins were determined by Western blot analysis after 6 h of OP treatment and are expressed as a percentage of the control after normalization to actin protein. For each experiment, significant differences were assessed between the two groups after one-way ANOVA and a Student-Newman-Keuls multiple-range test. ND, not determined. [a]$P < 0.05$, OP vs. control, SB-203580 vs. control, or LY-294002 vs. control; [b]$P < 0.05$, OP vs. OP + SB-203580 or OP vs. OP + LY-294002.

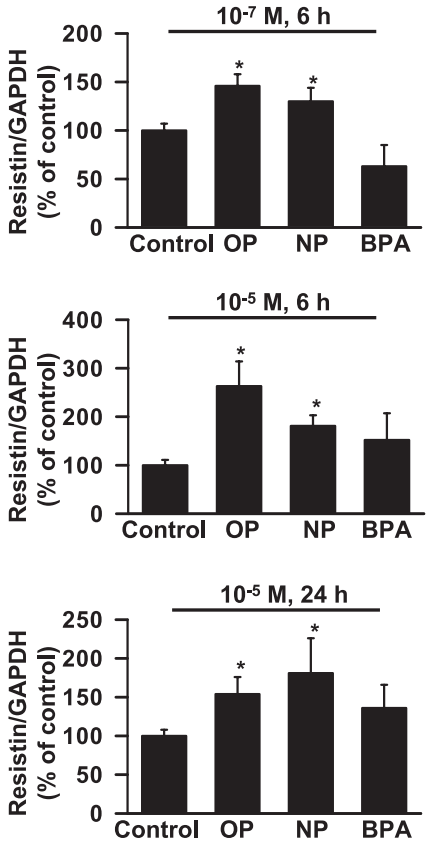


Fig. 6. Assessment of differences among OP, nonylphenol (NP), and bisphenol A (BPA) in altering resistin mRNA expression. Starved 3T3-L1 adipocytes were incubated in the presence or absence (control group) of 0.1 or 10 μM OP, NP, or BPA for 6 or 24 h. Levels of adipocyte resistin mRNA were analyzed by real-time PCR. Data are expressed as means ± SE from replicates of 3 experiments. *$P < 0.05$ vs. the control at a given time or dose.

In vivo effects of octylphenol on tissue and serum resistin, adiponectin, and leptin levels. To examine whether any of these in vitro effects of octylphenol could be observed in vivo, male C57BL/6J mice were daily injected subcutaneously for 3 days with 50 or 100 mg octylphenol/kg body wt, and resistin mRNA expression was then analyzed using real-time PCR (Fig. 8). A subcutaneous injection of 50 or 100 mg octylphenol/kg body wt increased the respective levels of resistin mRNA by 88% and 179% in epididymal adipose tissues relative to the control (Fig. 8*A*). Circulating levels of resistin were elevated by 22–34% after treatment with 50 and 100 mg octylphenol/kg body wt (Fig. 8*B*).

In a further demonstration of whether adiponectin and leptin are also regulated by octylphenol in vivo, changes in their mRNA expressions in epididymal adipose tissues were assessed by real-time PCR (Fig. 8, *C* and *E*). Doses of 50 or 100 mg octylphenol injected daily for 3 days were not or were only slightly effective in reducing adiponectin mRNA levels (Fig. 8*C*). Levels of serum adiponectin were significantly decreased by 38–42% after doses of 50 and 100 mg octylphenol/kg body wt (Fig. 8*D*). However, the expression of leptin mRNA in epididymal adipose tissues was reduced 3 days after an injection of 50 or 100 mg octylphenol/kg body wt (Fig. 8*E*). Also, octylphenol tended to decrease circulating levels of leptin protein (Fig. 8*F*).

In vivo effects of octylphenol on serum glucose levels. To further examine whether octylphenol-stimulated expression of the resistin gene in vivo is linked to glucose homeostasis, changes in serum glucose levels were assessed 3 days after daily subcutaneous injection of male C57BL/6J mice with 50 or 100 mg octylphenol/kg body wt (Fig. 9). We observed that injection of either dose of octylphenol caused increases in serum glucose levels of 51–62%.

DISCUSSION

The present study demonstrates that octylphenol stimulates resistin gene expression in 3T3-L1 adipocytes. The effects of octylphenol were dose and time dependent. It is likely that octylphenol upregulates resistin mRNA levels by stabilizing resistin mRNA. This conclusion is supported by the finding that treatment with octylphenol increased the basal half-life of resistin mRNA induced by Acti-D alone (Fig. 1*C*). Moreover, pretreatment of 3T3-L1 adipocytes with cycloheximide did not prevent octylphenol-stimulated resistin mRNA expression (data not shown), which suggests that new protein synthesis is not required for the effect of octylphenol. This suggestion is also supported by our observations that acute (6 h) exposure to octylphenol induced a 50% increase in resistin mRNA and that octylphenol increased the basal half-life of the intracellular resistin protein induced by cycloheximide. Taken together, octylphenol may directly stimulate the stability of resistin

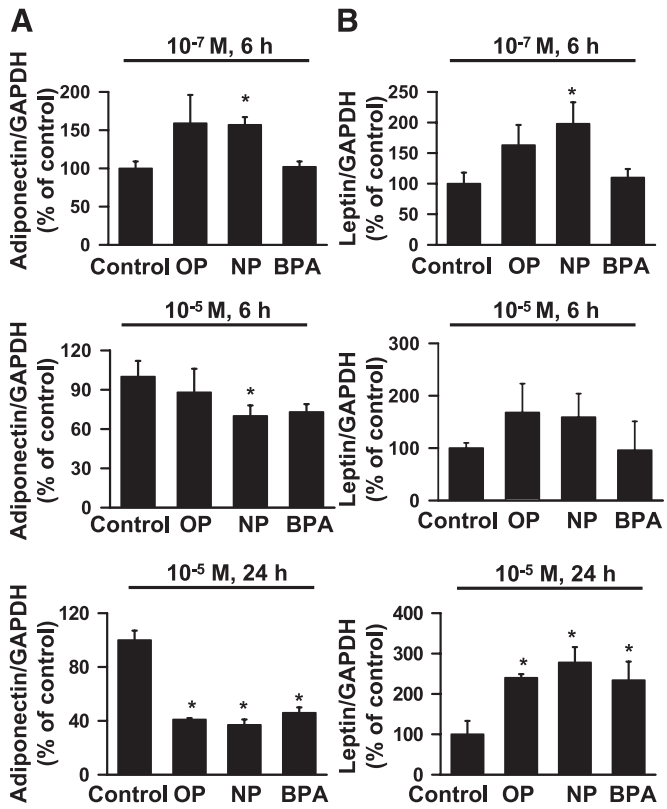


Fig. 7. Assessment of differences among OP, NP, and BPA in altering adiponectin (*A*) and leptin (*B*) mRNA expressions. Starved 3T3-L1 adipocytes were incubated in the presence or absence (control group) of 0.1 or 10 μM OP, NP, or BPA for 6 or 24 h. Levels of adipocyte adiponectin and leptin mRNA were analyzed by real-time PCR. Data are expressed as means ± SE from replicates of 3 experiments. *$P < 0.05$ vs. the control at a given time or dose.

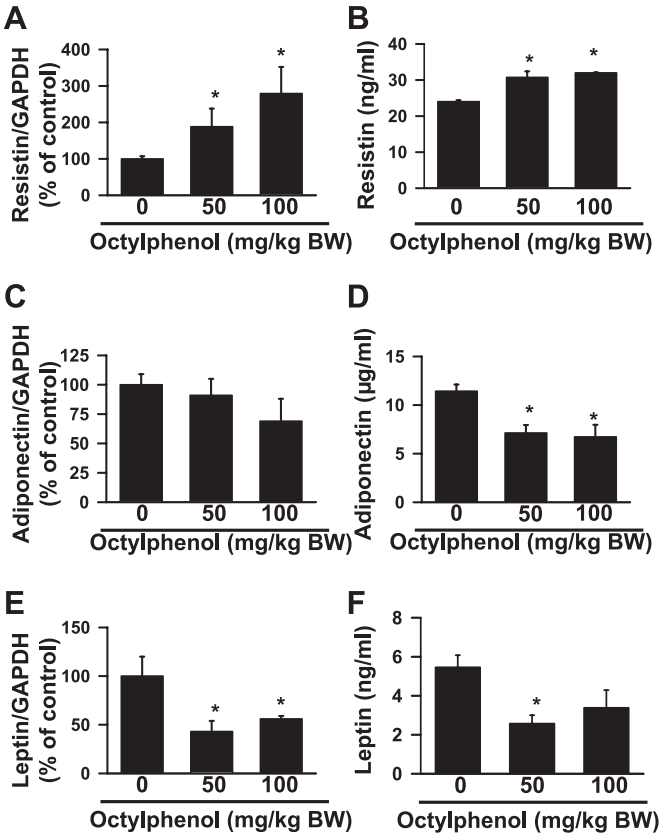


Fig. 8. In vivo effects of OP on resistin (*A* and *B*), adiponectin (*C* and *D*), and leptin (*E* and *F*) mRNA levels in murine epididymal adipose tissues (*A*, *C*, and *E*) as well as circulating levels of these hormones (*B*, *D*, and *F*). The OP compound [50 and 100 mg/kg body wt (BW) per day] was dissolved in DMSO and subcutaneously given to male C57BL/6J mice in a total volume of 0.1 ml/animal once in a day for 3 days. Levels of serum and adipose adipokines were measured with ELISA and real-time PCR, respectively. Data are expressed as means ± SE from replicates of 6 animals. $*P < 0.05$ vs. the control at a given dose.

mRNA and protein, or there may be an argument for the presence of preexisting resistin mRNA and protein. The latter contention is supported by recent discoveries that ERs are located on the membrane and in nuclei of 3T3-L1 adipocytes (34, 43) and that octylphenol can regulate the activity of ERs (48, 49) and kinases (8, 27). In higher eukaryotes, the control of mRNA stability results from a complex set of events, dependent on the stability of the poly(A) tail, polysomes, and the 3′-untranslated region with adenosine- and uridine-rich elements as well as on the activities of adenosine- and uridine-rich element-binding proteins, coding region-binding proteins, and RNase (41). For example, estradiol is known to regulate ER (42) and vitellogenin (5) mRNAs through altering nuclease activity associated with polyribosomes. Drawing firm conclusions as to whether any of these effects of E_2 can explain the effect of octylphenol on the half-life of adipocyte resistin mRNA will require further studies.

We attempted to search for the signaling proteins required for octylphenol's induction of resistin gene expression. It is evident from these data that ERα inactivation via the antiestrogen ICI-182780 (47) prevented the octylphenol-induced increases in resistin mRNA levels and ERα activity. This demonstrates that functional ERα is necessary for the effect of octylphenol. We also attempted to find the downstream signaling transducers of ERα involved in the activation of resistin mRNA expression. We observed herein that the specific inhibitor of ERK MAPKK U-0126 (18) significantly prevented octylphenol-increased levels of resistin mRNA and amounts of p-ERK proteins. None of the respective inhibitors of p38 MAPK and PI3K (Fig. 5), such as SB-203580 (16) and LY-294002 (7), prevented octylphenol-increased levels of resistin mRNA, but they respectively antagonized octylphenol-induced increases in the amounts of p-p38 and p-Akt proteins. These observations suggest that the stimulatory effect of octylphenol on resistin mRNA expression of 3T3-L1 adipocytes is mediated via a pathway that requires the activation of ERK MAPK, but not p38 MAPK and PI3K, activity. The ERK-dependent effect of octylphenol was also supported by our observations that ICI-182780 reduced octylphenol-stimulated MEK1 activity and resistin expression and by other findings that octylphenol activated ERK MAPK in MCF-7 cells (27) and GH_3/B6/F10 cells (8). It was evident that E_2 induced the rapid nuclear translocation of ERK MAPK together with quick ERK MAPK-dependent activation of some transcriptional factors (i.e., the cAMP response element-binding protein, activator protein-1, and ERα) in rat adipocytes, leading to transcriptional activation of E_2-responsive genes (i.e., c-*fos*) (13, 34, 43). Whether this mechanism explains the ERK-dependent effect of octylphenol on resistin gene expression was not demonstrated in this study.

The expression and secretion of resistin protein are differently regulated by certain hormones, such as E_2, insulin, IGF-I, growth hormone, dexamethasone, endothelin-1, and vitamin A (11, 12, 19, 25, 44, 45). Our previous study implied the involvement of p38 MAPK-dependent pathways in the basal and IGF-I-stimulated distribution of the resistin protein between intracellular and extracellular compartments (12), whereas the MEK1 pathway was involved in the E_2-stimulated distribution of the resistin protein (11). In the present report, we observed increased intracellular resistin protein contents and increased resistin protein release after 6 h of octylphenol treatment, whereas U-0126 inhibited octylphenol-stimulated ERK MAPKK activity and resistin protein expression and release. Neither SB-203580 nor LY-294002 significantly prevented octylphenol-stimulated resistin protein release by 3T3-L1 adipocytes. These observations suggest that octylphenol mod-

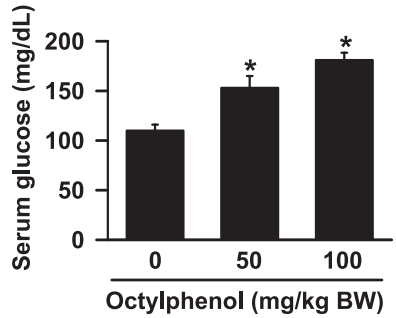


Fig. 9. In vivo effects of OP on serum glucose levels of male C57BL/6J mice. The OP compound (50 and 100 mg/kg BW per day) was dissolved in DMSO and subcutaneously given in a total volume of 0.1 ml/animal once in a day for 3 days. Six hours after the last injection, serum glucose levels were measured. Data are expressed as means ± SE from 6 animals. $*P < 0.05$ vs. the control at a given dose.

ifies the distribution of resistin protein between intracellular and extracellular compartments of 3T3-L1 adipocytes and that the way octylphenol signaling increases resistin protein expression and release from 3T3-L1 cells is similar to that by which it increases resistin mRNA levels; it is likely mediated through an ERK-dependent pathway. This is consistent with the MEK1-dependent effect of E_2 on stimulation of the resistin gene expression by 3T3-L1 adipocytes (11).

In support of this study, octylphenol at 100 nM for 6 h was found to induce a 69% increase in resistin mRNA levels from C3H10T1/2 mouse adipocytes (data not shown), which were obtained according to a previously published method (11). In addition, pretreatment of these cells with 1 μM ICI-182780 for 90 min prevented octylphenol-activated resistin mRNA expression. Moreover, octylphenol significantly stimulated a 207% increase in intracellular protein levels of C3H10T1/2 cells after 6 h of treatment, and the octylphenol activation of resistin protein expression was blocked by ICI-182780 pretreatment. These effects are similar to those observed for 3T3-L1 mouse adipocytes. In parallel with these in vitro observations, our in vivo findings showed that octylphenol stimulated resistin mRNA expression in epididymal adipose tissues and increased serum resistin levels when male C57BL/6J mice were injected daily with 50 or 100 mg octylphenol/kg body wt for 3 days.

It is interesting that the environmental estrogens (octylphenol, nonylphenol, and bisphenol A) were not entirely consistent in stimulating resistin expression by 3T3-L1 adipocytes. At the same dose and duration of treatment, octylphenol and nonylphenol were generally more effective than bisphenol A in changing the expression of adipocyte resistin. Bisphenol A requires a dose increase and an increase in the duration of treatment to stimulate resistin mRNA expression. The observed structure-specific effects of xenoestrogens on resistin expression suggest that octylphenol and nonylphenol may act differently from bisphenol A in the regulation of the expressions of adipokines. To strengthen this contention, we observed herein that nonylphenol at a 100 nM concentration for 6 h was like octylphenol in stimulating resistin, adiponectin, and leptin mRNA levels and that bisphenol A did not alter the expression of any of the adipocyte resistin, adiponectin, or leptin genes (Figs. 6 and 7). The fact that octylphenol stimulates adipocyte resistin gene expression through ERK-dependent and PI3K-independent pathways, the fact that nonylphenol, but not bisphenol A, produced rapid dose-dependent ERK1/2 phosphorylation in pituitary cancer cells (8), and the fact that bisphenol A accelerates adipogenesis of 3T3-L1 cells through a PI3K-dependent pathway (28) also indirectly support the different effects of octylphenol and nonylphenol from bisphenol A on the expressions of different adipokines. According to the nature of the unique structures of the three xenoestrogens tested (28), octylphenol and nonylphenol contains a single phenolic ring and a longer alkyl group on their aromatic ring, whereas bisphenol A consists of two phenolic rings and two methyl groups on the central carbon atom. Octylphenol and nonylphenol have a longer alkyl group, which has some conformational flexibility and a lower polarity, that may be important for interactions with other molecules. Other possible explanations for this discrepancy are that the distinct types of xenoestrogens bind to ERα or ERβ in 3T3-L1 adipocytes at varying levels (13, 43) and that coactivators (i.e., cAMP response binding protein and p300) and corepressors (i.e., silencing mediator of retinoid and thyroid receptors) of ERs required for the actions of xenoestrogens vary with estrogen species, ER forms, or the dose and duration of xenoestrogen treatment (28, 33).

In this study, our data indicate that different adipokines, i.e., resistin, adiponectin, and leptin, were distinctly regulated by octylphenol. This statement is supported by the in vivo findings that injection of male C57BL/6J mice with 100 mg octylphenol/kg body wt daily for 3 days caused significant increases in levels of adipose resistin mRNA and serum resistin protein and significant decreases in levels of adipose leptin mRNA and serum leptin protein as well as a trend of decreases in levels of adipose adiponectin mRNA and serum adiponectin protein. In support of these in vivo observations, our in vitro results obtained from 3T3-L1 adipocytes showed that octylphenol at 10 μM for 24 h increased steady-state levels of resistin mRNA and reduced levels of adiponectin mRNA. The observed adipokine-selective effect of octylphenol is comparable with those reported for the different effects of castration or E_2 replacement on plasma and perigonadal adipose resistin, adiponectin, and leptin levels in mice (21). Determining whether any of the distinct effects of octylphenol on different adipokines is due to differences in the basal or octylphenol-stimulated stabilities of these three adipokine mRNAs and proteins (31) requires further demonstrations. However, our study could not exclude the possibility that the genomic mechanism of actions of octylphenol (13, 43) on adiponectin and leptin may help explain its differential effects on adipokine expression. A possible explanation for the discrepancy of leptin mRNA levels caused by octylphenol in vitro and in vivo is that a variety of serum factors and hormones are present in the experimental condition of animals and changes in their levels caused by octylphenol may contribute to the expression of the adipose leptin gene and circulating levels of leptin. Whether any of these alterations in serum resistin, adiponectin, and glucose levels caused by octylphenol is responsible for an in vivo suppressive effect of octylphenol on adipose leptin gene expression was not determined in this study.

The toxicokinetics of *p*-tert-octylphenol in male rats has been reported (10). Blood octylphenol levels were detected as early as 10 min and reached their peak values of 50–100 ng/ml (~243–486 nM) 2 h after gavage administrations of 50 or 200 mg octylphenol/kg body wt. Therefore, octylphenol may have systemic effects (10). In that same experiment (10), octylphenol was detected at concentrations of 10 and 1,285 ng/g fat tissue (~49–6,220 nM if the density of fat tissues is assumed to be 1 g/cm^3). Accordingly, the doses (1–10,000 nM) of octylphenol used in our cell culture system and the doses (50 and 100 mg/kg body wt) of octylphenol administered to our animals were equal or close to higher physiological circulating octylphenol levels. Similarly, the levels of resistin released from octylphenol-stimulated adipocytes and C57BL/6J mice ranged from 50 to 80 ng/ml and from 32 to 35 ng/ml, respectively, which correspond to higher circulating resistin levels (23–25 ng/ml) and are compatible with the effective doses (30–20,000 ng/ml) of resistin needed to stimulate insulin resistance in cells or animals (45). Recent studies have also shown that resistin increases plasma glucose levels and impaired adipocyte, muscular, and hepatic glucose uptake (32, 33, 45), that adiponectin reduces plasma and hepatic glucose levels in mice (4) and improves glucose uptake and insulin sensitivity in 3T3-L1 adipocytes (20), and that leptin failed to stimulate

glucose transport in fat and muscle cells (40). Accordingly, our finding that injection of mice with 50 and 100 mg octylphenol/kg body wt increased serum glucose levels can be explained by the octylphenol-increased levels of the resistin hormone and by the octylphenol-decreased levels of the adiponectin hormone but not by changes in the leptin hormone. Changes in the production and secretion of resistin, adiponectin, and leptin induced by octylphenol, as well as increases in serum glucose levels caused by octylphenol, suggest a possible role of octylphenol in modulating insulin sensitivity or energy metabolism in animals. This needs further examination.

We conclude that octylphenol activation of resistin gene expression in 3T3-L1 adipocytes is likely mediated through decreases in mRNA and protein degradation. While these actions were shown to be mediated via ERα and ERK MAPKK pathways, signaling was demonstrated to likely be independent of p38 MAPK and PI3K pathways. However, an inhibitor of ERK MAPKK modified the octylphenol-stimulated distribution of the resistin protein between intracellular and extracellular compartments of 3T3-L1 adipocytes.

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