Importance of adipocyte cyclooxygenase-2 and prostaglandin E2–prostaglandin E receptor 3 signaling in the development of obesity-induced adipose tissue inflammation and insulin resistance

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ABSTRACT: We examined the involvement of adipocyte cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2)–prostaglandin E receptor (EP3)–mediated signaling during hypertrophy and hypoxia in the development of obesity-associated adipose tissue (AT) inflammation and insulin resistance. The experiments were conducted with high-fat diet (HFD)-induced obese rats, db/db mice, human subjects, and 3T3-L1 and the human Simpson-Golabi-Behmel syndrome (SGBS) adipocytes; the groups were treated with selective inhibitors of COX-2 [celecoxib 30 mg/kg, half maximal inhibitory concentration (IC50) ≈ 0.04 µM] and EP3 (L-798106 100 µg/kg, IC50 ≈ 0.5 µM) or a short interfering RNA. There were strong, positive correlations between adipocyte COX-2 and EP3 gene expressions and the AT TNF-α and monocyte chemotactic protein-1 contents and the homeostatic model assessment for insulin resistance in HFD-induced obese rats, as well as body mass index in human subjects. Treatment with COX-2 and EP3 inhibitors significantly reversed AT inflammatory gene and protein expressions (~50%) and impaired glucose and insulin tolerance in db/db mice. COX-2 inhibition diminished the chemotaxis of adipocytes isolated from HFD rats to macrophages and T cells. Targeting inhibition of adipocyte COX-2 and EP3 during hypertrophy and hypoxia reversed the release of the augmented proinflammatory adipokines and the diminished adiponectin and also suppressed NF-κB and hypoxia-inducible factor-1alpha transcription activation. These findings suggest that adipocyte COX-2 PGE2–EP3-mediated signaling is crucially involved in the development of obesity-associated AT inflammation and insulin resistance.—Chan, P.-C., Hsiao, F.-C., Chang, H.-M., Wabitsch, M., Hsieh, P. S. Importance of adipocyte cyclooxygenase-2 and prostaglandin E2–prostaglandin E receptor 3 signaling in the development of obesity-induced adipose tissue inflammation and insulin resistance. FASEB J. 30, 000–000 (2016). www.fasebj.org

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Adipose tissue (AT) inflammation has been suggested to play a central role in the pathogenesis of many obesity-associated complications, including insulin resistance (1), type 2 diabetes (2, 3), atherosclerosis (2–4), and non-alcoholic fatty liver disease (1). However, the underlying mechanisms of this process remain elusive.

Adipocytes in an obesity setting are characterized by hypertrophy and hypoxia, and they are important sources of inflammation (5–7). This inflammation is mediated by the production of a substantial number of cytokines and chemokines, including TNF-α (3, 5), IL-6 (8), monocyte chemotactic protein-1 (MCP-1) (9), and RANTES (10). These cytokines and chemokines are crucially involved in the initiation of the adipocyte-mediated inflammatory response in obese individuals. The capacity of the constitutive and regulated release of immune mediators from adipocytes demonstrates a causal link between the biology of adipocytes and immune cells, such as macrophages and T cells. Moreover, the synergistic effect of inflamed...
Adipocytes and AT immune cell has been implicated in the development of obesity-induced insulin resistance (11, 12).

Furthermore, cyclooxygenase-2 (COX-2) is highly expressed in AT (1, 13). Our recent study demonstrated that COX-2 activation in epididymal AT is strongly correlated with the development of AT inflammation, insulin resistance, and fatty liver in high-fat diet (HFD)-induced obese rats (1). Accordingly, up-regulated COX-2-mediated signaling has also been reported to be highly expressed in the subcutaneous AT of obese humans (13). Prostaglandin E₂ (PGE₂) is an endogenous lipid mediator that is produced from arachidonic acid via COX as the rate-limiting enzyme, and it acts on 4 types of prostaglandin E receptor (EP) subtypes (EP₁–EP₄) to exert its physiologic functions. Although obesity-related AT inflammation has been speculated to be primarily a result of adipose immune cell infiltration and the secretion of inflammatory cytokines, such as TNF-α and COX-2-derived PGE₂ (14, 15), the causal role of the adipocyte COX-2-mediated signaling pathway in the initiation and maintenance of AT inflammation has not been explored. Furthermore, the role of PGE₂–EP3 signaling in tissue inflammatory responses remains controversial (16, 17).

In the present study, we tested the hypothesis that adipocyte COX-2 and the PGE₂–EP3-mediated signaling pathway in the development of adipocyte hypertrophy and hypoxia are crucially involved in the initiation of AT inflammation in obesity. It is of clinical importance to understand the underlying mechanism of obesity-induced adipose inflammation and to identify a potential therapeutic target to prevent the development of obesity-associated complications.

**MATERIALS AND METHODS**

**Animals and diets**

Male Sprague-Dawley rats and male db/db mice and their non-diabetic controls (db/+m) aged 4 wk were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The animals were housed in an animal center at the National Defense Medical Center (Taipei, Taiwan), which is certified by the Association of Assessment and Accreditation of Laboratory Animal Care. The animal care methods and protocol design followed the guidelines and manual set by the Committee of the Care and Use of Laboratory Animals in this institute.

The rats were randomly assigned to 3 groups and fed a control regular diet (CRD; 5010 LabDiet laboratory autoclavable rodent diet; PMI Nutrition, Richmond, IN, USA), a 45% high-fat-enriched diet (HFD; D12451; Research Diet, Inc., New Brunswick, NJ, USA) ad libitum, or an energy-restricted diet for 4, 8, or 12 wk. The rats on the energy-restricted HFD were maintained at a similar body weight as the rats on a CRD. A comparison of the difference between the rats with energy-restricted and ad libitum diets reflected the effect of the HFD-induced weight gain. The rats fed an HFD ad libitum were further divided into 3 subgroups cotreated with vehicle (HHa), celecoxib (a selective COX-2 inhibitor; 30 mg/kg/d, oral gavage; Pfizer Inc., New York, NY, USA) (HHa-Cel), or nimesulide (a selective COX-2 inhibitor; 30 mg/kg/d, oral gavage; Helsinn Birex Pharmaceuticals, Dublin, Ireland) (HHa-Nim) (n = 8 per group). The db/db mice were treated with celecoxib (30 mg/kg/d, oral gavage; Pfizer), a high dose of L-798106 (an EP3 antagonist; 100 μg/kg/d, oral gavage; Tocris Cookson, Bristol, United Kingdom) (EP3Ahi), a low dose of L-798106 (50 μg/kg/d) (EP3Alow) or vehicle (saline) via oral gavage for 8 wk. A similar protocol was applied for the db/+m mice with the exception of the low dose L-798106-treated group. The effective drug doses were selected based on previous studies (1, 18, 19). Following euthanasia with an overdose of pentobarbital (100 mg/kg of body weight, i.p.) at the end of the experiment, blood and AT from the epididymis were harvested.

**Glucose and insulin tolerance tests**

For the oral glucose tolerance test, the mice were fasted overnight and orally administered glucose (gavage with 2 g glucose/kg body weight). For the intraperitoneal insulin tolerance test, the mice were unfed for 4 h, followed by the administration of insulin 0.75 U/kg (Actrapid; Novo Nordisk, Bagsvaerd, Denmark); blood samples were collected at 0, 30, 60, 90, and 120 min to determine the glucose levels. Blood glucose was assayed using the glucose oxidase method. The plasma insulin level was measured using a commercial rat enzyme immunoassay kit (Mercodia AB, Uppsala, Sweden).

**Isolation of adipocytes from AT and preparation of adipocyte-derived conditioned medium**

Human visceral AT (VAT; perigastric omentum) and epididymal AT of the rats, db/db, and db/+m mice were digested with 1.5 mg/ml type VII collagenase (Sigma-Aldrich, St. Louis, MO, USA) in HBSS, which contained 5% FBS. The adipocytes and stromal vascular cells (SVCs) were isolated as previously described (10, 20). An equal amount of isolated adipocytes from each experimental group was seeded in 1.2 ml of DMEM in a humidified incubator at 37°C and 5% CO₂ for 24 h to prepare adipocyte-derived conditioned medium (CM) (21). See Supplemental Data for further details. For some part of experiments, 10 μM L-798106 was added to the adipocyte-derived CM obtained from epididymal AT of db/db mice for 24 h.

**Assessment of adipose T cells and macrophages by flow cytometry**

Flow cytometry of the rat epididymal AT-derived SVCs was performed as previously described (9, 10). CD4 or CD8 T cells were identified as CD₃-positive/CD4-positive or CD₃-positive/CD8-positive cells, respectively. For the intracellular staining of T helper (TH)1 and TH2 cells, cells were labeled with conjugated anti-IFN-α and anti-IL-4 (BD Bioscience). For the assessment of macrophage distribution in the AT, we first incubated with a primary antibody specific to CD68 (Serotec, Oxford, United Kingdom), followed by Alexa Fluor 488-labeled goat-anti-rabbit IgG (A11070; Invitrogen, Carlsbad, CA, USA), measured with a BD Cytofix/Cytoperm kit (BD Biosciences), and labeled with a BD Cytotox/CTyperm kit (BD Biosciences), and labeled with conjugated anti-IFN-γ and anti-IL-4 (BD Bioscience). For the assessment of macrophage distribution in the AT, we first incubated with a primary antibody specific to CD68 (Serotec, Oxford, United Kingdom), followed by Alexa Fluor 488-labeled goat-anti-rabbit IgG (A11070; Invitrogen, Carlsbad, CA, USA), measured with a FACScan and analyzed using CellQuest software (BD Bioscience).

**Preparation of 3T3-L1 adipocyte hypertrophy and hypoxia treated with COX-2 inhibitor and EP3 antagonist or with the gene knockdown of COX-2 and EP subtypes**

The differentiation procedure of the 3T3-L1 cell (No. CL-173, American Type Culture Collection, Manassas, VA, USA) was performed as previously described (22, 23). To mimic adipocyte hypertrophy during the development of obesity, we designed an artificially hypertrophic adipocyte model by preloading 3T3-L1...
adipocytes with 300 μM palmitate discussed elsewhere (24, 25). This palmitate concentration has been suggested as the maximum palmitate concentration without the induction of cytotoxicity for the purpose of preparing artificially hypertrophied mature adipocytes in vitro (26, 27). A subset of the palmitate-treated differentiated 3T3-L1 adipocytes was placed in a hypoxia chamber that contained 1% O2, 5% CO2, and 94% N2 for 12 h at 37°C. In our hypertrophied adipocyte model, we added palmitate at a 3T3-L1 adipocyte well-differentiated status. Briefly, 300 μM palmitate was added to the culture medium on day 5 of adipocyte differentiation for 3 d, and samples of the culture medium were cotreated with 10 μM NS-398 (a selective COX-2 inhibitor, half maximal inhibitory concentration (IC50) ≈ 1.77 μM; Tocris), L-798106 (0.2, 1, or 10 μM, IC50 ≈ 0.5 μM; Tocris), or vehicle. To prepare COX-2 knockdown cells, lentivirus-based short hairpin COX-2 (TRCN67940 or TRCN67941) and control vectors were purchased from the National RNAi Core Facility (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). The 3T3-L1 adipocytes were infected with the lentivirus for 2 d in the presence of 8 μg/ml polybrene (Sigma-Aldrich) on d 3 of differentiation.

In another part of the experiments, RNA interference was used for knocking down the expression of the EP1, EP2, EP3, and EP4 genes in mature adipocytes during the development of adipocyte hypertrophy and hypoxia. On-targetPlus SmartPool short interfering RNA (siRNA) for EP1, EP2, EP3, and EP4 genes were purchased from Dharmacon (Thermo Scientific, Lafayette, CO, USA). The siRNA was transfected into the cell by using DharmaFECT 1 transfection reagents (Thermo Scientific) according to the instructions. See Supplemental Data for further details.

Coculture with 3T3-L1 adipocytes and macrophages or activated T cells

The coculture of 3T3-L1 adipocytes with RAW 264.7 macrophages or activated T cells isolated from mouse spleens was performed in a transwell system with a 0.4 μm porous membrane (Millipore, Billerica, MA, USA) to separate the treated 3T3-L1 adipocytes from the RAW 264.7 cells (1 × 106 cells/well) (28, 29) or T cells (5 × 105 cells/well) isolated from mouse spleens as previously described (10, 30). The medium was harvested following 24 h of incubation.

Macrophage chemotaxis assay

The macrophages extracted from normal rat alveoli were resuspended in DMEM with 5% FBS as previously described (31). T cells were purified from rat spleenocytes with immunomagnetic sorting via MACS technology with anti-T cell (OX52) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described (29). Rat adipocyte-derived CM was placed in the lower well of a 24 well chemotaxis chamber and separated from isolated rat spleen activated T cells (3 × 105 cells/well) or alveolar macrophages (1 × 106 cells/well) in the top well using a transwell system as previously described (10, 30). In the in vitro study, the conditioned medium from the 3T3-L1 adipocytes treated with palmitate alone or combined with hypoxia was placed in the lower well of a 12-well chemotaxis chamber and separated from the RAW 264.7 cells (1 × 106 cells/well) in the top well using a 5-μm porous membrane (Millipore). The chemotaxis assays were performed as previously described for the RAW264.7 macrophages (32).

Preparation of SGBS adipocyte hypertrophy and hypoxia with EP3 antagonist and gene knockdown

The human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes were a gift from Dr. Martin Wabitsch (Ulm University), and they were induced to differentiate to mature adipocytes as previously described (33). To mimic adipocyte hypertrophy during the development of obesity, we designed a hypertrophied adipocyte model by preloading SGBS adipocytes with palmitate. Briefly, 300 μM palmitate was added to the culture medium on d 10 of adipocyte differentiation for 3 d, and samples of the medium were treated with 100 nM recombinant human PGE2 (PeproTech, Rocky Hill, NJ, USA) or vehicle. In some part of experiments, 10 μL of L-798106, an EP3 antagonist was added to the medium. Besides, RNA interference was also used for knocking down the expression of the EP3 gene in mature adipocytes during the development of adipocyte hypertrophy and hypoxia. On-TargetPlus SmartPool siRNA for EP3 gene were purchased from Dharmacon (Thermo Scientific). See Supplemental Data for further details.

RNA isolation and gene expression analysis

The procedures for RNA purification and gene expression and the related analyses were performed as suggested by a previous study (18). RNA was isolated separately from rat epididymal adipocytes, human visceral adipocytes, 3T3-L1 adipocytes, and SGBS adipocytes using Trizol reagent (Applied Biosystems, Foster City, CA, USA). RNA was quantitated and reverse transcribed with a high capacity cDNA reverse transcription kit (Applied Biosystems). Real-time quantitative PCR was performed using TaqMan gene expression assays and the 7500 real-time PCR system (Applied Biosystems). The TaqMan probes used in this study are listed in Supplemental Table 1.

Measurement of adipokine levels

The levels of MCP-1 and RANTES in rat epididymal adipocyte CM were assessed using rat MCP-1 (Antigenex America Inc., Melville, NY, USA) and RANTES (PeproTech, Rocky Hill, NJ, USA) ELISA kits. The mouse MCP-1, RANTES, IL-6, TNF-α, and PGE2 proteins were quantified in the 3T3-L1 adipocyte supernatant and mouse epididymal adipocyte CM using commercial cytokine-specific ELISA kits (R&D Systems, Minneapolis, MN, USA) and cAMP levels measured by a Cyclic AMP EIA Kit (Cayman Chemicals, Ann Arbor, MI, USA). The human MCP-1 and IL-6 proteins were quantified in supernatant obtained from adipocytes isolated from human VAT and SGBS adipocytes using commercial cytokine-specific ELISA kits (R&D Systems) following the manufacturer’s instructions.

Western blot analysis

For the total protein extraction, the SGBS adipocytes were lysed in RIPA buffer that contained total protease inhibitor and phosphatase inhibitor (Invitrogen), and the lysates were subsequently centrifuged at 4°C and 12,000 g for 15 min. The protein concentration was determined using a protein assay kit (#500-0006; Bio-Rad, Hercules, CA, USA). An equal amount of sample was separated via SDS-PAGE and transferred to PVDF membranes (Millipore). An immunoblot analysis was performed with the indicated primary antibodies as follows: anti-p65 (#4764; Cell Signaling, Beverly, MA, USA) and anti-p-p65 (#8242; Cell Signaling). Following incubation with the corresponding secondary antibody, the reactive bands were detected using an ECL kit (Thermo Fisher Scientific, Rockford, IL, USA) with an ImageQuant LAS 4000 mini (GE Healthcare, Waukesha, WI, USA). The immunoblots were quantified using ImageQuant TL 7.0 software (GE Healthcare, Waukesha, WI, USA) and expressed as a ratio of the specific protein phosphorylation to the corresponding protein or specific protein to β-actin.

Statistical analysis

Statistical analysis was performed using repeated measurements of ANOVA followed by the Bonferroni test. Correlations were calculated using the Pearson correlation test. A value of P < 0.05
was considered a significant difference between means. Values are expressed as the means ± SEM.

RESULTS

Metabolic and biochemical characteristics of experimental rats and isolated adipocyte inflammatory responses from rat and human VAT

The average body weight was significantly increased in the HFa, HFa-Cel, and HFa-Nim rats compared with the CRD rats in a time-dependent manner (Fig. 1A). The increases in the fasting blood glucose, insulin levels, and the homeostatic model assessment for insulin resistance (HOMA-IR) in the HFa animals were significantly suppressed in the animals cotreated with COX-2 inhibitor for 8 and 12 wk (Fig. 1B–D). There were no differences in these parameters between the CRD and energy-restricted HFD rats. In the adipocytes isolated from rat epididymal AT, the adipocyte gene expression levels of COX-2 and EP3 were highly correlated with the AT TNF-α and MCP-1 levels, as well as the HOMA-IR value in the rats and the body mass index (BMI) in the human subjects (Table 1). The augmented mRNA expression levels of COX-2, MCP-1, TNF-α, RANTES, and C-C chemokine receptor (CCR)5 in the isolated epididymal adipocytes of the HFa rats were significantly attenuated following COX-2 inhibition in a time-dependent manner (Fig. 1E–I). The enhanced adipocyte hypoxia-inducible factor-$\alpha$-1alpha (HIF-$\alpha$) mRNA level in the HFa rats was also consistently suppressed with COX-2 inhibition at the end of wk 12 (Fig. 1F). These findings suggested that adipocyte COX-2-mediated signaling was causally linked with the transcript levels of MCP-1, TNF-$\alpha$, RANTES, CCR5, and HIF-$\alpha$ in the epididymal adipocytes isolated from the experimental rats. Moreover, the human obese subjects (age 30.7 ± 4.1 yr; BMI 43.7 ± 2.2 kg/m²) had significantly increased mRNA transcript levels of HIF-$\alpha$, MCP-1, and TNF-$\alpha$ but decreased adiponectin levels in the adipocytes isolated from VAT compared with the lean controls (age 44.5 ± 7.2 yr; BMI 23.7 ± 0.4 kg/m²; Supplemental Fig. 1A). The release of IL-6 and MCP-1 from the isolated adipocytes of obese human VAT was significantly suppressed following cotreatment with NS-398 (Supplemental Fig. 1B).

Chemokine levels in CM derived from isolated rat epididymal adipocytes and the COX-2-mediated effects on macrophage and T-cell chemotaxis and AT infiltration

The MCP-1 and RANTES levels were significantly increased in the CM derived from the isolated epididymal adipocytes of the HFa rats and were reversed in the samples from the HFa rats cotreated with the COX-2 inhibitor (Fig. 2A, B). Moreover, the addition of NS398 into the HFa adipocyte-derived CM also significantly suppressed the increased MCP-1 and RANTES release (Fig. 2A, B). The macrophage and T-cell migration were significantly increased while coincubated in CM derived from HFa adipocytes, but not in those from the rats with chronic COX-2 inhibition (HFa-Cel and HFa-Nim) or HFa adipocytes treated directly with NS-398 (Fig. 2C, D). The augmented PGE$_2$ level in the isolated epididymal adipocytes of the HFa animals was significantly attenuated following COX-2 inhibition (Fig. 2E). The macrophages isolated from SVCs of HFa epididymal AT exhibited a significant increase in the CD68-expressing cells (mean fluorescence intensity) compared with those of the CRD but not with those from the HFa-Cel and HFa-Nim rats at the end of the experiment (Fig. 2F). Moreover, we sought to examine AT macrophage heterogeneity by measuring adipose M1 and M2 macrophage markers using real-time PCR analysis. The data indicated that the expression of proinflammatory genes, such as NOS2 and TNF-$\alpha$, exhibited primarily in M1 macrophages were significantly decreased following COX-2 inhibition (Fig. 2G). By contrast, the diminished expression of the M2-specific genes IL-10 and macrophage galactose N-acetylgalactosamine specific lectin 1 in HFa was reversed with COX-2 inhibition (Fig. 2H). Chronic COX-2 inhibition significantly suppressed the increases in the adipose CD4* and CD8* T cells obtained from the HFa rats (Fig. 2I, J). The SVCs isolated from the experimental group had negligible intracellular levels of IFN-$\gamma$ and IL-4 proteins following brefeldin A administration to block secretion. However, stimulation with PMA and ionomycin resulted in an increase in the number of T$_\text{H}$ cells in the SVCs isolated from the HFa rats, which was diminished in the rats cotreated with COX-2 inhibitor (Fig. 2K). Accordingly, the increase in the T$_\text{H}$. T$_\text{M}$. cell ratio in HFa was also reversed when combined with COX-2 inhibitor treatment for 12 wk (Fig. 2L).

Characterization of COX-2 knockdown in 3T3-L1 adipocytes

A loss-of-function study was performed in 3T3-L1 adipocytes using lentivirus-derived shRNA sequence variants that targeted COX-2. Palmitate and hypoxic treatment resulted in augmented gene expression of COX-2 (Fig. 3A), as well as MCP-1, TNF-$\alpha$, HIF-$\alpha$, and NF-$\kappa$B p50 (Fig. 3C–F). Transfection with COX-2 shRNA or treatment with NS-398 markedly reversed the increase in the adipocyte COX-2 mRNA level and PGE$_2$ production (Fig. 3A, B), as well as the previously described gene expressions in the 3T3-L1 adipocytes treated with palmitate alone or combined with hypoxia (Fig. 3C–F). The suppressive effect of COX-2 inhibition was also noted in the release of MCP-1, RANTES, IL-6, and TNF-$\alpha$ into the medium from the palmitate- and hypoxia-treated cells (Fig. 3G–J).

Consistently, the migration of RAW264.7 macrophages was significantly increased when coincubated with palmitate-treated adipocytes and further augmented when combined with hypoxic treatment. The enhanced macrophage migration was significantly suppressed when coincubated with COX-2 shRNA or NS-398-pretreated 3T3-L1 adipocytes, especially following treatment with both palmitate and hypoxia (Fig. 4A, B). Additionally, the levels of MCP-1, IL-6, and TNF-$\alpha$ were significantly increased in the coculture medium of the RAW264.7 cells with palmitate-treated 3T3-L1 adipocytes and further increased when combined with hypoxic treatment. These
increases were significantly suppressed when 3T3-L1 adipocytes were pretreated with COX-2 shRNA transfection or the COX-2 inhibitor (Fig. 4C–E).

The levels of RANTES, IL-6, and TNF-α in the medium were markedly increased when the T cells were coincubated with 3T3-L1 adipocytes treated...
with palmitate alone or combined with hypoxia. These increases, especially regarding the RANTES level, were attenuated when the T cells were coincubated with 3T3-L1 adipocytes with COX-2 knockdown or inhibition (Fig. 4–H). Taken together, these data demonstrated that COX-2 activation during the development of adipocyte hypertrophy and hypoxia was important to recruit and interact with adipose immune cells to amplify the inflammatory responses in AT.

**Adipocyte PGE<sub>2</sub> receptor expression and the suppressive effect of EP3 antagonists on gene expression and the release of inflammatory adipokines from human and rat primary adipocytes and palmitate- and hypoxia-treated 3T3-L1 cells**

COX-2 activation results in the conversion of arachidonic acid to PGE<sub>2</sub>, which can subsequently bind to 1 of 4 G-protein linked receptors (EP1–EP4) (34). Of the 4 prostaglandin E receptor subtypes, EP3 was the only subtype to exhibit consistently increased mRNA expression in the adipocytes isolated from the HFD-induced obese rats (Fig. 5A), obese humans (Fig. 5B), 3T3-L1 adipocytes (Fig. 5C), and human SGBS adipocytes treated with palmitate alone or combined with hypoxia (Fig. 5D). Treatment with the EP3 antagonist L-798106 significantly attenuated the levels of MCP-1, RANTES, and TNF-α from the isolated rat epididymal adipocytes of the HFa rats (Fig. 5E–G). The specific role of EP3 in adipocytes was studied by siRNA in 3T3-L1 adipocytes during the development of hypertrophy and hypoxia. The knockdown of EP3 but not other EP receptors decreased the elevated mRNA expression of MCP-1 and IL-6, moderately increased the diminished mRNA expression of adiponectin in the palmitate- and hypoxia-treated 3T3-L1 adipocytes (Fig. 5H–J). L-798106 treatment significantly attenuated the secretion of MCP-1, RANTES, and TNF-α in the palmitate- and hypoxia-treated 3T3-L1 adipocytes to a similar level as the adipocytes treated with NS-398 (Supplemental Fig. 2). Furthermore, the transcript levels of MCP-1, HIF-1α, and TNF-α into medium were also diminished in the adipocytes treated with L-798106 or NS-398 (data not shown).

### TABLE 1. Pearson correlation coefficients between the epididymal adipocyte COX-2 and EP3 mRNA levels and the BMI in the human subjects and the HOMA-IR, epididymal adipose TNF-α, and MCP-1 contents in the HFD-induced obese rats

<table>
<thead>
<tr>
<th>Species</th>
<th>Adipocyte COX-2 mRNA level</th>
<th>Adipocyte EP3 mRNA level</th>
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<tr>
<td></td>
<td>Coefficient ($r^2$)</td>
<td>P value</td>
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<tr>
<td><strong>Human</strong></td>
<td></td>
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<tr>
<td>BMI</td>
<td>0.74</td>
<td>&lt;0.05</td>
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<tr>
<td><strong>Rat</strong></td>
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<tr>
<td>HOMA-IR</td>
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<td>&lt;0.05</td>
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<td>Adipose TNF-α (pg/g tissue)</td>
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<td>&lt;0.05</td>
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<tr>
<td>Adipose MCP-1 (pg/g tissue)</td>
<td>0.73</td>
<td>&lt;0.05</td>
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**Effects of a COX-2 inhibitor and EP3 antagonist on systemic insulin resistance and AT inflammation in db/db mice**

To delineate the suppressive effects of the selective COX-2 inhibitor and EP3 antagonist on systemic insulin resistance and AT inflammation, we administered celecoxib and an EP3 antagonist (50 and 100 μg/kg/d, orally) to the db/db mice and their nondiabetic controls (db/+m) for 8 wk. The mean body weight (Fig. 6A), tissue weights (Supplemental Fig. 3A), and food intake (Supplemental Fig. 3B) were not different among the db/db and db/db-Cel, db/db-EP3Ahi, and db/db-EP3Alow mice. The increased fasting blood glucose levels in the db/db mice were significantly suppressed in the mice cotreated with COX-2 inhibitor and a high dose of EP3 antagonist for 8 wk (Fig. 6B). However, celecoxib and the EP3 antagonist did not significantly affect the fasting insulin level (Fig. 6C). The insulin sensitivity, which was indicated by the HOMA-IR values, was significantly improved in the db/db-Cel and db/db-EP3Alow mice compared with the db/db mice (Fig. 6D). Consistently, the oral glucose and intraperitoneal insulin tolerance test results exhibited similar trends (Fig. 6E–H). Nevertheless, insulin sensitivity was not different among the db/+m, db/+m-Cel, and db/+m-EP3Ahi mice.

The mRNA levels of MCP-1, RANTES, IL-6, and TNF-α were significantly increased in the adipocytes isolated from the epididymal AT of the db/db mice compared with the db/+m mice (Fig. 6I–L). Oral administration of the COX-2 inhibitor or EP3 antagonist, especially a high dose, significantly suppressed these increased proinflammatory gene expressions in the adipocytes isolated from the epididymal AT of the db/db mice (Fig. 6I–L). Furthermore, the diminished adiponectin mRNA levels identified in the isolated epididymal adipocytes of the db/db mice were also reversed in the celecoxib, EP3Ahi, and EP3Alow mice (Fig. 6M). These previously described gene expressions in the epididymal AT exhibited similar trends as the expressions in the epididymal adipocytes of the experimental mice; however, the changes were more significant (data not shown). Accordingly, the increased IL-6 and TNF-α levels and the diminished adiponectin level in the CM derived from the db/db adipocytes were significantly reversed in those derived from the db/db mice with the COX-2 inhibitor or EP3 antagonist treatment (Fig. 6N–P). The addition of the EP3 antagonist L-798106 remarkably and directly
Figure 2. Chemokine production and chemotactic effects of rat adipocyte-CM on macrophages and T cells and the accumulation and genetic markers of macrophages and distribution of T-cell subtypes in the AT of experimental rats. A) MCP-1 and (B) RANTES levels in the CM of the adipocytes isolated from the CRD, HFr, HFa, HFa-Cel, HFa-Nim, and HFa + NS398 animals following a 12-wk observation period. Isolated HFa + NS398 and HFa adipocytes incubated with 10 μM NS398. The chemotaxis of (C) macrophages and (D) T cells was examined using adipocyte-CM derived from isolated adipocytes from the epididymal fat of the previously described groups following a 12-wk observation period. E) PGE$_2$ level in adipocytes isolated from the previously described groups following a 12-wk observation period. (F) Analysis of CD68$^+$ cells in the SVCs of the epididymal fat pads from the previously described groups at the end of a 12-wk observation period via flow cytometry. (G) mRNA expression of the M1 macrophages markers NOS2 and TNF-α and (H) mRNA expression of the M2 macrophage markers IL-10 and macrophage galactose N-acetyl-galactosamine-specific lectin 1. Quantification of (I) CD3$^+$ CD4$^+$, (J) CD3$^+$ CD8$^+$, and (K) Th1 cells in the epididymal fat pads of rats with 4-, 8-, or 12-wk observation periods and (L) the Th1:Th2 ratio in the 12-wk observation period. Data are presented as the total number of cells ($1 \times 10^4$) for each T-cell subtype. The data represent the means ± SEM. n = 8. *P < 0.05 vs. CRD; †P < 0.05 vs. HFa at the corresponding time.
Figure 3. mRNA and protein levels following COX-2 gene knockdown and selective COX-2 inhibition. A) COX-2 mRNA expression and (B) PGE2 production in culture medium of 3T3-L1 adipocytes treated with COX-2 shRNA transfection or NS-398 (10 μM), combined with palmitate alone or palmitate plus hypoxia. The gene expression levels of MCP-1 (C), TNF-α (D), HIF-1α (E), and NF-kB p50 (F), as well as the protein levels of MCP-1 (G), RANTES (H), IL-6 (I), and TNF-α (J) in the 3T3-L1 adipocytes pretreated with COX-2 shRNA or NS-398 (10 μM) followed by treatment with palmitate alone or palmitate plus hypoxia. Con, control; pal, 0.3 mM palmitate treatment for 3 d; pal + hypoxia, 300 μM palmitate treatment for 3 d plus hypoxia (1% O2) for 12 h prior to the end of the experiment. The data represent the means ± SEM. n = 6. *P < 0.05 vs. con; †P < 0.05 vs. pal; ‡P < 0.05 vs. pal + hypoxia.
reversed the changes in the IL-6, TNF-α and adiponectin levels in the db/db adipocyte-CM (Fig. 6N–P).

**EP3 signaling in human adipocytes**

Both the COX-2 and EP3 mRNA levels in the adipocytes isolated from the human VAT were positively correlated with the increases in the BMI of the human subjects ($P < 0.05$) (Table 1). The role of EP3 in adipocytes was first studied by EP3 siRNA and EP3 antagonist, L-798106 in SGBS adipocytes during the development of adipocyte hypertrophy and hypoxia. As shown in Supplemental Fig. 4, both EP3 siRNA and L-798106 treatment decreased mRNA expression of MCP-1 and IL-6, especially IL-6 and increased mRNA expression of adiponectin in the...
Figure 5. mRNA levels of adipocyte PGE2 receptors in adipocytes isolated from rats, adipocytes isolated from human subjects, 3T3-L1 adipocytes, and human SGBS adipocytes, in addition to the MCP-1, RANTES, and TNF-α levels in the CM of isolated rat adipocytes treated with or without L-798106. The EP1, EP2, EP3, and EP4 mRNA levels in the adipocytes isolated from the epididymal fat of HFD-induced obese rats (A) (n = 8), the visceral fat of obese humans (B) (n = 4–6), the palmitate-treated or palmitate plus hypoxia-treated 3T3-L1 adipocytes (C) (n = 6), and the human SGBS adipocytes (D) (n = 6). *P < 0.05 vs. con. The (E) MCP-1, (F) RANTES, and (G) TNF-α levels were measured. Values are expressed as the means ± SEM. n = 8 per group. *P < 0.05 vs. CRD; †P < 0.05 vs. HFa at the corresponding time point. The (H) MCP-1, (I) IL-6, and (J) adiponectin mRNA levels in 3T3-L1 adipocytes treated with either EP1, EP2, EP3, and EP4 siRNA, combined with palmitate alone or palmitate + hypoxia. Con, control; pal, 300 μM palmitate treatment for 3 d; pal + hypoxia, 3 μM palmitate treatment for 3 d plus hypoxia (1% O2) for 12 h prior to the end of the experiment. The values are expressed as the means ± SEM. n = 6 per group. *P < 0.05 vs. con; †P < 0.05 vs. pal; §P < 0.05 vs. pal + hypoxia.
palmitate- and hypoxia-treated SGBS adipocytes. We also performed some part of experiment in the presence of PGE2 in SGBS adipocyte. PGE2 administration significantly up-regulated the gene expression of EP3 and augmented the HIF-1α, MCP-1, and IL-6 expression and NF-κB p65 phosphorylation and down-regulated the gene expression of adiponectin, which were significantly suppressed via treatment with EP3 siRNA (Fig. 7A–G).

**DISCUSSION**

AT inflammation has been speculated to play a crucial role in the development of obesity-associated complications. However, the pathogenesis of AT inflammation remains unclear. This study aimed to clarify the role of endogenous COX-2 and PGE2–EP3-mediated signaling during the development of adipocyte hypertrophy and hypoxia in the etiology of obesity-associated AT inflammation and systemic insulin resistance. The present findings demonstrated strong correlations between adipocyte COX-2 and EP3 gene expressions and obesity-induced AT inflammation, as well as systemic insulin resistance in the rats fed an HFD and the BMI in human subjects. Both COX-2 and EP3 inhibition significantly reversed AT inflammation and improved glucose homeostasis and insulin sensitivity in the diet-induced and genetically obese rodent models, respectively. The ex vivo and in vitro studies suggest that the activation of adipocyte COX-2 and PGE2/EP3 signaling during hypertrophy and hypoxia substantially contributes to not only the increase in proinflammatory adipokine production but also the decrease in...
adiponectin production mainly via the activation of the NF-κB-mediated inflammatory pathway (Fig. 8). This response would further contribute to the development of adipose immune cell infiltration and AT inflammation and subsequently systemic insulin resistance.

Adipose immune cell infiltration has been demonstrated to be crucially involved in the pathogenesis of AT inflammation (9, 35–37). However, the initial role and the mechanism that underlies adipocyte hypertrophy and hypoxia in the development of the inflammatory response in AT remain unknown. The present study provides the first compelling evidence that during the development of adipocyte hypertrophy and hypoxia, adipocyte COX-2 activation is crucially involved in adipocyte-derived inflammatory cytokine and chemokine production. The COX-2 mediated releases of adipokines would further facilitate the recruitment of immune cells and escalate the inflammatory response in AT. Accordingly, COX-2 activation (rather than COX-1) in VAT, rather than subcutaneous AT, skeletal muscle, or liver, has been demonstrated to strongly correlate with obesity-induced insulin resistance and AT inflammation in a rat model of obesity (1, 18). COX-2 inhibition also increases AT expression of the anti-inflammatory adipokine adiponectin in a rat model of chronic inflammation (38). The diminishment of macrophage-dependent inflammation has recently been demonstrated in the AT of COX-2-deficient mice combined with a significant reduction in the body weight and percent body fat (39). Taken together, our current findings further clarify that adipocyte COX-2 mediated signaling, especially in epididymal AT, in the development of obesity-associated hypertrophy and hypoxia not only initializes but also significantly promotes the inflammatory reaction in AT.

PGE2 has been demonstrated to be involved in inflammation and inflammatory immune diseases via distinct receptors (41). Moreover, the role of EP3-mediated signaling in tissue inflammatory reactions varies across different experimental models. For example, PGE2 has been demonstrated to elicit vascular permeability and edema formation via EP3 on mast cells (42) and was involved in stroke injury through the enhancement of...
inflammatory and apoptotic reactions in the ischemic cortex (16). A recent study has indicated that the expression of EP3 was higher than other receptors, such as EP1 (undetectable), as well as EP2 and EP4, examined in the AT of mice (43). However, no report has specifically focused on its role in adipocytes during the development of obesity-associated AT inflammation and insulin resistance. In the present study, we demonstrated that EP3 was up-regulated in the obese primary adipocytes isolated from the HFD-induced obese rats and human subjects, as well as 3T3-L1 and human SGBS adipocytes during the development of adipocyte hypertrophy and hypoxia. Furthermore, in the genetically obese db/db mice, treatment with the EP3 antagonist significantly reversed the obesity-induced AT inflammation and systemic insulin resistance similar to treatment with the selective COX-2 inhibitor. Taken together, it is suggested that the augmentation of COX-2 and PGE2 EP3 mediated signaling in obese adipocytes plays a crucial role in the development of obesity-associated AT inflammation and subsequent insulin resistance.

The effects of the EP3 antagonist (both low and high doses) on the prevention of AT inflammation in db/db mice are similar compared with mice treated with a COX-2 inhibitor. MCP-1 and RANTES have been reported to be important chemoattractants for immune cell infiltration (10, 35). Both the COX-2 inhibitor and EP3 antagonist attenuated the gene expressions of MCP-1 and RANTES in the adipocytes isolated from the epididymal AT of the diet-induced and genetically obese rodent models. The blockade of both COX-2 and EP3-mediated signaling significantly suppressed MCP-1 and RANTES release from the palmitate- and hypoxia-treated 3T3-L1 adipocytes, which mimic adipocyte hypertrophy and hypoxia in the state of obesity. Accordingly, both adipose T-cell and macrophage infiltration have been considered to be the crucial components in the development of obesity-related AT inflammation.
Obesity

Adipocyte hypertrophy and hypoxia

\[\text{UP} \quad \text{COX-2-PGE2-EP3 signaling}\]

\[\text{UP} \quad \text{Nfkb}\]

Pro-inflammatory adipokines: TNF-\(\alpha\), IL-6, RANTES, MCP-1

Anti-inflammatory adipokines: Adiponectin

- Adipose tissue immune cell infiltration
- Adipose tissue inflammation
- Insulin resistance

**Figure 8.** Schematic presentation of adipocyte COX-2 and PGE\(_2\)/EP3-mediated signaling pathways linked to AT inflammation and insulin resistance.

and these factors were significantly reversed when treatment was combined with chronic COX-2 inhibition. These data further support that adipocyte COX-2 and PGE\(_2\)-EP3-mediated signaling in the state of obesity occurs, at least in part, through increasing the production of adipocyte chemoattractants, such as MCP-1 and RANTES, to promote adipose immune cell infiltration.

From a mechanistic aspect, EP3 knockdown via shRNA diminished the increased HIF1-\(\alpha\) mRNA level and the NF-\(\kappa\)B activity in the SGBS adipocytes treated with palmitate alone or combined with hypoxia. Accordingly, AT hypoxia in the development of obesity directly promotes chronic AT inflammation primarily through the activation of transcription factors (NF-\(\kappa\)B and HIF-1\(\alpha\)) in adipocytes and macrophages (44). Moreover, an increase in the constitutive NF-\(\kappa\)B activity in 3T3-L1 adipocytes has been demonstrated to potentially lead to adipokine overproduction during adipocyte hypertrophy (24). Thus, our observation suggests that the activation of NF-\(\kappa\)B and HIF-1\(\alpha\) may be primarily responsible for the EP3-mediated inflammatory reaction in the development of adipocyte hypertrophy and hypoxia.

It has also been demonstrated that the gene expressions of all known EP receptor subtypes are exhibited in mouse epididymal AT. Moreover, the mRNA levels of EP2, EP3, and EP4 were increased in mouse epididymal AT in EP4\(^{+/+}\) mice fed an HFD (45). The present study demonstrated that only EP3 was significantly and consistently up-regulated in the obese primary adipocytes isolated from the HFD-induced obese rats and human subjects, as well as the murine and human cell lines during the development of adipocyte hypertrophy and hypoxia. The discrepancy between the previous and current findings may, at least in part, attribute to the different sample sources from AT and adipocytes, respectively, which would not affect our data interpretation. In addition, Tang et al. reported that PGE\(_2\)-EP4 signaling plays an important anti-inflammatory role in the regulation of AT inflammation (45). Interestingly, our current data also demonstrated that the up-regulation of EP3 was accompanied by the down-regulation of EP4 in the obese primary adipocytes isolated from the HFD-induced obese rats and human subjects. Therefore, it is possible that the counterbalance between adipocyte EP4 and EP3 is important in the pathogenesis of AT inflammation especially in the late stage of obesity. However, this finding requires further investigation.

Although the present result indicated the importance of COX-2–PGE\(_2\)–EP3-dependent mechanism in the development of insulin resistant phenotype in these mice, it could not completely reverse the pathogenesis of insulin resistance in this obese model. The pathway other than COX-2–PGE\(_2\)–EP3-mediated signaling could also be involved in this db/db-associated insulin resistant phenotype. For example, obesity-associated oxidative stress induced damage also plays a significant part in the development of insulin resistance, \(\beta\)-cell dysfunction, impaired glucose tolerance, and type 2 diabetes mellitus (46). In addition, several inflammatory signaling other than COX-2–PGE\(_2\)–EP3-mediated pathway have been reported to associate with obesity and the development of insulin resistance such as SOCS proteins (47), TLR4 (48) and JNK1, a serine/threonine protein kinase (49).

Our present result indicated the beneficial effect of COX-2 inhibitor and EP3 antagonist on obesity-induced dysregulation of glucose metabolism mainly by improving insulin resistance using the oral glucose tolerance test and intraperitoneal insulin tolerance test. However, the action of these treatments on improving obesity-associated \(\beta\)-cell dysfunction could not be completely excluded. It is well established that increased PGE\(_2\) production and EP3 expression in islets negatively regulate insulin secretion and mediate \(\beta\)-cell dysfunction in diabetic mouse. Moreover, suppression of EP3 signaling by L-798106 specifically enhanced \(\beta\)-cell function in diabetic human islets (50).

Nevertheless, Fain et al. reported that exogenous PGE\(_2\) could stimulate lipolysis and adipocyte leptin production under dexamethasone treatment. In addition, these authors also indicated that the endogenous formation of PGE\(_2\) does not regulate basal lipolysis and leptin release (51). Accordingly, our observation suggests that the inhibition of COX-2- and PGE\(_2\)-EP3-mediated signaling does not appear to affect the intracellular cAMP levels and subsequent lipolysis (43) during the development of adipocyte hypertrophy and hypoxia (data not shown). These findings indicate that the potential effects of COX-2 and EP3-mediated lipolysis were not activated during adipocyte hypertrophy and hypoxia. It is suggested that the PGE2 EP3-mediated inflammatory response in fat is independent of its effect on lipid metabolism.

The COX-2 and EP3 mRNA levels were positively correlated with the BMI in the human subjects, which thereby provides evidence for the association between adipocyte COX-2 and PGE\(_2\)-EP3 signaling and obesity-related AT inflammation in human subjects. However, we could not obtain sufficient VAT for the lean controls; thus, we were unable to compare the COX-2 and
EP3-mediated effects on adipokine production in isolated adipocytes from VAT between obese and lean subjects. This concept is intriguing and should be further investigated.

In summary, adipocyte COX-2 activation during the development of hypertrophy and hypoxia plays a pivotal role in the initiation and maintenance of obesity-associated AT inflammation primarily through PGE2–EP3-mediated signaling to activate NF-κB and the HIF-1α-mediated inflammatory pathway, which subsequently affects the development of systemic insulin resistance. Thus, adipocyte COX-2-mediated PGE2–EP3 signaling could be a promising therapeutic target for the prevention and treatment of obesity-associated insulin resistance and diabetes mellitus.

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