M S . Thesis

Ginsenoside Rc Inhibits Urban Particulate Matter 2.5-induced Cyclooxyenase-2 Expression in 3T3-L1 Mouse Preadipocytes and Human Adipose Stem Cells

Graduate School, Keimyung University
School of Medicine

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Nivethasri Lakshmana Perumal

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1. Introduction

1.1. Background of The Study:

In these years, particulate matter (PM) has gained attention and becoming a significant environmental and global health problem due to its adverse impact on human health and global warming (1). PM is categorized into PM0.1, PM2.5, and PM10, which refer to the aerodynamic diameter of fine particles in it less than or equal to 0.1, 2.5, and 10 μm, respectively (2). Among those, PM2.5 is found to be more harmful to human health and hard to control (3).

PM2.5 causes human diseases by inducing an inflammatory response, production of reactive oxygen species (ROS), and autophagy and apoptosis (4). Cyclooxygenase-2 (COX-2) is an inflammatory enzyme whose expression is highly induced in many cells upon exposure to external and internal stimuli (5). Notably, recent *in vivo* and *in vitro* studies have introduced that PM2.5 exposure induces high COX-2 expression in HaCaT human epidermal keratinocytes (9) and RAW264.7 mouse macrophages (10). Moreover, there is evidence that PM2.5 exposure in rodents causes oxidative stress, inflammation, and insulin resistance in white adipose tissue (WAT), resulting in macrophage mobilization to WAT, and increase of the expression and phosphorylation levels of c-Jun N-terminal kinase (JNK), nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) (11). Preadipocytes and differentiated adipocytes are the primary type of cells present in WAT and they secrete an array of adipocytokines, immunomodulatory, and

inflammatory mediators (12). To date, PM2.5 regulation of COX-2 expression in white (pre)adipocytes is not fully understood. Assuming that COX-2 is an inflammatory enzyme and obesity is defined as chronic inflammation, any natural substance inhibiting PM2.5-induced COX-2 expression in white (pre)adipocytes is regarded as a potential anti-obesity agent.

Ginsenosides are the main active compounds in Panax ginseng C.A. Mayer which have been used as a folk medicine to treat various symptoms in Asia, including Korea (13). Mounting evidence indicates that ginsenosides have sedative, neuroprotective, anti-stress, anti-cancerous, and anti-inflammatory effects (14). However, until now, ginsenosides regulation of PM2.5-induced COX-2 expression in (pre)adipocytes is not reported. In this study, I investigated whether the exposure of urban PM2.5 (PM2.5U), a standardized PM2.5 product with a mixture of liquid and solid particles in the atmosphere, leads to up-regulation of COX-2 expression in undifferentiated and differentiated adipocytes of 3T3-L1 mouse preadipocytes and human adipose stem cells (hASCs), and ginsenosides (Rc, Ro, and Rd) inhibit it. Here I report firstly that PM2.5U exposure at 50 μg/mL rapidly induces COX-2 expression by activating JNK-1/2 in undifferentiated and differentiated adipocytes of 3T3-L1 cells or hASCs, but ginsenoside Rc at 30 μM strongly inhibits it.

1.2. Aim of The Study:

1. To investigate whether PM2.5U exposure induces COX-2 expression in undifferentiated and differentiated adipocytes of 3T3-L1 cells or hASCs.

2. To delineate molecular and signaling mechanisms underlying PM2.5U-induced COX-2 expression in undifferentiated and differentiated adipocytes of 3T3-L1 cells or hASCs.

3. To check whether ginsenoside Rc attenuates PM2.5U-induced COX-2 expression in undifferentiated and differentiated adipocytes of 3T3-L1 cells or hASCs.

2. Materials and Methods

2.1. Cell Culture:

3T3-L1 mouse white preadipocytes (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagles' Medium (DMEM) (Welgene, Daegu, Korea) containing 10% heat-inactivated fetal calf serum (FCS) (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Welgene) at 37℃ in a humidified atmosphere of 5% CO2. The adipocyte differentiation of 3T3-L1 preadipocytes was induced by changing the medium to DMEM containing 10% heat-inactivated fetal bovine serum (FBS) (Welgene), 0.5 mM IBMX (M) (Sigma), 0.5 μM dexamethasone (D) (Sigma), and 5 μg/mL insulin (I) (Sigma) for 2 days. The first differentiation medium was replaced with DMEM supplemented with 10% FBS and 5 μg/mL insulin for additional 3 days. The cells were further fed with DMEM containing 10% FBS for3 days. hASCs were freshly isolated from the abdominal subcutaneous adipose tissue of a female patient admitted to Keimyung University Dongsan Hospital (KUDH), Daegu, Republic of Korea. The Ethics Committee of KUDH approved the study protocol (No. 2021-02-063-012), and informed consent was obtained from the patient. hASCs isolated were cultured in DMEM/Nutrient Mixture F-12 (DMEM/F-12) media supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.25 μg/mL fungizone. The adipocyte differentiation of hASCs was induced by changing the medium to adipocyte differentiation medium (DM-2) (Zenbio, Durham, NC, USA) for 7 days. The medium was changed, and cells were fed with adipocyte maintenance medium (AM-1) (Zenbio) for 5 to 7 days.

2.2. Materials:

PM2.5U was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). PM2.5U was dissolved in DMSO, prepared in 25 μg/mL stock solution, and stored at 4℃ before use. Ginsenosides Rc, Rd, and Ro were purchased from SelleckChem (Houston, TX, USA). Primary antibodies for phosphorylated (p)-extracellular signal-regulated kinase (ERK)-1/2, total (T)-ERK-1/2, p-JNK-1/2, T-JNK-1/2, p-p38 mitogen-activated protein kinase (MAPK), T-p38 MAPK, and inhibitory kappa B-α (IκB-α) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). An anti-COX-2 polyclonal antibody was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). TNF-α was purchased from PeproTech (Cranbury, NJ, USA). Anti-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Cell Count Analysis:

Undifferentiated and differentiated adipocytes of 3T3-L1 cells or hASCs that were treated without or with PM2.5U in the absence or presence of ginsenosides (Rc, Rd, Ro) or SP600125, a pharmacological inhibitor of JNK-1/2 at the indicated concentrations and times were stained with 0.4% trypan blue dye (Gibco, Grand Island, NY, USA). Based on the concept that lives cells have intact cell membranes that cannot be stained with trypan blue dye, the number of surviving cells was counted using a phase-contrast microscope. The cell count assay was done in triplicates. Data are mean ± standard error (SE) of three independent experiments.

2.4. Preparation of Whole-Cell Lysates:

After treatments, undifferentiated and differentiated adipocytes of 3T3-L1 cells or hASCs were washed with PBS and lysed in a modified RIPA buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, proteinase inhibitor cocktail (1x)]. Whole-cell lysates were collected and centrifuged at 13,000 rpm for 15 min at 4℃. The resultant supernatant was saved, and its protein concentration was measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

2.5. Western Blot Analysis:

An equal amount of proteins (50 μg) was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Co., Bedford, MA, USA) by electrotransfer. The membranes were washed with Tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (v/v) Tween-20 (TBST) and blocked with TBST containing 5% (w/v) non-fat dried milk. The membranes were incubated with primary antibodies of p-ERK-1/2 (1:2,000),T-ERK-1/2 (1:2,000), p-JNK-1/2 (1:2,000), T-JNK-1/2 (1:2,000), p-p38 MAPK (1:2,000),T-p38 MAPK (1:2,000), IκB-α(1:2,000) or β-actin (1:10,000) at 4℃ for 3 h or overnight. The membranes were washed with TBST and incubated with secondary antibodies coupled to horseradish peroxidase for an additional 2 h. The membranes were washed with TBST and exposed to enzyme-linked chemiluminescence (ECL) reagents to detect the image or

signal of the target protein (Advansta, San Jose, CA, USA). The expression levels of control β-actin protein verified equal loading of proteins.

2.6. RT-PCR Analysis:

After treatments, total RNA from undifferentiated and differentiated adipocytes of 3T3-L1 cells or hASCs was extracted using RNAiso Plus (TaKaRa, Kusatsu, Shiga, Japan). Three μg of total RNA was used to make complementary DNA (cDNA) using a random hexadeoxynucleotide primer and reverse transcriptase. PCR then amplified the single-strand cDNA with a specific sense and antisense primer of COX-2 or actin. The primer sequences used for PCR were mentioned in Table 1. Expression levels of control actin mRNA were used as an internal control to evaluate the relative expression of COX-2 mRNA.

2.7. Statistical Analysis:

The cell count analysis was measured in triplicate and repeated three times. The results were expressed as mean ± standard error (SE). One-way ANOVA was used to compare the different significance. All significance testing was established on a $p<0.05$.

Table 1. Antibodies Used for Western Blot Analysis

Antibodies	Dilution used	Source	Catalog no.
COX-2	1:2,000	Santa Cruz Biotechnology	SC-19999
IκB-α	1:2,000	Cell signaling	#9242
p-JNK-1/2	1:2,000	Cell signaling	#9251
T-JNK-1/2	1:2,000	Cell signaling	#9252
p-ERK-1/2	1:2,000	Cell signaling	#9101
T-ERK-1/2	1:2,000	Cell signaling	#9102
p-p38 MAPK	1:2,000	Cell signaling	#9211
T-p38 MAPK	1:2,000	Cell signaling	#9212
β-actin	1:10,000	Sigma	A5441
Goat anti-rabbit IgG-HRP	1:2,000	Jackson ImmunoResearch Laboratories	111-035-045
Goat anti-mouse IgG-HRP	1:2,000	Jackson ImmunoResearch Laboratories	115-035-062

Table 2. Primer Sequences Used for PCR Analysis

Cell lines	Primer	Sequences
3T3-L1	COX-2	sense 5'TTGAAGACCAGGAGTACAGC3' antisense 3'GGTACAGTTCCATGACGTCG5'
	β-actin	sense 5'TCATGAAGTGTGACGTTGACATCCGT3' antisense 3'CCTAGAAGCATTTGCGGTGCACGATG5'
	COX-2	sense 5'CCGGACAGGATTCTATGGAG3' antisense 3'CAATCATCAGGCACAGGAGG5'
	β-actin	sense 5'GTCTTTGAGACCTTCAA3' antisense 3'GTCTTTGCGGATGTCCACG5'
hASCs	COX-2	sense 5'TTGAAGACCAGGAGTACAGC3' antisense 3'GGTACAGTTCCATGACGTCG5'
	β-actin	sense 5'TCATGAAGTGTGACGTTGACATCCGT3' antisense 3'CCTAGAAGCATTTGCGGTGCACGATG5'
	COX-2	sense 5'CCGGACAGGATTCTATGGAG3' antisense 3'CAATCATCAGGCACAGGAGG5'
	β-actin	sense 5'GTCTTTGAGACCTTCAA3' antisense 3'GTCTTTGCGGATGTCCACG5'

3. Results

3.1. PM2.5U exposure at 50 μg/mL rapidly induces COX-2 expression in 3T3-L1 preadipocytes with no cytotoxicity:

To first see whether PM2.5U modulates COX-2 expression in 3T3-L1 preadipocytes, cells were grown in the absence or presence of PM2.5U at different concentrations (5, 25, and 50 μg/mL) for 4 h. TNF-α, a known inducer of COX-2 in 3T3-L1 (pre)adipocytes (Yadav and Jang, 2021), was included as a positive control herein. As shown in Fig. 1A and B, results of Western blot and RT-PCR analysis demonstrated that the exposure of PM2.5U at 25 or 50 μg/mL for 4 h markedly up-regulated COX-2 expression at the protein and mRNA levels in 3T3-L1 preadipocytes, respectively. Notably, levels of COX-2 induced by PM2.5U at 25 or 50 μg/mL for 4 h in 3T3-L1 preadipocytes were similar to those caused by TNF-α at 10 ng/mL for 4 h. Control actin protein and mRNA expressions remained constant under these experimental conditions. Distinctly, the exposure of diesel PM2.5 (PM2.5D), the solid material in exhausts of diesel vehicles at 5, 25, or 50 μg/ml for 4 h did not induce COX-2 expression in 3T3-L1 preadipocytes (data not shown). These results indicate the ability of PM2.5U to induce COX-2 expression in 3T3-L1 preadipocytes selectively. Next, using cell count analysis, I probed whether PM2.5U or TNF-α at doses tested influences the growth (survival) of 3T3-L1 preadipocytes. As shown in Fig. 1C, 4 h treatment with PM2.5U or TNF-α at doses tested were not cytotoxic to 3T3-L1 preadipocytes. Owing

to the maximal effect to induce COX-2 expression in undifferentiated 3T3-L1 cells, I selected the 50 μg/mL concentration of PM2.5U for further studies. Notably, results of kinetic studies, as shown in Fig. 1D and E, further revealed that PM2.5U exposure at 50 μg/mL for 2 h caused a maximal induction of COX-2 expression at the protein and mRNA levels in 3T3-L1 preadipocytes. These results demonstrate the ability of PM2.5U exposure at 50 μg/mL to rapidly and strongly elevate COX-2 expression in 3T3-L1 preadipocytes.

3.2. Ginsenoside Rc at 30 μM reduces the PM2.5U-induced COX-2 expression in 3T3-L1 preadipocytes with no cytotoxicity:

Next, we explored whether three different ginsenosides, including Rc, Rd, and Ro, modulate the PM2.5U-induced COX-2 expression in 3T3-L1 preadipocytes. To this end, cells were treated without or with PM2.5U (50 μg/mL) in the absence or presence of Rc, Rd, or Ro at different doses for 2 h. Strikingly, among the ginsenosides tested, Rc at 30 μM most strongly inhibited the air pollutant-induced COX-2 protein expression in 3T3-L1 preadipocytes (Fig. 2A). Ro or Rd at doses tested had no or little effect on it (Fig. 2B and C). The data of RT-PCR analysis further demonstrated Rc's capability at 30 μM to vastly suppress the PM2.5U-induced COX-2 mRNA expression in 3T3-L1 preadipocytes (Fig. 2D). Control actin protein and mRNA expressions remained unchanged under these experimental conditions. Furthermore, as shown in Fig. 2E, results of the MTS assay displayed that 2 h treatment with PM2.5U and Rc at doses applied did not alter the proliferation (viability) of 3T3-L1 preadipocytes. The chemical

structure of ginsenoside Rc is shown in Fig. 2F.

3.3. PM2.5U exposure induced the phosphorylation of JNK-1/2 and p38 MAPK, but ginsenoside Rc selectively blocks the PM2.5U-induced JNK-1/2 phosphorylation in 3T3-L1 preadipocytes:

COX-2 expression induced by external or internal stimuli is primarily influenced by the phosphorylation (activation) of multiple protein kinases and transcription factors, including the members of mitogen-activated protein kinases (MAPKs) and NF-κB (Klein, et al. 2007). This promptly led us to investigate the effects of PM2.5U (50 μg/mL) and/or ginsenoside Rc (30 μM) on the phosphorylation (activation) of ERK-1/2, JNK-1/2, p38 MAPK, and the expression of IκB-α, a cytosolic inhibitory protein of NF-κB in 3T3-L1 preadipocytes. As shown in Fig. 3, compared with control (no PM2.5U), PM2.5U exposure at 50 μg/mL for 1 h led to an elevation of the phosphorylation of JNK-1/2 and p38 MAPK, but not ERK-1/2, in 3T3-L1 preadipocytes. Distinctly, PM2.5U exposure at 50 μg/mL for 1 h did not modulate IκB-α expression (or proteolysis). However, treatment with ginsenoside Rc at 30 μM considerably blocked the PM2.5U-induced JNK-1/2 phosphorylation in 3T3-L1 preadipocytes. Ginsenoside Rc treatment at 30 μM did not primarily affect the PM2.5U-induced p38 MAPK phosphorylation, as assessed by less T-p38 MAPK). Expression levels of total JNK-1/2, ERK-1/2, p38 MAPK, and control actin remained unchanged under these experimental conditions.

3.4. SP600125, a phosphorylation inhibitor of JNK-1/2

blocks the PM2.5U-induced COX-2 expression and JNK-1/2 phosphorylation in 3T3-L1 preadipocytes:

I next probed the role of JNK-1/2 in the PM2.5-induced COX-2 expression in 3T3-L1 preadipocytes using SP600125, a specific JNK-1/2 inhibitor. As shown in Fig. 4A, the results of the immunoblotting analysis demonstrated that treatment with SP600125 at 25 μM effectively blocked the PM2.5U-induced COX-2 protein expression in 3T3-L1 preadipocytes. Moreover, the data of RT-PCR analysis revealed the ability of SP600125 to strongly suppress the PM2.5U-induced COX-2 mRNA expression in 3T3-L1 preadipocytes (Fig. 4B). As further shown in Fig. 4C, SP600125 treatment at 25 μM almost completely blocked the PM2.5U-induced JNK-1/2 phosphorylation in 3T3-L1 preadipocytes. Control actin protein, mRNA, and total JNK-1/2 protein expression levels remained constant under these experimental conditions.

3.5. PM2.5U exposure also induces COX-2 expression in a JNK-1/2-dependent manner in differentiated 3T3-L1 cells, but ginsenoside Rc suppresses it:

Little is known about PM2.5U and ginsenoside Rc regulation of COX-2 expression and JNK-1/2 phosphorylation in differentiated or mature adipocytes. I next questioned whether the short-term exposure of PM2.5U at 50 μg/mL further induces COX-2 expression and JNK-1/2 phosphorylation in differentiated 3T3-L1 cells and ginsenoside Rc interferes with it. Profoundly, as shown in Fig. 5A and B, PM2.5U

exposure at 50 μg/mL for 2 h led to a marked elevation of COX-2 expression at the protein and mRNA levels in differentiated 3T3-L1 cells. Still, treatment with ginsenoside Rc at 30 μM suppressed it. Furthermore, as shown in Fig. 5C, PM2.5U exposure at 50 μg/mL for 1 h also resulted in high phosphorylation of JNK-1/2 in differentiated 3T3-L1 cells, but ginsenoside Rc treatment at 30 μM blocked it. Moreover, as shown in Fig. 5D and E, treatment with SP600125, a JNK-1/2 inhibitor, down-regulated the PM2.5U-induced COX-2 expression at the protein and mRNA levels in differentiated 3T3-L1 cells. The efficacy of SP600125 to inhibit the PM2.5U-induced JNK-1/2 phosphorylation in differentiated 3T3-L1 cells was shown in Fig. 5F. Expression levels of control actin protein and mRNA, as well as total JNK-1/2 protein, remained constant under these experimental conditions. Characteristically, while 3T3-L1 preadipocytes have no lipid droplets (LDs), mature 3T3-L1 adipocytes are filled with many LDs after being differentiated for several days. Compared with 3T3-L1 preadipocytes (D0), the degree of differentiated adipocytes of 3T3-L1 cells on 8 days (D8) was confirmed by a phase-contrast microscope (Fig. 5G).

3.6. PM2.5U exposure further induces COX-2 expression in a JNK-1/2-dependent manner in hASCs and differentiated adipocytes of hASCs, but ginsenoside RC interferes with it:

Human adipose stem cells (hASCs) are differentiated into mature adipocytes through adipogenesis. Currently, PM2.5U and ginsenoside Rc regulation of COX-2 expression and JNK-1/2 phosphorylation in hASCs and differentiated adipocytes of hASCs is unknown. This promptly led us to isolate hASCs from human adipose tissues and investigate the effects of PM2.5U and ginsenoside Rc on COX-2 expression and JNK-1/2 phosphorylation in hASCs and differentiated hASCs. Notably, as shown in Fig. 6A and B, the exposure of PM2.5U at 50 μg/mL for 2 h could elevate COX-2 expression at the protein and mRNA levels in hASCs.

However, treatment with ginsenoside Rc at 30 μM effectively suppressed it. In addition, as shown in Fig. 6C, PM2.5U exposure at 50 μg/mL for 1 h caused a marked increase in JNK-1/2 phosphorylation in hASCs, and ginsenoside Rc treatment (30 μM) blocked it. Similar to hASCs, as shown in Fig. 6E and F, the exposure of PM2.5U (50 μg/mL) for 2 h increased COX-2 expression at the protein and mRNA levels in differentiated adipocytes of hASCs and treatment with ginsenoside Rc at 30 μM intensely repressed it. Moreover, as shown in Fig. 6C, PM2.5U exposure at 50 μg/mL for 1 h caused robust JNK-1/2 phosphorylation in differentiated adipocytes of hASCs, but ginsenoside Rc treatment at 30 μM essentially interfered with it. Expression levels of control actin protein and mRNA, as well as total JNK-1/2 protein, remained unchanged under these experimental conditions. Compared with hASCs (D0), the degree of differentiated adipocytes of hASCs on 12 days (D12) was confirmed by a phase-contrast microscope (Fig. 6G).

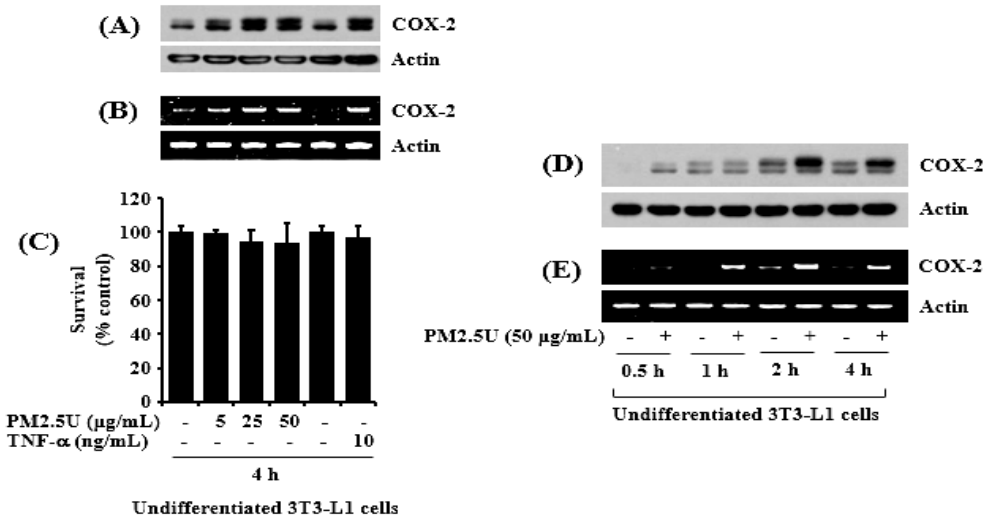

Figure 1. Effect of PM2.5U exposure at different concentrations and times on COX-2 expression in undifferentiated 3T3-L1 preadipocytes. (A-B) 3T3-L1 preadipocytes were treated without or with PM2.5U or TNF-α at the indicated concentrations for 4 h. Whole-cell lysates and total RNA from the conditioned cells were extracted and analyzed by Western blotting (A) and RT-PCR (B) analysis, respectively. (C) 3T3-L1 preadipocytes were treated without or with PM2.5U or TNF-α at the designated concentrations for 4 h. The number of surviving cells in the conditioned cells, which cannot be stained with trypan blue dye, was counted under a microscope. Data are mean ± standard error (SE) of three independent experiments. (D, E) 3T3-L1 preadipocytes were treated without or with PM2.5U at 50 μg/mL for 0.5, 1, 2, or 4 h. times. At each time point, whole-cell lysates and total RNA from the conditioned cells were extracted and analyzed by Western blotting (D) and RT-PCR (E) analysis, respectively.

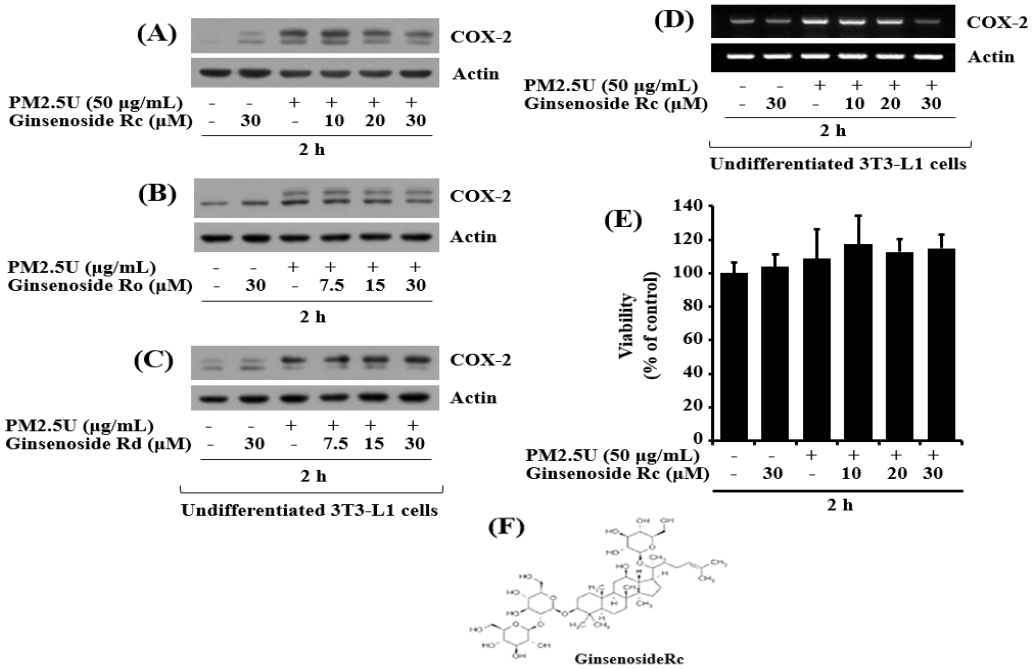

Figure 2. Effects of ginsenoside Rc, Ro, and Rd on the PM2.5U-induced expression of COX-2 in undifferentiated 3T3-L1 preadipocytes. (A-C) 3T3-L1 preadipocytes were treated without or with PM2.5U (50 μg/mL) in the absence or presence of ginsenoside Rc (A), Ro (B), and Rd (C) at the indicated doses for 2 h. Whole-cell lysates from the conditioned cells were extracted and analyzed by Western blotting analysis. (D-E) 3T3-L1 preadipocytes were treated without or with PM2.5U (50 μg/mL) in the absence or presence of ginsenoside Rc at the indicated concentrations for 2 h. Total RNA from the conditioned cells was extracted and analyzed by RT-PCR analysis (D). The viability of the conditioned cells was measured by MTS assay. Data are mean ± SE from three independent experiments with three replicates. (F) The chemical structure of ginsenoside Rc.

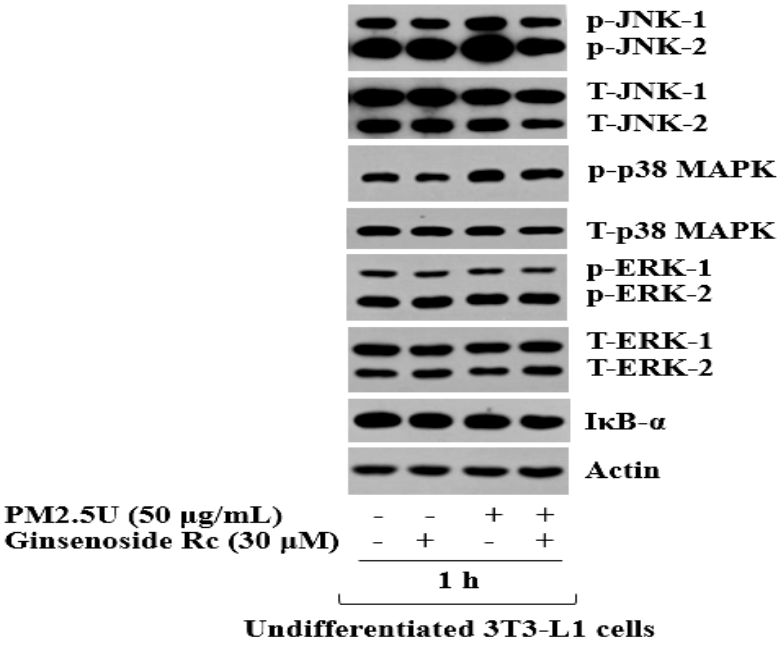

Figure 3. Effects of PM2.5U and/or ginsenoside Rc on the phosphorylation and expression of MAPKs and IkB-a in undifferentiated 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were treated without or with PM2.5U (50 μg/mL) in the absence or presence of ginsenoside Rc (30 μM) for 1 h. Western blotting analysis extracted and analyzed whole-cell lysates from the conditioned cells. p-JNK-1, phosphorylated JNK-1; T-JNK-1, total JNK-1; p-JNK-2, phosphorylated JNK-2; T-JNK-2, total JNK-2; p-p38 MAPK, phosphorylated p-p38 MAPK; T-p38 MAPK, total p38 MAPK; p-ERK-1, phosphorylated ERK-1; T-ERK-1, total ERK-1; p-ERK-2, phosphorylated ERK-2; T-ERK-2, total ERK-2.

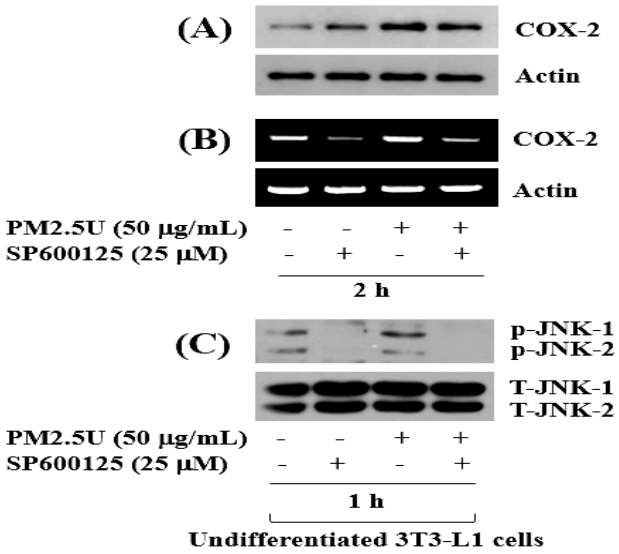

Figure 4. Effects of SP600125, a JNK-1/2 inhibitor on the PM2.5U-induced COX-2 expression and JNK-1/2 phosphorylation in undifferentiated 3T3-L1 preadipocytes. (A, B) 3T3-L1 preadipocytes were treated without or with PM2.5U (50 μg/mL) in the absence or presence of SP600125 (25 μM), a selective inhibitor of JNK-1/2, for 2 h. Whole-cell lysates and total RNA from the conditioned cells were extracted and analyzed by Western blotting (A) and RT-PCR (B) analysis, respectively. (C) 3T3-L1 cells preadipocytes were treated without or with SP600125 (25 μM) for 1 h. Western blotting analysis extracted and analyzed whole-cell lysates from the conditioned cells.

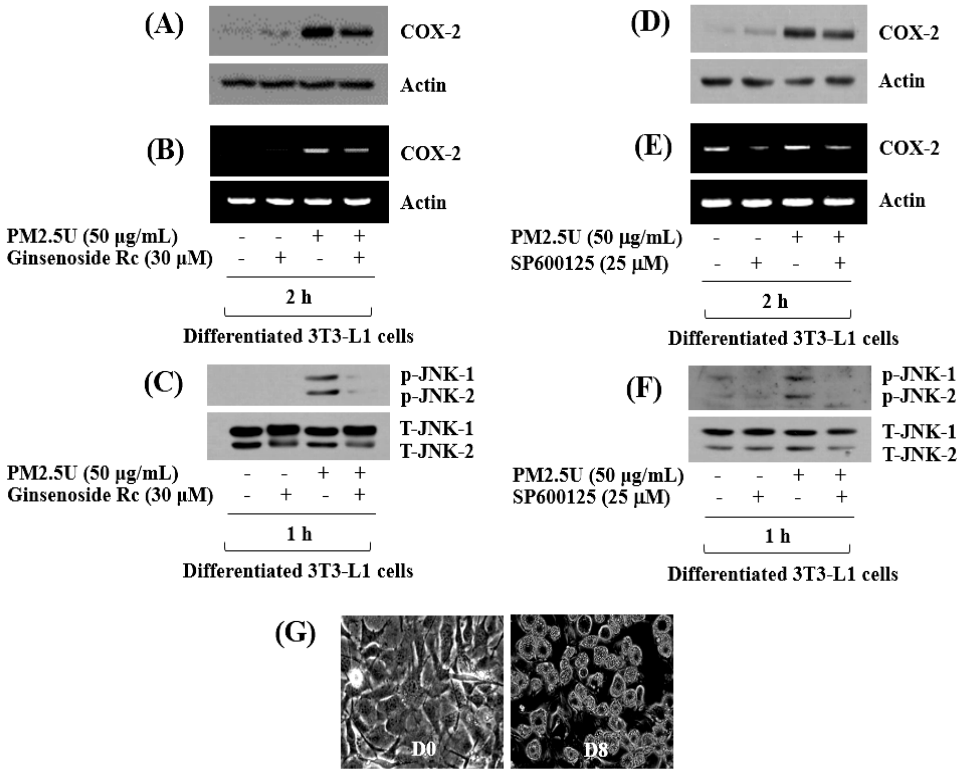

Figure 5. Effects of PM2.5U, ginsenoside Rc, and/or SP600125 on COX-2 expression and JNK-1/2 phosphorylation in differentiated 3T3-L1 adipocytes. (A-C) 3T3-L1 preadipocytes were induced to differentiate with an induction medium containing MDI, insulin, and FBS for 8 days. On day 8 (D8), differentiated 3T3-L1 cells were treated without or with PM2.5U (50 μg/mL) in the absence or presence of ginsenoside Rc (30 μM) for additional 2 h (A, B) or 1 h (C). Whole-cell lysates and total RNA from the conditioned cells were extracted and analyzed using Western blotting (A, C) and RT-PCR (B) analysis, respectively. (D-F) 3T3-L1 preadipocytes were induced to differentiate with an induction medium containing MDI, insulin, and FBS for 8 days. On D8,

differentiated 3T3-L1 cells were treated without or with PM2.5U in the absence or presence of the JNK-1/2 inhibitor SP600125 (25 μM) for an additional 2 h (D, E) or 1 h (F). Whole-cell lysates and total RNA from the conditioned cells were extracted and analyzed by Western blotting (D, F) and RT-PCR (E) analysis, respectively. (G) The images of 3T3-L1 preadipocytes before differentiation (D0) and differentiated 3T3-L1 cells (D8) were taken by a phase-contrast microscope.

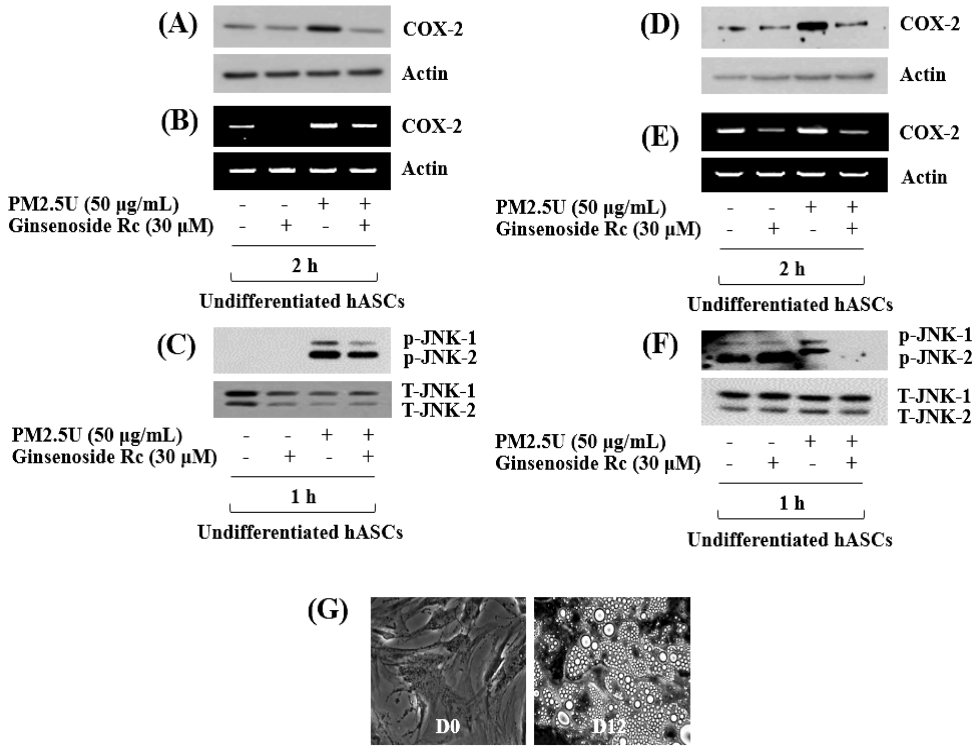

Figure 6. Effects of PM2.5U and/or ginsenoside Rc on COX-2 expression and JNK-1/2 phosphorylation in hASCs and differentiated adipocytes of hASCs. (A-C) hASCs were treated without or with PM2.5U (50 μg/mL) in the absence or presence of ginsenoside Rc (30 μM) for 2 h (A, B) or 1 h (C). Whole-cell lysates and total RNA from the conditioned cells were extracted and analyzed using Western blotting (A, C) and RT-PCR (B) analysis, respectively. (D-F) The adipocyte differentiation of hASCs was induced using adipocyte differentiation and maintenance medium for 12 days. Differentiated adipocytes of hASCs were treated without or with PM2.5U (50 μg/mL) in the absence or presence of ginsenoside Rc (30 μM) for an additional 2 h

(D, E) or 1 h (F). Whole-cell lysates and total RNA from the conditioned cells were extracted and analyzed using Western blotting (D, F) and RT-PCR (E) analysis. (G) The images of hASCs before differentiation (D0) and differentiated adipocytes of hASCs (D12) were taken by a phase-contrast microscope. (D0) and differentiated adipocytes of hASCs (D12) were taken by a phase-contrast microscope.

4. Discussion

In recent years, PM2.5-induced inflammatory pathologies in the lung by its airway invasion and other organs by its inflow into the blood vessel, which may further support the air pollutant-induced systemic inflammation, have become a global concern (15). Accordingly, adipose inflammation is closely linked to the development of obesity (16). Thus, any substance that increases or decreases adipose inflammation is considered obesity (adipose inflammation) inducing or suppressing material. To date, little is known about the effect of PM2.5 exposure on adipose inflammation and ginsenoside Rc regulation of the process. Here I demonstrate, for the first time, that PM2.5U exposure at 50 μg/mL induces inflammatory COX-2 expression by activating JNK-1/2, but, ginsenoside Rc at 30 μM interferes with it in undifferentiated and differentiated adipocytes of 3T3-L1 cells or hASCs.

Multiple lines of evidence illustrate that PM2.5 exposure up-regulates COX-2 expression in various types of cells, including human monocytic cells (THP-1) (17), mouse microglial cells (BV2) (18), mouse macrophages (RAW 264.7) (19), human lung alveolar epithelial cells (A549) (20), and human epidermal keratinocytes (HaCaT) (21). However, PM2.5 regulation of inflammatory COX-2 expression and molecular and signaling mechanisms associated with it in (pre)adipocytes is not fully understood. As aforementioned, COX-2 is an inducible enzyme that converts its substrate, arachidonic acid, into prostaglandins (PGs), an inflammation and pain-related hormone (22). Induction of COX-2 expression and enzymatic activity is regulated at multiple levels, including transcription,

post-transcription, translation, and co-translation (N-glycosylation) (21). It is well documented that COX-2 expression is highly induced in cells exposed to numerous extracellular and endogenous stimuli, including endotoxins, heavy metals, ultraviolet, growth factors, and pro-inflammatory cytokines (5, 22). There is mounting evidence that the agonist-induced cellular COX-2 expression is greatly influenced by not only the expression of many transcription factors such as NF-κB, activator protein 1 (AP-1) but also the phosphorylation (activation) of multiple signaling components, including ERK-1/2, JNK-1/2, p38 MAPK, protein kinase B, and protein kinase C (23, 24, 25, 26). Notably, recent *in vivo* and *in vitro* studies have introduced that COX-2 induced by PM2.5 exposure is regulated by the expression and phosphorylation of NF-κB, signal transducer and activator of transcription 3 (STAT3), c-Jun, toll-like receptor-4, IκB-α kinase, Janus-activated protein kinase (JAK), and ROS (16, 27), which thus point out that the NF-κB/IκB-α and JAK/STAT pathways are crucial for the air pollutant-induced COX-2 expression. The suppression of inhibitor of IκB-α phosphorylation, IκB-α degradation, nuclear translocation of p65, and transcription of the reporter gene are signs that the NF-κB signaling pathway is active (28).

In this study, I have also demonstrated that the short-term exposure of PM2.5U, but not PM2.5D, at 50 μg/ml leads to robust COX-2 expression at both protein and mRNA levels in undifferentiated and differentiated adipocytes of 3T3-L1 cells. These results indicate that the PM2.5U-induced COX-2 expression in 3T3-L1 (pre)adipocytes is rapid and is due to the COX-2 transcriptional and translational up-regulation. Accordingly, NF-κB is a pivotal transcription factor of the agonist-induced cellular COX-2 expression (25), and its cellular expression and nuclear localization and activity is greatly

affected by the expression levels of IκB-α, a cytosolic inhibitory protein of NF-κB (29). Given that PM2.5D triggers the NF-κB-dependent COX-2 expression in particular cells mentioned above (18, 17), it was reasonable to investigate the effect of PM2.5U exposure on IκB-α expression in undifferentiated 3T3-L1 cells. Distinctly, the present study has shown that PM2.5U exposure at 50 μg/ml for 30 min did not affect IκB-α expression in undifferentiated 3T3-L1 cells. These results imply that, unlike PM2.5D, PM2.5U does not elicit IκB-α proteolysis in 3T3-L1 preadipocytes. Thus, the PM2.5U-induced COX-2 transcriptional up-regulation in these cells is not through the NF-κB/IκB-α but via other transcription factors or signaling components.

As aforementioned, many signaling components, including ERK-1/2, JNK-1/2, and p38 MAPK, play positive roles in the agonist-induced COX-2 expression in many types of cells, including 3T3-L1 (pre)adipocytes. Until now, little is known about the PM2.5 regulation of these members of the MAPK family in (pre)adipocytes, including hASCs. Notably, I have found that the short-term exposure of PM2.5U (50 μg/ml) further leads to a marked elevation of the phosphorylation of JNK-1/2, but not p38 MAPK and ERK-1/2, in 3T3-L1 preadipocytes, pointing out the specificity. Furthermore, I have shown the capability of PM2.5 exposure to stimulate JNK-1/2 phosphorylation in differentiated adipocytes of 3T3-L1 cells. Notably, the pharmacological inhibition study herein has demonstrated that SP600125, a selective inhibitor of JNK-1/2 that interferes with the PM2.5-induced JNK-1/2 phosphorylation, further blocks the air pollutant-induced COX-2 expression at the protein and mRNA levels in undifferentiated and differentiated adipocytes of 3T3-L1 cells. These results thus suggest that the PM2.5U-induced COX-2 transcriptional

and translational up-regulation in 3T3-L1 (pre)adipocytes seen in this study is partly mediated by activating JNK-1/2 and its downstream component.

Given that JNK-1/2 activation leads to up-regulation of c-Jun, one of the transcription factor components of AP-1 (30), that participates in the agonist-induced cellular COX-2 transcriptional up-regulation, it is conceivable that activation of JNK-1/2 is crucial for the PM2.5U-induced COX-2 expression in undifferentiated and differentiated adipocytes of 3T3-L1 cells herein. Another key finding of the current study is the capability of ginsenoside Rc at 30 μM to inhibit the PM2.5U-induced COX-2 expression and JNK-1/2 phosphorylation in undifferentiated and differentiated adipocytes of 3T3-L1 cells. It is thus speculative that ginsenoside Rc down-regulation of the PM2.5U-induced COX-2 expression in undifferentiated and differentiated adipocytes of 3T3-L1 cells is associated with its ability to inhibit JNK-1/2 phosphorylation in response to PM2.5U exposure.

Human adipose stem cells (hASCs) are adipoblasts or preadipocytes and become differentiated into mature adipocytes (31). In this study, I have isolated and utilized hASCs and the differentiated adipocytes of hASCs to further test PM2.5U regulation of COX-2 expression and ginsenoside Rc control of the process. Strikingly, I herein have observed that the short-term exposure of PM2.5U also leads to high COX-2 expression at both protein and mRNA levels in hASCs and the differentiated adipocytes of hASCs, pointing out that PM2.5U can induce COX-2 transcriptional and translational up-regulation in human (pre)adipocytes and it is not limited to 3T3-L1 mouse (pre)adipocytes. Notably, the present study has further shown the ability of PM2.5U exposure at 50 μg/mL to increase JNK-1/2

phosphorylation in hASCs and the differentiated adipocytes of hASC. It is of interest further demonstrated herein that ginsenoside Rc at 30 μM inhibits the PM2.5U-induced COX-2 expression and JNK-1/2 phosphorylation in human (pre)adipocytes. These results collectively point out that PM2.5U and ginsenoside Rc regulation of the PM2.5U-induced COX-2 expression and JNK-1/2 phosphorylation is not restricted to 3T3-L1 (pre)adipocytes and thus seems to be a general event occurring in (pre)adipocytes regardless of the origins and differentiation status of fat cells.

5. Summary

This is the first report demonstrating that PM2.5U exposure induces robust COX-2 expression by activating JNK-1/2, but ginsenoside Rc suppresses it in undifferentiated and differentiated adipocytes of 3T3-L1 cells or hASCs. This work advocates that PM2.5U is an inducer of COX-2 , and ginsenoside Rc is an inhibitor of it in fat cells. Thus, the natural phytochemical ginsenoside Rc can be applied as an anti-inflammatory agent against PM2.5U-induced adipose inflammation and related pathologies.

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Ginsenoside Rc Inhibits Urban Particulate Matter 2.5-induced Cyclooxygenase-2 Expression in 3T3-L1 Mouse Preadipocytes and Human Adipose Stem Cells

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(Abstract)

Ginsenoside Rc is an active component in Panax ginseng with many pharmacological properties. Particulate matter 2.5U (PM2.5U) is an environmentally hazardous material leading to the induction of an acute inflammatory response in the human body. However, until now, whether PM2.5U induces the expression of inflammatory cyclooxygenase-2 (COX-2) and ginsenoside Rc inhibits it in fat cells remains elusive. Here I investigated the effect of PM2.5U on COX-2 expression and ginsenoside Rc regulation of PM2.5-induced COX-2 expression in 3T3-L1 mouse preadipocytes and human adipose stem cells (hASCs). Strikingly, treatment with PM2.5U (50 μg/ml, 2 h) up-regulated COX-2 expression in undifferentiated and differentiated

adipocytes of 3T3-L1 cells or hASCs. Of further interest, treatment with ginsenoside Rc (30 μM) abrogated PM2.5-induced COX-2 expression in these cells. Importantly, while treatment with PM2.5U (50 μg/ml, 1 h) led to the hyperphosphorylation (hyperactivation) of c-jun N-terminal protein kinase-1/2 (JNK-1/2) in in undifferentiated and differentiated adipocytes of 3T3-L1 cells or hASCs, ginsenoside Rc greatly blocked it. Of note, treatment with SP600125, a selective JNK-1/2 inhibitorblocked the PM2.5-induced JNK-1/2 activation and COX-2 expression in these cells. Taken together, these results demonstrate firstly that PM2.5U induces high COX-2 expression by activating JNK-1/2 in undifferentiated and differentiated adipocytes of 3T3-L1 cells or hASCs, and ginsenoside Rc inhibits PM2.5U-induced COX-2 expression by inhibiting JNK-1/2 in these cells. This study advocates that PM2.5U is an inducer of COX-2 and ginsenoside Rc is a suppressor of it in (pre)adipocytes.

Ginsenoside Rc의 3T3-L1 마우스 지방전구세포 및 사람 지방줄기세포에서 초미세먼지 PM2.5U 유도 cyclooxygenase-2 발현 억제 효과

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(초록)

Ginsenoside Rc는 파낙스 인삼에 함유된 활성 성분의 하나로 항암, 항염증 및 항산화 등의 많은 약리학적 특성을 가지고 있다. PM2.5U는 입자의 직경이 2.5 μM 미만의 대기 중에 존재하는 초미세먼지를 말하며 체내 유입 시 여러 장기에 급성 염증 반응을 촉진한다. Cyclooxygenase-2(COX-2)는 염증효소단백질로 세포내 과발현 시 prostaglandins 과량 생성을 통해 염증 반응을 매개한다. 현재까지 지방(전구)세포에서 PM2.5U의 COX-2 발현 유도 효과/기전 및 ginsenoside Rc의 PM2.5U 유도 COX-2 발현 억제 효과/기전이 매우 불분명한 상태이다. 이에 본 연구에서는 마우스 지방전구세포(3T3-L1) 및 사람 지방줄기세포(hASCs)에서 PM2.5U의COX-2 발현 유도 효과 및 기전 규명, 그리고 ginsenoside Rc의 PM2.5U 유도 COX-2 발현 증가에 대한 억제 효과 및 기전을 조사하였다. 놀랍게도, PM2.5U(50 μg/ml, 2시

간) 처리 시 미분화 및 분화3T3-L1 지방전구세포와 hASCs에서 높은 COX-2 발현이 관찰되었다. 흥미롭게도, Rc(30 μM)처리 시 이들 세포에서의 PM2.5U 유도 COX-2 발현 증가가 억제되었다. 중요하게도, PM2.5U(50 μg/ml, 1시간) 처리 시 미분화 및 분화 3T3-L1 지방전구세포와 hASCs에서 JNK-1/2 단백질 과인산화(과활성화)가 관찰되었으나, Rc(30 μM)처리 시 이들 세포에서의 PM2.5U 유도 JNK-1/2 단백질 과인산화가 억제되었다 한편, JNK-1/2 화학저해제 SP600125(25 μM)처리 시 미분화 및 분화 3T3-L1 지방전구세포와 hASCs에서의 PM2.5U 유도 JNK-1/2 단백질 과인산화 및 COX-2 과발현이 모두 크게 억제되었다. 결론적으로, 본 연구를 통해 미분화 및 분화 3T3-L1 지방전구세포와 hASCs에서 PM2.5U는 JNK-1/2 단백질 과인산화를 통해 COX-2 과발현을 유도하며, ginsenoside Rc는 PM2.5U 유도 JNK-1/2 단백질 과인산화 방어를 통해 PM2.5U 유도 COX-2 과발현을 억제함을 알 수 있었다. 이 연구결과는 PM2.5U가 지방(전구)세포에서 새로운 (비만)염증반응 촉진 물질로 작용할 수 있고, ginsenoside Rc는 PM2.5U 매개 (비만)지방염증의 신규방어 소재(물질)로서의 개발/적용 가능성을 제시한다.