Sulforaphane inhibits PDGF-induced proliferation of rat aortic vascular smooth muscle cell by up-regulation of p53 leading to G1/S cell cycle arrest

Su-Hyang Yoo, Yong Lim, Seung-Jung Kim, Kyu-Dong Yoo, Hwan-Soo Yoo, Jin-Tae Hong, Mi-Yea Lee, Yeo-Pyo Yun

Abstract

Vascular diseases such as atherosclerosis and restenosis artery angioplasty are associated with vascular smooth muscle cell (VSMC) proliferation and intimal thickening arterial walls. In the present study, we investigated the inhibitory effects of sulforaphane, an isothiocyanate produced in cruciferous vegetables, on VSMC proliferation and neointimal formation in a rat carotid artery injury model. Sulforaphane at the concentrations of 0.5, 1.0, and 2.0 μM significantly inhibited platelet-derived growth factor (PDGF)-BB-induced VSMC proliferation in a concentration-dependent manner, determined by cell count. The IC50 value of sulforaphane-inhibited VSMC proliferation was 0.8 μM. Sulforaphane increased the cyclin-dependent kinase inhibitor p21 and p53 levels, while it decreased CDK2 and cyclin E expression. The effects of sulforaphane on vascular thickening were determined 14 days after the injury to the rat carotid artery. The angiographic mean luminary diameters of the group treated with 2 and 4 μM sulforaphane were 0.25 ± 0.1 and 0.09 ± 0.1 mm², respectively, while the value of the control groups was 0.40 ± 0.1 mm², indicating that sulforaphane may inhibit neointimal formation. The expression of PCNA, maker for cell cycle arrest, was decreased, while that of p53 and p21 was increased, which showed the same pattern as one in in-vitro study. These results suggest that sulforaphane-inhibited VSMC proliferation may occur through the G1/S cell cycle arrest by up-regulation of p53 signaling pathway, and then lead to the decreased neointimal hyperplasia thickness. Thus, sulforaphane may be a promising candidate for the therapy of atherosclerosis and post-angiography restenosis.

1. Introduction

Abnormal proliferation of vascular smooth muscle cells (VSMCs) plays a critical role in the occurrence of atherosclerosis [28]. Regulation of cell cycle is an important mechanism for the inhibition of cell proliferation [11]. The cell cycle is regulated by the coordinated action of cyclin-dependent kinases (CDKs), in association with their specific regulatory cyclin proteins. Thus, functional activation of CDK–cyclin is required for cell cycle progression [2,32]. The kinase activity of these CDK–cyclin complexes is inhibited by two classes of cyclin-dependent kinase inhibitors (CKIs), including INK4 family (p16INK4a and p15INK4b) and cip family (p21cip1 and p27kip1) [25,26,29]. The p53 was known to be an up-regulation of p21 in cell cycle arrest. Several findings have demonstrated that the product of the p53 is responsible for the G1 checkpoint. In response to genotoxic stress and drugs, the level of p53 protein was increased, and a transient arrest of cell cycle progression in the G1 phase occurred [20,21], or a trigger apoptosis was triggered [2,27]. Thus, induction of p53 has been considered to be an effective strategy for molecular target therapy of atherosclerosis. VSMC proliferation induced by platelet-derived growth factor (PDGF) has been considered to be crucial for the development of vascular diseases. PDGF interacts with its receptor in VSMCs. PDGF-BB can activate PLC gamma, Akt, and ERK1/2 signaling pathways to induce the cell proliferation, cell migration, and angiogenesis of VSMCs [4]. Cruciferous vegetables, particularly broccoli, contain glycosinolates and glucoraphanin, which are metabolized sulforaphane [30]. Sulforaphane inhibits carcinogenesis in many cancers including breast cancer [17], angiogenesis [1], and adipocyte differentiation [9], and induces cell cycle arrest [13] and apoptosis [23]. However, the effects of sulforaphane on VSMCs proliferation have not yet been clarified. Thus the aim of this study was to elucidate the effects of sulforaphane on PDGF-BB-stimulated RASMC proliferation and neointimal thickness, as well as its mechanism.
2. Methods

2.1. Chemicals and reagents

The L-sulforaphane (99%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). PDGF-BB was obtained from Upstate Biotechnology. The p-ERK1/2, p-Akt, p-PLCγ1, ERK1/2, Akt, and PLCγ1 anti-bodies were supplied by Cell Signaling Technology Inc. The PDGFR-β and p-PDGFR-β antibodies were obtained from Upstate Biotechnology. The p21, p53, cyclin E, CDK2, α-actin, and PCNA antibodies were obtained from Santa Cruz Biotechnology Inc. All other chemicals used in this study were purchased from Sigma Chemical Co.

2.2. Cell culture

Rat aortic smooth muscle cells (RASMCs) were isolated by enzymatic dispersion according to the modified method of Chamley et al. The cells were cultured in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 8 mM HEPES, and 2 mM l-glutamine at 37 °C and 5% humidity in a CO₂ incubator. The purity of the RASMC culture was confirmed by immunocytochemical localization of α-smooth-muscle actin. RASMCs were used at passages 4–8 in these experiments.

2.3. Cell proliferation

Cell proliferation was measured by direct counting [18]. Initially, the cells were seeded into 12-well culture plates at 4 × 10⁴ cells/mL and cultured in DMEM containing 10% FBS at 37 °C for 24 h. When the cells reached 70% confluence, the medium was replaced with serum-free containing sulforaphane. Cells were stimulated with 25 ng/mL PDGF-BB, and then trypsinized with trypsin-EDTA. Cells were counted using a hemocytometer and microscope.

2.4. Apoptosis analysis

Briefly, VSMCs in a 6-well plate at 4 × 10⁴ cells/well were pre-incubated in serum-free medium containing a mixture DMEM medium in the presence and absence of 0.5, 1.0, and 2.0 μM of sulforaphane for 24 h. The cells were then trypsinized, collected, rinsed twice with cold PBS, resuspended in 100 μL of a binding buffer, and incubated with 2 μL FITC-labeled annexin-V and 2 μL propidium iodide (PI, stock solution 50 μg/mL) for 15–25 min in the dark at room temperature. The apoptotic cells were determined using the Modfit LT program (Verify Software House, ME, USA).

2.5. Immunoblotting

Immunoblotting was performed as previously described [14]. Briefly, RASMCs were incubated in serum-free DMEM containing various concentrations of sulforaphane for 24 h, and then stimulated with 25 ng/mL PDGF-BB for 1 min for PDGFR-β, 5 min for ERK1/2, and 15 min for Akt and PLCγ1 phosphorylation assays. For the assay of cyclin E, CDK2, PCNA, p53, and p21 expressions, the cells were stimulated with 25 ng/mL PDGF-BB for 24 h. The primary antibodies and their dilution factors used in this study were as follows: p-ERK1/2 (1:1000), p-Akt (1:1000), p-PLCγ1 (1:1000), p-PDGFR-receptorβ (1:1000), CDK2 (1:1000), cyclin E (1:1000), p21 (1:200), PCNA (1:1000), and p53 (1:1000).

2.6. Cell cycle progression analysis

Cell cycle was determined by flow cytometric analysis after propidium iodide (PI) staining as previously described. Cells were harvested, fixed in 70% ethanol, and stored at −20 °C. Cells were then washed twice with ice-cold PBS and incubated with RNase and PI, a DNA-intercalating dye. After the staining, 10,000 cells per experiment were analyzed using the FACS Calibur® system (Becton Dickinson). The cell cycle progression was determined using the Modfit LT program (Verify Software House, ME, USA).

2.7. Animals

The present study was performed on Sprague–Dawley rats as described previously [18]. Male Sprague–Dawley rats (Samtako Bio Korea Co., Ltd., Korea) weighing about 260 g were fed a normal chow diet and given water. All protocols were approved by the Chungbuk National University Animal Care and Use Committee (CBNUA-452-12-01).

2.8. Surgical intervention

The rat carotid artery balloon injury model [31] was modified. Animals were anesthetized with tiletamine plus zolazepam (Zoletil; Virbac, Carros, France), and the right carotid artery was surgically exposed. An A2F Fogarty balloon embolectomy catheter (Baxter, McGraw Park, IL) was advanced along the length of the common carotid artery and retracted under mild balloon inflation pressure [10]. Sulforaphane and ethanolic solutions were added to the cooled 30% pluronic gel solution. Rats were divided into four treatment groups: normal (n = 5), control (vehicle alone; n = 10), 2 μM sulforaphane (n = 10), and 4 μM sulforaphane (n = 10). Immediately following balloon injury, 100 μL sulforaphane (2–4 μM) or vehicle solutions were applied to the exposed adventitial surface of an approximately 10-mm segment of the injured carotid artery.

2.9. Morphometric analysis and immunohistochemistry

Carotid arteries were perfusion-fixed with 10% buffered formalin. Carotid artery sections (4 μm) were stained with hematoxylin–eosin, and an investigator blind to the experimental procedure performed morphometric analyses of the three individual sections from the middle of each arterial segment exposed to pluronic gel. For immunohistochemical staining, sections were deparaffinized, rehydrated with PBS and incubated for 30 min at 37 °C with primary PCNA (1:200), p53 (1:100) and p21 antibodies (1:100), followed by staining with a labeled streptavidin biotin kit (DAKO Corp.). Section were analyzed by using a IX71 microscope (OLYMPUS, Tokyo, Japan) connected to a DP72 camera (OLYMPUS, Tokyo, Japan) which allowed projection of the entire observed field onto a computer monitor. Cross-sectional areas (lumen, intima and media) and artery area (in mm²) were measured with the aid of a Cell Sense Image System (OLYMPUS, Tokyo, Japan). The intima was defined as the layer between the endothelial lining of the lumen and the internal elastic lamina; the media, as the layer between the internal and external elastic lamina; and the adventitia, as the layer between the external elastic lamina and the edge of the loose fibroadipose tissue surrounding the carotid arteries [24].

2.10. Statistical analysis

The experimental results are expressed as the mean ± S.E.M. One-way analysis of variance (ANOVA) was used for multiple comparisons, followed by a Dunnett’s test. P values < 0.05 and <0.01 were considered significant.

3. Results

3.1. Effect of sulforaphane on PDGF-BB-induced proliferation of RASMCs

The effects of sulforaphane on cell growth and DNA synthesis of PDGF-BB-induced RASMCs were examined. The numbers of RASMCs
Fig. 1. Effect of sulforaphane on PDGF-BB-induced proliferation of RASMCs. RASMCs were pre-cultured in serum-free medium in the presence or absence of sulforaphane (0.5–2.0 μM) for 24 h, and then stimulated with 25 ng/mL PDGF-BB for 24 h. (A) The cells grown in 12 well culture plates were trypsinized and counted using a hemocytometer. (B) Cells grown in 6 well culture plates were harvested, stained with annexin-V-FITC, and analyzed by flow cytometry. Lower left, upper left and right sections in each panel represent viable, necrotic and apoptotic cells, respectively. Data are expressed as a mean ± S.E.M. (*P < 0.05, **P < 0.01 vs. stimulus control).

Fig. 2. Effect of sulforaphane on PDGF-BB-induced cell cycle progression in RASMCs. RASMCs were pre-cultured in a serum-free medium in the presence or absence of sulforaphane (0.5–2.0 μM) for 24 h, and then stimulated with 25 ng/mL PDGF-BB for 24 h. The cells were harvested by trypsinization. Then, the cells were fixed with 70% ethanol at 4 °C for 24 h, and then incubated at 4 °C overnight, after adding PI staining solution. Each item is derived from a representative experiment in which the data from at least 10,000 events were obtained. The data are representative of at least three independent experiments with similar results.
were counted after treating with sulforaphane at the concentrations of 0.5, 1.0, and 2.0 μM (Fig. 1A). The numbers of PDGF-BB-stimulated RASMCs were increased by approximately 1.9 folds, compared to those of the unstimulated group. Sulforaphane inhibited the PDGF-BB-induced increase in the number of RASMCs in a concentration-dependent manner. The number of PDGF-BB-stimulated cells treated with 2.0 μM sulforaphane was decreased to that of the unstimulated cells. However, sulforaphane at 0.5–2.0 μM did not cause apoptotic cell death (Fig. 1B).

3.2. Effect of sulforaphane on cell cycle progression of RASMCs

Flow cytometric analysis demonstrated that sulforaphane affected cell cycle progression of RASMCs induced by PDGF-BB (Fig. 2). Serum deprivation of RASMCs for 24 h resulted in approximately 88.9% synchronization of the cell cycle in the G0/G1 phase. After RASMCs were treated with PDGF-BB, the percentage of cells in the S phase increased from 4.5 ± 0.1% to 32.3 ± 1.0%, indicating that the cell proliferation may be activated by PDGF-BB. Interestingly, sulforaphane arrested
the cell cycle progression of RASMCs induced by PDGF-BB. When RASMCs were treated with PDGF-BB plus sulforaphane at 0.5, 1.0, and 2.0 μM, the percentage of cells in the S phase were 26.4 ± 1.0%, 22.4 ± 1.5%, and 12.4 ± 1.5%, respectively. Sulforaphane decreased the population of cells in the S phase in a concentration-dependent manner. Proliferating cell nuclear antigen (PCNA) functions the cell cycle progression from early G1 to S phase. The expression of PCNA was decreased. RASMCs were treated with sulforaphane at 0.5, 1.0, and 2.0 μM in the presence of PDGF-BB (Fig. 3). Thus, these results suggest that sulforaphane may suppress the proliferation of RASMCs through cell cycle arrest in G1–S phase.

3.3. Effect of sulforaphane on the expression of cell cycle regulatory proteins and PDGF-BB signaling pathway in RASMCs

Cell cycle progression is regulated through a complex network of cell cycle regulatory proteins, such as cyclins, CDKs, p21, and p53. To investigate the mechanism of cell cycle arrest by sulforaphane, the effects of sulforaphane on the expressions of cyclin E, CDK 2, p21, and p53 were determined. Sulforaphane decreased the PDGF-BB-stimulated expression levels of cyclin E and CDK 2 in a concentration-dependent manner (Fig. 4A). For clarifying the molecular target of sulforaphane in the upstream signaling pathway, we measured the expression levels of p21 and its upstream signaling molecule p53 in RASMCs treated with PDGF-BB plus sulforaphane (0.5, 1.0 and 2.0 μM). Sulforaphane increased the PDGF-BB inhibition expression levels of p21 and p53 up to the level of the untreated cells (Fig. 4B).

For determining whether sulforaphane may inhibit the early signaling pathway of PDGF-BB, we measured the expression levels of PDGF-Rβ, p-PLCγ1, p-Akt, and p-ERK1/2 in RASMCs treated with sulforaphane in the presence of PDGF-BB. Sulforaphane did not change the level of PDGF-BB-stimulated PDGF-Rβ, Akt, and PLCγ1 phosphorylation (Fig. 5). However, only 2.0 μM sulforaphane in the presence of PDGF-BB showed the significant difference of p-ERK1/2 expression from the PDGF-BB-alone-treated cells (Fig. 5B). These results suggest that p53 may be a molecular target for the inhibition of RASMC proliferation by sulforaphane, not the early signaling pathway.

3.4. Effect of sulforaphane on neointimal formation in the carotid artery injury model

To evaluate the effects of sulforaphane on neointimal formation, the area of newly formed intima was measured in the balloon-induced arterial vascular injury rat model. The neointimal formation in the groups treated with sulforaphane was significantly reduced compared to the balloon angioplasty group from the morphological observation (Fig. 6). Areas of neointima in the groups treated with sulforaphane at 2 and 4 μM were approximately 0.25 and 0.09 mm², respectively (Table 1). Areas of lumen in angioplasty group were significantly decreased compared with the normal control, indicating that lumen space may be constricted after balloon injury. Areas of lumen in the 2 μM sulforaphane-treated groups were increased by 2.8 folds, compared with balloon angioplasty group.

Immunohistochemical staining for PCNA, p21 and p53 was performed on sections from arteries explant at 14 days after balloon injury. Expression of PCNA in the balloon injury group was increased, but it in sulforaphane-treated group was decreased (Fig. 7). Expressions of p53 and p21 were increased compared with angioplasty group (Fig. 7). These results showed the same pattern as that in in-vitro study (Figs. 3, 4, 7).

Thus, the results from the areas of neointima and lumen, as well as the morphological observation of neointimal formation clearly indicate that sulforaphane may inhibit the neointimal formation in the carotid artery.

4. Discussion

Abnormal proliferation of vascular smooth muscle cells (VSMCs) in arterial walls acts as a critical contributing factor in the pathogenesis of atherosclerosis and restenosis after angioplasty, and also in the
Sulforaphane inhibited the expression of vascular adhesion molecule-1 in tumor necrosis factor (TNF)-α-stimulated VSMCs, and then blocked their migration \[15,16\]. Sulforaphane also inhibited PDGF-BB-induced cell proliferation \[16\]. In this study, we further investigated the molecular mechanism sulforaphane on the PDGF-induced proliferation of RASMCs. We demonstrated that sulforaphane potently inhibited PDGF-BB-stimulated proliferation without apoptosis through cell cycle arrest of RASMCs (Figs. 1, 2). Anti-proliferative activity of sulforaphane was shown in human ovarian and colon cancer cells \[5,12\]. Sulforaphane inhibited the expression of PCNA and cyclin E/CDK 2 in PDGF-BB-stimulated RASMCs (Figs. 3, 4A). The complex of cyclin E/CDK 2 formed in late G1 phase of cell cycle directs entry into S phase \[20,21\]. The protein levels of cyclin E and CDK 2 in quiescent VSMCs were low, and when the cells were stimulated with PDGF-BB, their expression levels were increased (Fig. 4A). Sulforaphane inhibited the PDGF-BB-induced increase in the level of cyclin E and CDK 2, and up-regulated the expression level of p21, the upstream regulatory protein in RASMCs (Fig. 4B). In porcine arteries, p21 induction was directly associated with the suppression of arterial smooth muscle cell proliferation after balloon catheter injury \[25,32\]. The expression level of p53, the upstream signaling molecule of p21 \[20,21\], in RSMCs treated with PDGF-BB plus sulforaphane, was increased (Fig. 4B).

We further studied the effects of sulforaphane on PDGF-signaling pathway in PDGF-BB-stimulated RASMCs. PDGF is a potent growth factor produced by VSMCs, endothelial cells, and platelets in injured vascular wall \[4,22\]. Once PDGF-BB is bound to its receptor in smooth muscle cells, three major signal transduction pathways including p-PLCγ1, p-Akt, and p-p-ERK1/2 can be activated \[14\]. In this study, we found that sulforaphane had no effect on the expressions of p-PDGF-Rβ, p-PLCγ1, and p-Akt (Fig. 6). Thus, the present results suggest that sulforaphane may inhibit the PDGF-BB-stimulated proliferation of RASMCs, through the blocking of p53 signaling pathway followed by cell cycle arrest.

In clinical study, single doses of about 200 μmol of broccoli sprout isothiocyanates (largely sulforaphane) given into human volunteers have reached peak concentration of 0.943–2.27 μmol/L in plasma at development of hypertension \[18\]. Sulforaphane inhibited the expression of vascular adhesion molecule-1 in tumor necrosis factor (TNF)-α-stimulated VSMCs, and then blocked their migration \[15,16\]. Sulforaphane also inhibited PDGF-BB-induced cell proliferation \[16\]. In this study, we further investigated the molecular mechanism sulforaphane on the PDGF-induced proliferation of RASMCs. We demonstrated that sulforaphane potently inhibited PDGF-BB-stimulated proliferation without apoptosis through cell cycle arrest of RASMCs (Figs. 1, 2). Anti-proliferative activity of sulforaphane was shown in human ovarian and colon cancer cells \[5,12\]. Sulforaphane inhibited the expression of PCNA and cyclin E/CDK 2 in PDGF-BB-stimulated RASMCs (Figs. 3, 4A). The complex of cyclin E/CDK 2 formed in late G1 phase of cell cycle directs entry into S phase \[20,21\]. The protein levels of cyclin E and CDK 2 in quiescent VSMCs were low, and when the cells were stimulated with PDGF-BB, their expression levels were increased (Fig. 4A). Sulforaphane inhibited the PDGF-BB-induced increase in the level of cyclin E and CDK 2, and up-regulated the expression level of p21, the upstream regulatory protein in RASMCs (Fig. 4B). In porcine arteries, p21 induction was directly associated with the suppression of arterial smooth muscle cell proliferation after balloon catheter injury \[25,32\]. The expression level of p53, the upstream signaling molecule of p21 \[20,21\], in RSMCs treated with PDGF-BB plus sulforaphane, was increased (Fig. 4B).

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**Table 1**

Comparison of histomorphometric indices in the balloon-injured carotid artery after treating with sulforaphane.

<table>
<thead>
<tr>
<th></th>
<th>Normal control (n = 5)</th>
<th>Balloon angioplasty (n = 10)</th>
<th>Sulforaphane (μM) 2 (n = 10)</th>
<th>4 (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area of carotid artery (mm²)</td>
<td>0.60 ± 0.06***</td>
<td>0.80 ± 0.04</td>
<td>1.05 ± 0.06***</td>
<td>0.81 ± 0.05</td>
</tr>
<tr>
<td>Area of lumen (mm²)</td>
<td>0.40 ± 0.15***</td>
<td>0.20 ± 0.02</td>
<td>0.56 ± 0.08***</td>
<td>0.54 ± 0.07***</td>
</tr>
<tr>
<td>Area of neointima (mm²)</td>
<td>0.40 ± 0.03</td>
<td>0.25 ± 0.10</td>
<td>0.09 ± 0.13***</td>
<td></td>
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</table>

The rat carotid artery coated with sulforaphane has been maintained for 14 days after the balloon-inflated injury. After sacrificing the animals, the areas of intima, lumen, and media were measured. The values are the average of similar experiments using the number of animals indicated in parenthesis and expressed as mean ± S.E.M.

*** P < 0.001 vs. balloon angioplasty.
1 h after feeding, and declined quickly [33], indicating that micro-molar levels of sulforaphane may be transient. In this study, we coated sulforaphane to the damaged artery, and kept the rats alive for 14 days. Thus, sulforaphane is necessary to be administered repeatedly to maintain micro-molar levels in plasma for more than two weeks for the inhibition of neointimal formation. Neointimal hyperplasia occurred when arterial walls were injured [14,19]. Sulforaphane was known to inhibit neointimal formation by targeting adhesion molecule through the suppression of NF-κb/GATA6 [15]. We showed that sulforaphane inhibited neointimal formation on the rat arterial injury model (Fig. 6 and Table 1). Patterns of PCNA, p53 and p21 expression in sulforaphane-treated carotid arteries of rats were same as those in cultured RASMCs (Figs. 3, 4, 7). However, the recovery of the denuded endothelial cell layer within the damaged carotid artery by sulforaphane was not known. Exposure of neural crest cells to sulforaphane was shown to induce Nrf2 activation, and its expression was decreased in Nrf2-deficient mice after treatment with butyl-hydroxyanisole [7,8]. In response to oxidative stress, two-phase Nrf2 expression was regulated by p53 and p21 [6]. In the induction phase, p53 enhanced the protein level of Nrf2 in a p21-dependent manner. In the repression phase, Nrf2-mediated cell survival was inhibited by p53. Thus, sulforaphane may stimulate Nrf2 expression through p53 signaling pathway.

In conclusion, these results suggest that sulforaphane may inhibit neointimal hyperplasia through the suppression of smooth muscle cell proliferation via p53 signaling pathway, followed by cell cycle arrest. Sulforaphane may be a promising candidate for the treatment of atherosclerosis and cardiovascular disease.

Acknowledgments

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References


