Original Contribution

Mechanism by which avenanthramide-c, a polyphenol of oats, blocks cell cycle progression in vascular smooth muscle cells

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Abstract

Previously, we reported that avenanthramide-c (Avn-c), one of the major avenanthramides, polyphenols of oats, inhibited the serum-induced proliferation of vascular smooth muscle cells (SMC), which is an important process in the initiation and development of atherosclerosis. In the present study, we further investigated its cell cycle inhibitory mechanism. Rat embryonic aortic smooth muscle cell line A10 was used in this study. Flow cytometry analysis revealed that treatment of A10 cells with 80 μM Avn-c arrested the cell cycle in G1 phase as indicated by an increase in the number of cells in G1 phase and a decrease in the number of cells in S phase. This cell cycle arrest was associated with a decrease in the phosphorylation of retinoblastoma protein (pRb), whose hyperphosphorylation is a hallmark of the G1 to S transition in the cell cycle. The inhibition of pRb phosphorylation with Avn-c was accompanied by a decrease in cyclin D1 expression and an increase in cyclin-dependent kinase inhibitor p21cip1 expression, without significant changes in p27kip1 expression. Furthermore, Avn-c treatment increased the expression level and stability of p53 protein, which could account for the increase of p21cip1 expression. Our results demonstrate for the first time that Avn-c, which is a unique polyphenol found in oats, arrests SMC proliferation at G1 phase by upregulating the p53-p21cip1 pathway and inhibiting pRB phosphorylation. This inhibitory effect of Avn-c on SMC proliferation is an additional indication for the potential health benefit of oat consumption in the prevention of coronary heart disease beyond its known effect through lowering blood cholesterol.

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Keywords: Avenanthamide; Smooth muscle cell; Proliferation; Cyclin D1; p53; pRb; p21cip1

Introduction

The proliferation of vascular smooth muscle cells (SMC) plays pivotal roles in the development and progression of atherosclerosis and restenosis following angioplasty [1–4]. Vascular SMC comprise the medial layer of the vascular wall, whereas the intimal layer of the artery contains a few SMC, which are scattered within the intimal extracellular matrix. Under normal conditions, SMC are maintained in a quiescent and contractile state, whereas, during the chronic inflammation of an artery, which causes a continuous production of pro-inflammatory cytokines, SMC undergo a phenotypic transformation from a quiescent contractile state to a more synthetic phenotype and then activate, proliferate, and migrate to the intimal layer, causing intimal thickening of the arterial wall [5–7]. These changes in the characteristics of SMC, which may elicit inflammatory reactions, also occur during restenosis after angioplasty or following the application of a stent in an artery [8]. These conditions are major problems that limit the long-term clinical success of these procedures in approximately 20–40% of the cases [9,10]. Therefore, in addition to lowering blood cholesterol and triglyceride levels, inhibition of arterial SMC proliferation through drugs and bioactive components of healthy food is considered of great benefit in the maintenance of vascular homeostasis and in the prevention of atherosclerosis and restenosis following angioplasty or stent application.

Abbreviations: SMC, smooth muscle cells; Avns, avenanthramides; FBS, fetal bovine serum; pRb, retinoblastoma protein; DMEM, Dulbecco’s modified Eagle’s medium; PI, propidium iodide; DMSO, dimethyl sulfoxamine; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PPRB, phospho-prb; ECL, enhanced chemiluminescence; CDK, cyclin-dependent kinase; MAP, mitogen-activated protein.

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was dose-dependently inhibited by Avn-c [20]. In the current bovine serum (FBS)-induced cell proliferation of vascular SMC development of atherosclerosis. We have also found that fetal cytokines and chemokines [19], which are important in the 

They also reduce production of several inflammatory adhesion molecules and their adhesion to monocytes extracted from oats inhibit human aortic endothelial cell expres-
sion among the Avns [18]. We have reported that Avns their bioavailability has been demonstrated in hamsters [16] and present in other cereal grains. Avns are present in relatively high concentrations in the whole grain (up to 200 mg/kg) [15], and more recently in humans [17]. Avn-c (Fig. 1), one of the three major Avns found in oats, has the highest in vitro antioxidant activity among the Avns [18]. We have reported that Avns extracted from oats inhibit human aortic endothelial cell expression of adhesion molecules and their adhesion to monocytes [19]. They also reduce production of several inflammatory cytokines and chemokines [19], which are important in the development of atherosclerosis. We have also found that fetal bovine serum (FBS)-induced cell proliferation of vascular SMC was dose-dependently inhibited by Avn-c [20]. In the current study we sought to determine the molecular mechanism by which Avn-c blocks SMC proliferation in vitro. Since the progression of the cell cycle in SMC is strictly regulated by pRb phosphorylation status [21], we focused on the signaling molecules p53, p21cip1, p27kip1, and cyclin D1, which are known to regulate pRb phosphorylation [22–24].

Materials and methods

Materials

A10 rat embryonic aortic smooth muscle cell line and its culture media, Dulbecco’s modified Eagle’s medium (DMEM), were purchased from ATCC (Manassas, VA). FBS was purchased from Gibco (Grand Island, NY). Propidium iodide (PI) and DNase-free RNase were obtained from Sigma (Saint Louis, MO). Monoclonal antibody against pRB (14001A) was obtained from Pharmingen (San Diego, CA). Antiphosphorylated pRb and anti-p53 antibodies were from Cell Signaling (Beverly, MA). Anti-p21cip1 antibody (C-19, sc-397) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Cyclin D1 and anti-p27kip1 antibodies were from Sigma. ECL Western assay kit (RPN 2108) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). The biocinchoninic acid protein assay kit was purchased from Pierce Chemical Co. (Rockford, IL). Avn-c was synthesized from caffeic acid and 5-hydroxyanthranilic acid as described [18,20].

Cell culture

We used A10 cells, a primary rat embryonic aorta cell line, which is commonly used as a model for human vascular SMC. These cells not only maintain the phenotype of vascular SMC but also have some advantages over primary cultures including morphological and biochemical stability. Further, compared to human vascular SMC, A10 cells have a longer lifespan and can be used for 10 to 20 passages [25]. A10 cells were grown in DMEM containing 10% (v/v) FBS, penicillin (0.075 mg/mL), streptomycin (0.05 mg/mL), and 20 mM Hepes (pH 7.4), in 95% humidified air and 5% CO₂. Stock cells were trypsinized and subcultured every 5 days at a 1:10 dilution.

Avn-c treatment and sample preparation

Avn-c was dissolved in dimethyl sulfoxamine (DMSO) and added to the cell culture medium with a maximum final DMSO concentration of 0.1%, which showed no cytotoxicity and cell proliferative effect as determined by trypan blue exclusion assay [20]. A10 cells were cultured into 60 mm dishes. After reaching an 80% confluent condition, cells were treated with different concentrations of Avns for 20 h. Total cell extracts were harvested with 2× protein sample buffer (0.14 M Tris/HCl (pH 6.8), 22.4% glycerol, 6% SDS, 0.02% bromophenoxy bromide, and 10% β-mercaptoethanol) by scraping the cells. The genomic DNA was sheared by passing it through a 21-gauge syringe needle several times. The homogenized cell lysates were boiled for 5 min and centrifuged at 12,000 rpm for 5 min. Protein half-life studies were performed as described before [26]. Briefly, cells were treated with Avn-c for 4–6 h, cycloheximide (20 μg/mL) was then added to inhibit further protein synthesis. Cells were harvested in 2X protein sample buffer at 0, 30, and 60 min after cycloheximide addition. Protein samples were analyzed by Western blot as described below.

Cell cycle distribution by flow cytometry

Cells at exponentially growing phase (~50% confluence) were cultured in the absence or presence of 80 μM Avn-c for 48 h. Cells were then harvested by trypsin/EDTA treatment, washed with PBS, and fixed with 70% ethanol at 4 °C for 1 h. Prior to analysis, cells were washed again with PBS and stained with 1.0 mL of propidium iodide solution containing 25 μg/mL PI and 10 μg/mL RNase in PBS at 4 °C for 30 min in the dark as previously described [27]. Cell cycle distributions were then
analyzed by flow cytometry using fluorescence-activated cell sorting analysis (FACSCalibur, Becton–Dickinson, Rockville, MD). To calculate the percentage of cells in respective phases of the cell cycle, the DNA content frequency histograms were deconvoluted using the ModFit software. Based on the DNA content, the cell cycle can be distinguished from G1 to S and G2-M phase.

**Immunoblotting for p53, p21cip1, p27kip1, cyclin D1, and pRB protein expression levels and pRB phosphorylation**

Protein from 5 × 10^4 cells (25 μg) was subjected to electrophoresis on 7.5–10% SDS–polyacrylamide gels and electrotransferred to nitrocellulose membrane. Nonspecific binding was blocked by incubating with Tris-buffered saline (TBS) [137 mM NaCl and 15 mM Tris–HCl (pH 7.6)] containing 5% nonfat milk (w/v) for 1 h at room temperature and then probed overnight with primary antibodies against p53, p21cip1, p27kip1, cyclin D1, pRB, and phospho-pRB (ppRB). After washing in TBS–Tween 20 buffer (0.075% v/v), the membranes were incubated with horseradish-peroxidase-conjugated anti-IgG antibody. Proteins bound to the primary antibodies were detected with ECL detection reagents.

**Statistical analysis**

Results are presented as mean ± SE. The statistical analyses were performed using analysis of variance (ANOVA) and Newman–Keuls multiple comparison test for the assessment of significance. *p < 0.05 is considered statistically significant.

**Results**

**Effects of Avn-c on cell cycle distribution**

In our previous study, we showed that Avn-c inhibited FBS-induced cell proliferation as determined by [3H]thymidine incorporation and cell number counting [20]. Here we further investigated the effect of Avn-c on cell cycle distribution by flow cytometry assay. As shown in Fig. 2, in nontreated A10 cells at exponential growth phase, about 40% of the cells were in S phase while 49.7% of cells were in G0/G1 phase. When the growing cells were treated with 80 μM Avn-c for 48 h, the population of cells in S phase decreased to 25.7% while the population of cells in G0/G1 phase increased to 66.9%. This result indicated that Avn-c inhibited the cell proliferation of SMC and arrested the cell cycle at G1 phase.

**Effects of Avn-c on phosphorylation of pRB**

Based on the above findings that treatment of A10 cells with Avn-c arrests the cell cycle at G1 phase, we next analyzed the effect of Avn-c treatment on cell cycle regulatory molecules involved in the progression of cells from G1 to S phase. pRB, the product of the retinoblastoma tumor suppressor gene, is a major cell cycle regulator and its hyperphosphorylation (ppRb) is necessary for G1–S phase transition [21].
To elucidate the mechanism by which Avn-c inhibits the cell cycle progression, the changes in pRb phosphorylation status were measured using specific antibodies against pRb and phospho-pRb. In quiescent cells, as shown in Fig. 3Aa, most of the pRb is hypophosphorylated (lane 1). After the stimulation with 10% FBS, the amount of hyperphosphorylated forms (ppRb) markedly increased after 20 h (lane 2). Avn-c treatment significantly and dose-dependently attenuated the FBS-induced pRb phosphorylation as shown in Fig. 3A (lanes 3–5) and Fig. 3B. To ensure that the observed effect was not due to Avn-c decreasing the total amount of pRb, the expression of the total amount of pRb was assessed by anti-pRb antibody, which recognizes both hyper- and hypophosphorylated pRb. As shown in Fig. 3Ab, the expression of the total amount of pRb did not change significantly (as also shown in Fig. 3B) upon FBS stimulation and Avn-c treatment, which indicates that the inhibitory effect of Avn-c was due only to the inhibition of phosphorylation of pRb and not to the inhibition of the total pRb protein level. Fig. 3Ac shows the β-actin expression as a loading control.

Effects of Avn-c on cyclin D1 expression in A10 cells

The pRb phosphorylation takes place partly through the action of the cyclin D/CDK complex. Cyclin D is expressed upon mitogen stimulation, and it becomes associated with cyclin-dependent kinase to form the active cyclin D/CDK complex, which is responsible for the phosphorylation of pRb in G1 phase [28]. Since pRb phosphorylation is inhibited by Avn-c treatment, we examined whether this inhibitory effect is through inhibition of cyclin D1 expression. After 20 h stimulation of quiescent A10 cells with FBS, the amount of cyclin D1 expression was measured by Western blotting using polyclonal anti-cyclin D1 antibodies. As shown in Fig. 4A, in quiescent cells, cyclin D1 expression was undetectable. After 20 h of FBS stimulation and Avn-c treatment, which indicates that the cells entered from a quiescent state to a proliferating state. Avn-c treatment (0–120 μM) attenuated the FBS-induced cyclin D1 expression, and its effect appears to be dose dependent (Fig. 4B).

Effect of Avn-c on p53 expression

Induction of the tumor suppressor p53 has been implicated in control of cell growth of SMC both in vitro [29,30] and in vivo
Furthermore, there are reports showing that some polyphenolic antioxidants from green tea or red wine can increase p53 protein levels [26]. We therefore examined the effect of Avn-c treatment on p53 protein levels. Following a 24-h treatment of the A10 cells with a range of Avn-c concentrations (0–120 μM) in the presence of FBS, the level of p53 protein in cell extracts was assessed by Western blot analysis. The Avn-c treatment of A10 cells dose-dependently increased the amount of p53 protein (Fig. 5A). After exposure to 40, 80, and 120 μM Avn-c, the p53 protein level increased by 1.8-, 3-, and 5-fold, respectively, as shown in Fig. 5B.

p53 protein level is mainly regulated at the posttranslational level via control of protein stability [33]. To determine whether an increase in protein stability by Avn-c treatment might have contributed to the increased amount of p53 as observed above, we compared the half-life of p53 protein in Avn-c-treated and untreated A10 cells. A10 cells were treated with 0 or 80 μM Avn-c for 6 h, followed by 20 μg/mL cycloheximide treatment to block further protein synthesis. Whole-cell extracts were prepared at different time points (0, 30, and 60 min) and p53 protein levels were measured by Western blot. In the untreated control cells, the amount of p53 decreased to less than 50% after 30 min and decayed to less than 10% after 60 min (Fig. 5C).

This indicates that the half-life of p53 in A10 cells is within 20–30 min, which is consistent with values observed in other cells for wild-type p53 protein [33]. Treatment of A10 cells with Avn-c resulted in a higher expression of p53 protein compared with untreated control, which is consistent with our above findings (Fig. 5C). More than half of the p53 protein remained 1 h after cycloheximide treatment. Thus, exposure of A10 cells to Avn-c increases the half-life of p53. It appears that Avn-c treatment of A10 cells results in a significant stabilization of p53 protein, which might account for the increase in the expression of p53 protein levels by Avn-c.

**Effect of Avn-c on p21cip1 expression**

p21cip1 is the main target of p53-mediated cell cycle inhibition [28]. Upregulation of p21cip1 has also been shown to be one of the main mechanisms involved in the prevention of pRb phosphorylation, which causes G1 cell cycle arrest [30]. Thus, by Western blot, we examined the effect of Avn-c on p21cip1 protein expression. As shown in Fig. 6A, in growing phase cells upon FBS stimulation, p21cip1 expression level is low. Avn-c treatment markedly increased its expression level, and this effect was dose dependent (Fig. 6B). Probing the same membrane with anti-β-actin antibody showed that the observed p21cip1 protein levels were not due to protein loading (Fig. 6A).

**Effect of Avn-c on p27kip1 expression**

p21cip1 is the major transcriptional target of p53 and plays an important role in controlling G1 arrest. However, p53 has also been shown to regulate the other members of the CDK inhibitor family, such as p27kip1 [34]. Therefore, we also examined the effect of Avn-c on p27kip1 expression level. As shown in Fig. 6A, contrary to p21cip1, Avn-c had no significant effect on the expression of p27kip1 protein.

**Discussion**

Proliferation of SMC in the injured arterial wall during the development of atherosclerosis significantly contributes to the intimal thickening and restenosis following angioplasty and stent application [35]. Our in vitro cell culture results show for the first time that synthetically prepared Avn-c, a polyphenol found uniquely in oats, inhibits FBS-induced SMC proliferation by arresting the cell cycle at G1 phase. This inhibitory effect of Avn-c is mediated by several key growth-regulatory proteins that are known to play important roles in cell proliferation including pRb, cyclin D1, p21cip1m, and p53.

Mitogen-induced vascular SMC proliferation has been reported to be inhibited by many agents such as retinoids [24], sodium salicylate [23], and doxazocin [22]. Most of these agents are inhibitors of pRb phosphorylation. pRb is the major negative regulator of cell division and exerts most of its effect in the first two thirds of the G1 phase of the cell cycle [21]. Hypophosphorylated pRb binds to the transcription factor E2F-1, resulting in an inhibition of E2F-dependent transcriptions...
including the genes for thymidine kinase and dihydrofolate reductase, which are important for the cell cycle progression [36]. In contrast, phosphorylation of pRb leads to the inactivation of its growth-inhibitory effects by releasing E2F and causes cell cycle entering into the S phase [21]. Our data clearly show that Avn-c blocks the FBS-induced phosphorylation of pRb in a dose-dependent manner. Since phosphorylation of pRb occurs at the late G1 phase [37,38], the inhibition of pRb phosphorylation by Avn-c explains the cell cycle arrest at G1 phase.

A key molecule that regulates the phosphorylation of pRb is cyclin D1, which is required for cell cycle progression from G1 to S phase [39,40]. The expression of cyclin D1 increases in response to mitogenic stimulation and rapidly declines when growth factors or mitogens are withdrawn [28]. We observed response to mitogenic stimulation and rapidly declines when growth factors or mitogens are withdrawn [28]. The expression of cyclin D1, which is required for cell cycle progression from G1 to S phase [39,40]. The expression of cyclin D1 increases in response to mitogenic stimulation and rapidly declines when growth factors or mitogens are withdrawn [28]. We observed response to mitogenic stimulation and rapidly declines when growth factors or mitogens are withdrawn [28]. The expression of cyclin D1, which is required for cell cycle progression from G1 to S phase [39,40]. The expression of cyclin D1 increases in response to mitogenic stimulation and rapidly declines when growth factors or mitogens are withdrawn [28]. We observed response to mitogenic stimulation and rapidly declines when growth factors or mitogens are withdrawn [28].

whether Avn-c can affect these early response signals remains to be elucidated.

p21cip1 is a universal inhibitor of CDK, which phosphorylates pRb. Overexpression of p21cip1 by means of adenoviral gene delivery effectively prevented neointimal formation [42–44]. Upon FBS stimulation of quiescent A10 cells, and Avn-c dose-dependently suppressed the expression of this cell cycle regulatory protein. Transcriptional regulation of cyclin D1 is a key event in G1 progression [28]. Activation of Ras increases the cyclin D promoter activity [28,41], and the mitogen-activated protein (MAP) kinase is thought to be involved in this Ras-mediated transcription regulation [41]. Whether Avn-c can affect these early response signals remains to be elucidated.

The control of proliferation of vascular SMC has been reported to be regulated by p53 [26,45]. p53 is a key tumor suppressor protein and transcription factor of p21cip1 [46–48]. It has been shown that transfection of bovine SMC with exogenous p53 caused the cell cycle to be arrested at G1 phase [29]. However, induction of cell cycle arrest is mediated mainly by upregulating p21cip1. In FBS-stimulated growing cells, the p21cip1 expression level was low. Upon Avn-c treatment, the expression of p21cip1 increased in a dose-dependent manner but had no effect on p27kip1 level. Therefore, we suspect that changes in p53 levels with Avn-c might be responsible for the increase of p21cip1 level, which causes cell cycle arrest at G1 phase. We found that Avn-c at 120 μM increased p53 protein level approximately five-fold (Fig. 5B), which is in line with earlier data showing polyphenol upregulation of p53 expression [26,49,50]. Posttranslational modifications of p53 are important for stabilization and activation of this cell cycle regulatory protein [33,51]. Hofmann and Sonenshein [26] reported that the green tea polyphenol, epigallocatechin-3-gallate, increased p53 protein levels in SMC by increasing its stability. We found that Avn-c treatment of A10 cells increased the half-life of p53 from about 20 min to more than 1 h. Evidence indicates that nitric oxide (NO) is capable of stabilizing and accumulating p53 expression levels [52], and NO is considered to be an important modulator in regulation of cell proliferation and apoptosis. Earlier, we have shown that Avn-c upregulated NO production by SMC in a dose-dependent manner [20]. Thus, it is possible that the increase in p53 protein level by Avn-c might be due to Avn-c upregulation of NO production.

This increased stability of p53 with Avn-c treatment might have contributed to the upregulation of p21cip1 expression. In addition to p53-dependent expression, a p53-independent, Erk-1/-2 MAP kinase cascade-dependent mechanism is also known to regulate p21cip1 expression [53]. A similar study reported by Martin et al. [54] showed that delphinidin, a polyphenol of fruits and vegetables, inhibited endothelial cell proliferation by upregulating p21cip1 and activation of ERK-1/-2. Since Avn-c is also a polyphenol, it may upregulate p21cip1 independent of Avn-c upregulation of p53, which needs to be investigated.

In summary, these in vitro studies demonstrated the molecular mechanisms by which Avn-c effectively arrested the proliferation of SMC at G1 phase. This growth-inhibitory effect is attained by Avn-c acting on multiple steps upstream of pRb, inhibition of cyclin D1 expression and upregulation of p21cip1 expression, which our data show at least partly to be due to the increased stability of p53. At present, the primary target of Avn-c on SMC is not known and needs further elucidation. Nevertheless, the inhibitory effect of Avn-c on SMC proliferation is one potential mechanism by whichavenanthramides of oats may contribute to the health benefit effect of regular oat consumption in the prevention of cardiovascular disease.

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