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Avenanthramide, a polyphenol from oats, inhibits vascular smooth muscle cell proliferation and enhances nitric oxide production

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Abstract

The proliferation of vascular smooth muscle cells (SMC) and impaired nitric oxide (NO) production are both crucial pathophysiological processes in the initiation and development of atherosclerosis. Epidemiological data have indicated that diets rich in whole grain foods are associated with a reduced risk of developing atherosclerosis. Avenanthramides are polyphenols found exclusively in oats (*Avena sativa* L.). The present study was conducted to examine the effect of synthetically prepared avenanthramide-2c on the proliferation of SMC and NO production by SMC and human aortic endothelial cells (HAEC). Avenanthramide-2c significantly inhibited serum-induced SMC proliferation. At a concentration of 120 μM, avenanthramide-2c inhibited more than 50% of SMC proliferation, as measured by [^{3}H] thymidine incorporation, and increased the doubling time of rat SMC line (A10) from 28 to 48 h. Treatment of human SMC with 40, 80, and 120 μM avenanthramide-2c inhibited cell number increase by 41, 62, and 73%, respectively. In addition, avenanthramide-2c treatment significantly and dose-dependently increased NO production in both SMC and HAEC. At a concentration of 120 μM, avenanthramide-2c increased NO production by three-fold in SMC, and by nine-fold in HAEC. These increases were in parallel with the up-regulation of mRNA expression for endothelial NO synthase (eNOS) in both vascular SMC and HAEC. These results suggest that the avenanthramides of oats may contribute to the prevention of atherosclerosis through inhibition of SMC proliferation and increasing NO production.

Keywords: Avenanthramide; Atherosclerosis; Vascular smooth muscle cell; Endothelial cell; Cell proliferation; Nitric oxide; eNOS mRNA; Oats

1. Introduction

The fiber and antioxidant components of cereal grains are believed to be the major contributing factors to their health benefits in the prevention of cardiovascular disease [1–3]. Epidemiological evidence indicates that a higher intake of oats is associated with a reduced risk of coronary heart disease, which is the major cause of death in Western countries [4,5]. This effect of oats (*Avena sativa* L.) has been mainly attributed to its cholesterol-lowering effect. However, the consumption of oats also improves endothelial function [5]. Although the mechanism of this effect is not fully known, it might be through modulation of nitric oxide (NO) production by the vessel wall. In addition to containing soluble fibers, which contribute to the reduction of cholesterol, oats, like all monocot cereal grains, contain a number of phytochemicals possessing a phenolic moiety [6] with the capability to scavenge free radicals and thus exhibit antioxidant activity in vitro. Moreover, oats contain a unique group of low-molecular weight soluble phenolic compounds, the avenanthramides [7–9], which are not present in other cereal grains. Avenanthramides extracted from oats or synthetically prepared exhibit antioxidant activities [7,10,11]. It is important to note that avenanthramides constitute by far the major unbound phenolic antioxidants present in the oat kernel [12,13] including the bran and sub-aleurone layers [10]. Avenanthramide-2c, one of three major avenanthramides in oats, comprises about one-third of the total avenanthramide concentration in oat grain, and this avenanthramide has the

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highest antioxidant activity in vitro [10]. Bioavailability of avenanthramides has been demonstrated in hamsters [14] and recently in humans [15].

Earlier, we showed that a mixture of avenanthramides extracted from oats inhibited expression of several adhesion molecules including ICAM-1, VCAM-1, and E-selectin by human aortic endothelial cells (HAEC) [16]. We also reported that avenanthramides can inhibit the expression of pro-inflammatory cytokines IL-6, IL-8, and MCP-1 in activated HAEC. Since IL-8 is known to enhance smooth muscle cell (SMC) proliferation [17] and thus contributes to the development of atherosclerosis, we hypothesized that avenanthramides may have an inhibitory effect on SMC proliferation.

Several studies showed that impaired NO synthesis is often present in atherosclerosis [18,19]. Although endothelial cells are by far the main site of vascular NO synthesis, vascular SMC do express endothelial NO synthase (eNOS) and produce NO. Phenotypic changes that occur in SMC during lesion development may also impair the expression of eNOS and the production of NO by SMC [20]. Therefore, in this study we sought to examine the potential inhibitory effect of avenanthramides on SMC proliferation and NO biosynthesis by endothelial cells and SMC. To this end, supplementing SMC with synthetically prepared avenanthramide-2c inhibited SMC proliferation. Avenanthramide-2c also increased expression of eNOS mRNA and the production of NO by both HAEC and SMC.

2. Materials and methods

2.1. Materials

A10 cell line, derived from rat embryonic aortic smooth muscle cells, and Dulbecco's modified Eagle's medium (DMEM) were purchased from American Type Culture Collection, ATCC (Manassas, VA). Human aortic smooth muscle cells (SMC) and human aortic endothelial cells (HAEC) and the culture medium were from Cambrex (Walkersville, MD). [^{3}H] Thymidine was from DuPont-New England Nuclear (Boston, MA). Fetal bovine serum (FBS) and other tissue culture reagents were purchased from Gibco BRL (Grand Island, NY). RNeasy Mini Kit was from QIAGEN (Valencia, CA). All RT-PCR reagents were purchased from Invitrogen (Carlsbad, CA), and 4,5-diaminofluorescein (DAF-2) was from Sigma Chemicals (St. Louis, MO).

2.2. Synthesis of avenanthramide

Avenanthramide-2c [*N*-(3′,4′-dihydroxycinnamoyl)-5-hydroxyanthranilic acid] (Fig. 1) was synthesized from caffeic acid and 5-hydroxyanthranilic acid as described by Peterson et al. [10], using the method described by Collins [7]. The purity of the avenanthramide was verified by HPLC, and the structure was confirmed to be authentic by HPLC retention times, UV spectra, and NMR spectroscopy. The ^{1}H NMR spectra corresponded with those reported by Collins [7].

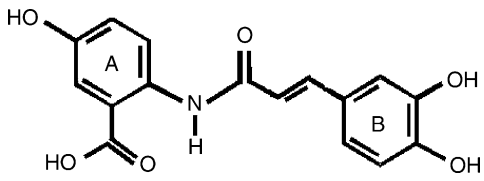

Fig. 1. Chemical structure of synthetic avenanthramide-2c.

2.3. Cell culture

A10 cells were grown in DMEM with 10% (v/v) FBS, 4.5 g/L glucose, 4 mM L-glutamine, 1.0 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 100 units/mL penicillin/streptomycin and 20 mM Hepes pH 7.4. Cells were generally used within 8–18 passages after their arrival. Human SMC were cultured in SMBM medium (Cambrex) containing 10% FBS and used between passages 4 and 5. HAEC were cultured in EBM-2 medium supplemented with 2% FBS, 1 μg/mL hydrocortisone, and 0.01 μg/mL epidermal growth factor. All cell cultures were maintained at 37 °C in a humidified incubator supplied with a 95% air and 5% CO_2 atmosphere. Avenanthramide was dissolved in dimethyl sulfoxide (DMSO) and added to the cell culture medium with a maximum final DMSO concentration of 0.1%. This showed no cytotoxicity as determined by trypan blue exclusion assay.

2.4. Measurement of vascular SMC proliferation

2.4.1. [^{3}H] Thymidine incorporation

A10 or human SMC were seeded into a 24-well plate at equal density. After reaching 80% confluence, the cells were synchronized to a quiescent condition by serum starvation for 48 h. Cells were then stimulated with 10% of FBS in the absence or presence of different concentrations of avenanthramide for 24 h. Cells were pulsed with 1.0 μCi/mL of [^{3}H] thymidine during the last 4 h of the incubation. After washing the cells with cold PBS, DNA was precipitated with 10% trichloroacetic acid, solubilized with 0.1 N sodium hydroxide, and counted in a scintillation counter. Each concentration point was done in triplicate wells, and at least three independent experiments were performed. Results are expressed as the standard error of the mean (mean ± S.E.M.).

2.4.2. Cell number determination

A10 or human SMC cells were seeded into 24-well or 12-well plates. After 4 h different concentrations of avenanthramide were added to different wells in triplicate. At different time points (2, 3 and 4 days) the cells were trypsinized and the total cell numbers were counted using a hemocytometer. Trypan blue exclusion test was carried out to determine the cell viability.

2.5. Nitric oxide (NO) production assay

NO production was measured by the 4,5 diaminofluorescein (DAF-2) fluorescence assay as described with modifications [21]. Briefly, SMC and HAEC were seeded into 24-well plates and allowed to grow to 90% confluence. The cells were pretreated with different concentrations of avenanthramides for 24 h. The culture medium of cells treated with avenanthramide was then changed to PBS, and the culture medium of non-treated cells was changed to PBS either containing 100 μM L-arginine and 1 μM bradykinin (positive control), or containing 100 μM L-arginine, 1 μM bradykinin and 1 μM L-NAME (negative control). Then 0.1 μM of DAF-2 was added to each well. After another 2 h of incubation, 200 μL of the supernatants were transferred to black microplates and their fluorescence was measured with a fluorescence microplate reader set for excitation at 485 nm and emission at 538 nm.

2.6. Measurement of eNOS expression

Real-time PCR was used to determine the effect of avenanthramide-2c on eNOS mRNA expression level. Total RNA was extracted using RNeasy Mini Kit (QIAGEN) and quantified spectrophotometrically at 260 nm. Twenty microliters of first-strand cDNA was synthesized from 1.5 μg of total RNA by using 300 ng of random hexanucleotide primers and 200 units of SuperScript II (Invitrogen) at 42 °C for 1 h followed by heat inactivation of reverse transcriptase at 70 °C for 15 min. PCR primers were designed based on the published gene sequences and were synthesized by Tufts University Core Facility (Boston, MA). The primer sequences used for eNOS were: 5′-ATCCTGGCAAGCCCTAAGACC-3′ (upstream) and 5′-TGGTAGCGTTTGCTGATCCCG-3′ (downstream). Beta-actin was used as an internal control with the primers: 5′-TTGTAACCAACTGGGACGATATGG-3′ (upstream), 5′-CACAATGCCAGTGGTA-CGACC-3′ (downstream). Fifty microliters of PCR reaction mixture contained 5 μL cDNA, up- and downstream primers (200 nM), and 25 μL of SYBR Green PCR master mix. Real-time PCR was performed on an ABI 4400 for amplification for 40 cycles with a cycle program of 95 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. The calculated threshold cycle (CT) value for each transcript was normalized against the corresponding β-actin CT value.

2.7. Statistical analysis

Results are presented as mean ± S.E.M. The statistical analyses were performed using analysis of variance (ANOVA) and Student's–Newman–Keuls test to determine the statistical differences between the control and avenanthramide treatments. When the results of two or more treatment groups were compared, we applied one-way or two-way ANOVA tests. *P*-values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Effect of avenanthramide on FBS-induced cell proliferation of vascular SMC

First [^{3}H] thymidine incorporation into DNA was measured to determine the effect of avenanthramide-2c on cell proliferation. Stimulation of quiescent human SMC with 10% FBS induced a six- to seven-fold increase in [^{3}H] thymidine incorporation vs. unstimulated control cells ($P < 0.001$). Treatment with avenanthramide-2c inhibited FBS-induced DNA synthesis and thus cell proliferation (Fig. 2a). At a concentration of 120 μM, avenanthramide-2c inhibited cell proliferation by 50% without significant cytotoxicity, as determined by the trypan blue cell viability assay (Fig. 2b).

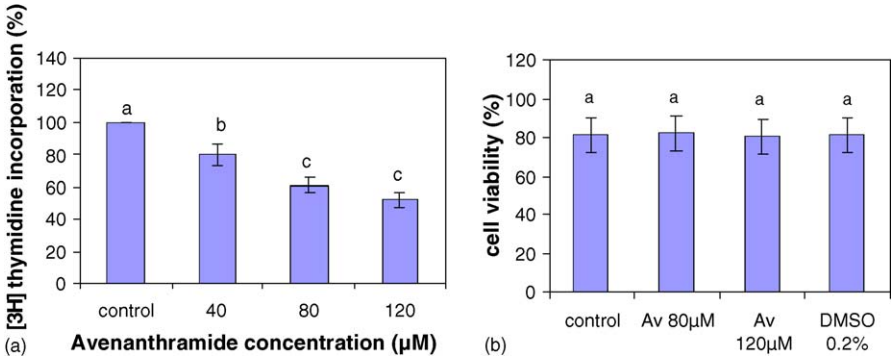

Fig. 2. Effect of avenanthramide-2c on FBS-induced DNA synthesis and viability of human vascular SMC. (a) [^{3}H] Thymidine incorporation. Confluent human vascular SMC were starved for 48 h and stimulated with 10% FBS in the presence or absence of different concentrations of avenanthramide-2c. Twenty-four hours later, [^{3}H] thymidine was added and cell proliferation was determined by measuring its incorporation into acid-precipitated material. (b) The viability of human SMC exposed to DMSO and avenanthramide-2c. Serum-starved cells were stimulated with 10% FBS, 0.2% DMSO, or different concentrations of avenanthramide-2c for 24 h at 37 °C. Cytotoxicity was measured by the trypan blue exclusion test. Data are means ± S.E.M. from three separate experiments, each conducted in triplicate. Bars not sharing the same character are significantly different ($P < 0.05$) from each other.

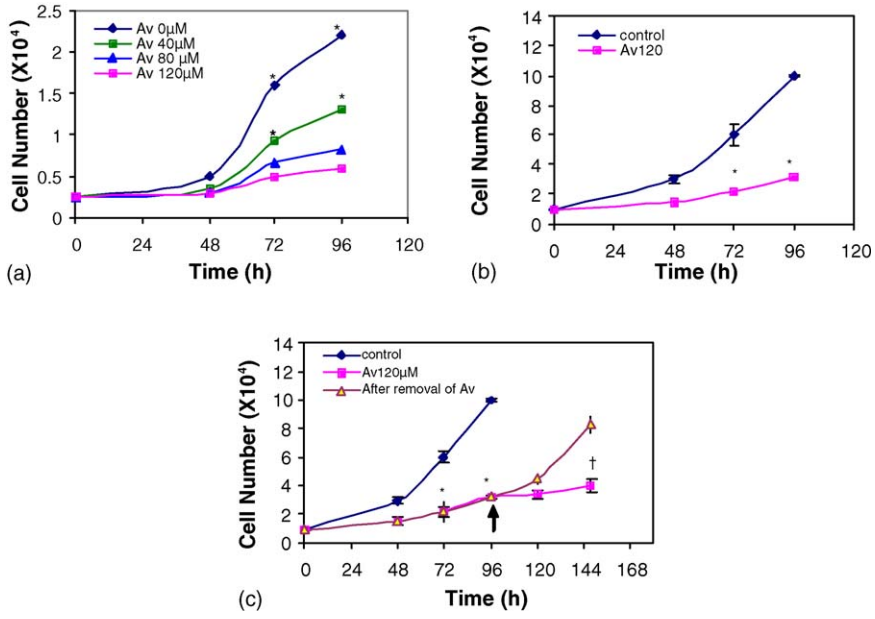

Fig. 3. Effect of avenanthramide-2c on FBS-induced vascular SMC proliferation. (a) Human vascular SMC were seeded into 24-well plate at a density of 0.2×10^4 cells/mL/well. After 4 h, cells were treated with different concentrations of avenanthramide-2c and the culture medium was changed daily. At each indicated time point, the viable cell number was determined by the trypan-blue exclusion test. $^*P < 0.05$ compared to other groups. (b) A10 cells were seeded into 12-well plates at a density of 1×10^4 cells/mL/well. After 4 h, avenanthramide-2c was added to culture medium to a concentration of 120 μM, and at each indicated time point the viable cell number was determined by the trypan-blue exclusion test. $^*P < 0.05$, avenanthramide treated cells vs. control. (c) A10 cells were seeded into 12-well plates at the density of 1×10^4 cells/mL/well. After 4 h, avenanthramide-2c was added to the culture medium to a concentration of 120 μM. The avenanthramide-containing medium was changed daily. After 96 h (arrow) avenanthramide was removed from medium of a set of culture dishes by washing with PBS two times and cells were cultured in fresh medium for another 48 h. The viable cell number was determined by the trypan-blue exclusion test. $^*P < 0.05$, control vs. cells supplemented with 120 μM avenanthramide. $^\dagger P < 0.05$, avenanthramide supplemented cells vs. cells after removal of avenanthramide. Data are the means ± S.E.M. from three separate experiments, each conducted in triplicate.

The results observed with the [^{3}H] thymidine incorporation as an index for DNA synthesis were verified by measuring increase in cell number. As shown in Fig. 3, 10% FBS caused a rapid increase in cell numbers; the doubling times for human SMC and A10 during the linear growth phase are calculated to be 28 and 33 h, respectively. The addition of 120 μM avenanthramides to A10 cells attenuated cell proliferation, and its doubling time increased to more than 48 h (Fig. 3b). For human SMC, we measured the effect of different concentrations of avenanthramides on cell growth. As shown in Fig. 3a, avenanthramide inhibited the cell number increase in a dose-dependent manner at 40 and 80 μM. After 96 h of treatment, 40, 80, and 120 μM of avenanthramide-2c inhibited cell number growth by 41, 62, and 73%, respectively. This result is consistent with those obtained from [^{3}H] thymidine incorporation. When avenanthramide was removed from the cell culture medium of A10 cells, cell proliferation resumed (Fig. 3c), indicating that the inhibitory effect was reversible.

3.2. Effect of avenanthramide on NO production

In addition to its vasodilation effect, NO has antiatherosclerotic properties by preventing platelet aggregation, leukocyte adhesion, SMC proliferation, and the expression of genes involved in atherogenesis. In this study, we used human SMC and HAEC to determine if avenanthramide-2c can influence NO production levels. As shown in Fig. 4a, avenanthramide treatment dose-dependently and significantly increased NO production in human SMC and at concentration of 120 μM, it increased NO production by threefold. In HAEC the stimulatory effect of avenanthramide-2c reached a maximum at a concentration of 80 μM with a nine-fold increase in NO production. No further significant increase was observed with the 120 μM treatment (Fig. 4b).

3.3. Effect of avenanthramide on eNOS mRNA level

Since NO is catalyzed by eNOS, the role of avenanthramide-2c in the induction of eNOS expression level was examined by quantitative real-time PCR. As shown in Fig. 5, eNOS mRNA in both human SMC and HAEC was upregulated by avenanthramide treatment. Compared with the control, treatment with 80 μM avenanthramide-2c increased the eNOS mRNA expression level by 2.2- and 2.4-fold in human SMC and HAEC, respectively. The enhancing effect is dose-dependent and the pattern is consistent with the results obtained for NO production (Fig. 4).

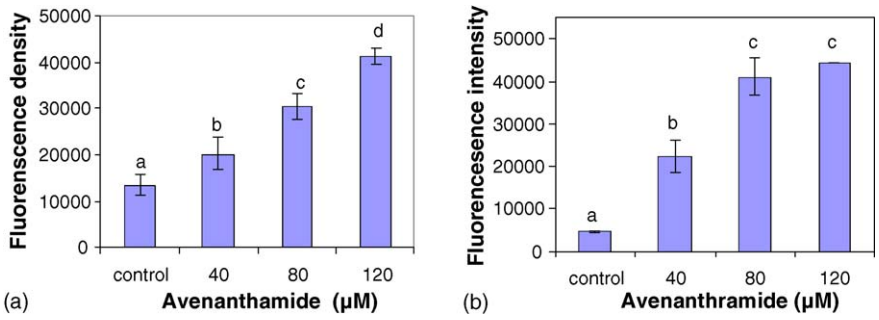

Fig. 4. Effect of avenanthramide-2c on NO production. Human vascular SMC (a) and HAEC (b) were seeded into 24-well plates and cultured to a 90% confluent condition in 10% FBS culture medium. The cells were pretreated with different concentrations of avenanthramide-2c for 24 h The culture medium of cells treated with avenanthramide was then changed to PBS, and the culture medium of non-treated cells was changed to PBS containing either 100 μM L-arginine and 1 μM bradykinin (positive control), or 100 μM L-arginine, 1 μM bradykinin and 1 μM L-NAME (negative control). Then DAF-2 was added to each well to a concentration of 0.1 μM. After another 2 h of incubation, the fluorescence was measured at 485 nm excitation and 538 emission with a fluorescence microplate reader. Data are means ± S.E.M. from three separate experiments, each in triplicate. Bars not sharing the same character are significantly different ($P < 0.05$) from each other.

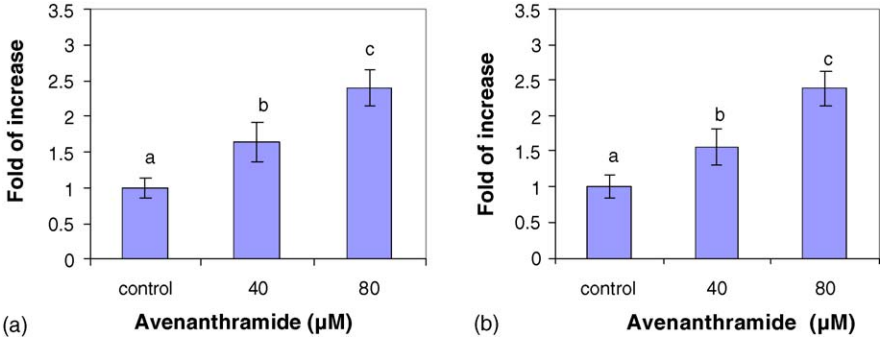

Fig. 5. Effect of avenanthramide-2c on eNOS mRNA expression measured by real-time PCR. (a) eNOS mRNA expression level in human vascular SMC. (b) eNOS mRNA expression level in HAEC. Data are means ± S.E.M. from three separate experiments, each in triplicate and expressed as fold increase compared with control. Bars not sharing the same character are significantly different ($P < 0.05$) from each other.

4. Discussion

In the present study, we found that synthetically prepared avenanthramide-2c, one of the major forms of avenanthramides found in oats, significantly inhibited A10 and human SMC proliferation in response to serum stimulation. This inhibition was reversible and dose-dependent at concentrations of 40 and 80 μM. We also found that avenanthramide-2c significantly increased NO production and eNOS mRNA expression levels in both SMC and HAEC. Both effects are known to contribute toward preventing the progression of coronary heart disease and have not previously been attributed to this class of compounds [1–3,20,22]. Our results provide experimental evidence consistent with the findings that regular consumption of oats is associated with a reduced risk of coronary heart disease [4,5,23].

It appears that the growth inhibitory effects of avenanthramides are reversible. When we removed avenanthramides from the culture medium and washed the cells with PBS twice, the increase in cell numbers did not differ significantly from those cells treated with avenanthramides during the 24 h period (Fig. 3c). However, after 48 h, the cell number of washed cells was increased significantly compared with the cells treated with avenanthramides. Therefore, the inhibitory effect of avenanthramides on cell proliferation remains for some time following exposure until it is metabolized or the cell recovers from the avenanthramide effect.

Vascular SMC in healthy vessel walls are commonly quiescent, and the intimal layer contains only a few of these cells scattered within the intimal extracellular matrix. However, in injured blood vessels and as fatty streaks evolve into more complicated atheroma lesions, these cells undergo a phenotypic transformation. They become activated and then they proliferate and migrate to the intimal layer where they accumulate lipids and participate in plaque formation [22]. The inhibition of this process is considered to be of great benefit in the maintenance of vascular homeostasis and in the prevention and development of atherosclerosis as well as restenosis following angioplasty. In our present study, we found that avenanthramide-2c could significantly inhibit serum-induced SMC proliferation from both human aorta and rat (A10) cells indicating that a common pathway(s) of serum-induced cell proliferation is probably hindered by avenanthramide exposure. A10 cells possess both normal and transformed SMC phenotypes, which are present in atherosclerotic lesions [24]. Serum contains several growth factors including platelet-

derived growth factor and basic fibroblast growth factor, which can induce vascular SMC proliferation in vivo and in vitro. There is mounting evidence showing that in SMC, the cell proliferation signaling pathways are redox-sensitive, and that antioxidants suppress cellular hyper-proliferation [25–28]. Avenanthramides are polyphenols from oats with known in vitro antioxidant activity [10]. It is plausible that these compounds modulate the cell cycle and proliferation through either antioxidant activity or through direct interaction with cellular signaling molecules that regulate cell proliferation. The mechanisms by which avenanthramide-2c inhibits SMC cycle progression and determines the primary molecular targets in cell cycle signaling pathways are currently under investigation in our laboratory.

While endothelial cells are by far the main site of vascular NO synthesis, vascular SMC also produce NO, which is important in regulating vascular function [29,30]. In this study, we found that avenanthramide-2c significantly increased production of NO by both SMC and HAEC, and this increase was found to be parallel with the up-regulation of mRNA for eNOS, which is the predominant form in both vascular SMC and endothelial cells. NO biosynthesis pathways play a major role in determining the structure and function of the vessel wall during normal physiologic conditions and during phenotypic changes that occurs in SMC in atherosclerosis [20]. Reduced bioavailability of NO is thought to contribute to an increase in blood pressure and to the development of atherosclerosis [31,32]. The mechanism(s) by which avenanthramide-2c up-regulates eNOS expression and NO production requires further elucidated.

The antiatherosclerotic effects of avenanthramide-2c reported here are consistent with our earlier findings of the potential health benefit of oat avenanthramides on cardiovascular disease (CVD). Previously we reported that these polyphenols extracted from oats reduced expression of adhesion molecules by endothelial cells and adhesion to monocytes in culture and that they suppressed production of pro-inflammatory cytokines and chemokines in activated endothelial cells [16]. These findings, and data from our current study, suggest that avenanthramides from oats are important dietary polyphenols that can contribute to the prevention of CVD.

The plasma bioavailability of avenanthramides following oral ingestion of avenanthramides has been demonstrated in hamsters by Chen et al. [15]. They also reported oat phenolic extract containing avenanthramides reduced in vitro copper-induced oxidation of low density lipoproteins from these animals with a synergetic effect with vitamin C. Ji et al. [33] reported the in vivo biological effect of avenanthramides on exercising rats. They reported that avenanthramide supplementation increased superoxide dismutase (SOD) activity in skeletal muscle, liver, and kidneys. It further increased glutathione peroxidase activity in the heart and attenuated exercise-induced reactive oxygen species. These observations indicate that these compounds are absorbed and incorporated into the plasma lipoproteins and different tissues and that they exert some in vivo biological effects. More recently, the plasma bioavailability of avenanthramides has been demonstrated in human volunteers [15]. While these are only a limited number of in vivo observations, they provide a basis for the potential in vivo effects of avenanthramides on the prevention of atherosclerosis as we observed in an in vitro cell culture system in this study. Further, we used a relatively high concentration of avenanthramides in our cell culture studies to demonstrate their biological effects on SMC and HAEC. However, it is plausible to observe a comparable, beneficial effect with relatively low concentrations of avenanthramides when they are introduced into the diets of animals or humans over a long period of the time. In addition, these compounds have the potential to be developed for therapeutic applications as drugs by slightly modifying their chemical structure (such as methyl ester substitution on A ring) to increase their bioavailability. These compounds have also the potential application to be developed as slow-release agents delivered in polymer fiber coated stents to the occluded vessels, thus eliminating the reduced bioavailability of these compounds due to intestinal absorption and metabolism.

In summary, we found that synthetically prepared avenanthramide-2c, which is one of the major avenanthramides found exclusively in oats, is effective in reducing mitogen-stimulated SMC proliferation and is capable of increasing NO production by SMC and HAEC in an in vitro cell culture system. Our findings may have both dietary and therapeutic implications in relation to the prevention of CVD. For example, regular inclusion of oats in the daily diet may not only provide a benefit from its soluble fiber content in the reduction of cholesterol, it would also provide these polyphenols, which we have demonstrated as having several antiatherogenic and anti-inflammatory activities. In addition, it may have the potential to be developed as anti-inflammatory and anti-atherogenic drug.

Acknowledgments

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