

Bioflavonoids Effectively Inhibit Smooth Muscle Cell-Mediated Contraction of Collagen Matrix Induced by Angiotensin II

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Abstract: Plant-derived bioflavonoids have been recognized to support arterial wall structural integrity and interfere with a variety of proatherosclerotic stimuli. In this study we tested the effects of bioflavonoids on the contractile activity of cultured human aortic smooth muscle cells (SMC) embedded in a 3-dimensional type I collagen matrix. Collagen I solution mixed with human aortic SMC in 24-well plates were allowed to form gels. Tested compounds were added to the wells, and the gels were set afloat by gentle tapping. Digital photographs of the gels were taken after 24 hours of incubation at 37°C. The area of contracted gel was measured and expressed as a percentage of the control gel area from 3 or more replicates. Expression of matrix metalloproteinase (MMP-2) in conditioned media was assessed by gel zymography. Different classes of bioflavonoids showed variable efficiency in inhibiting angiotensin II (ATII)-dependent collagen gel contraction by SMCs. An increase in the number of galate groups per catechin molecule was associated with increased inhibition of angiotensin II-dependent collagen gel contraction by SMC. Antioxidants (N-acetyl cysteine and ascorbic acid) did not inhibit collagen gel contraction. Bioflavonoid inhibition of collagen gel contraction by SMC correlated with inhibition of matrix metalloproteinase-2 expression. Bioflavonoids participate in the regulation of SMC-mediated contraction and have a strong potential in counteracting pathophysiological effects of ATII. Bioflavonoid activity depends on structural characteristics and can be related to extracellular matrix integrity.

Key Words: bioflavonoids, human aortic smooth muscle cell, collagen gel contraction

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Hypertension is one of the leading causes of disability and death from stroke, heart attack, and kidney failure. Worldwide, approximately 972 million adults were diagnosed with hypertension in 2000. By 2025 the number of cases is predicted to increase by ~60% to a total of 1.56 billion.¹

Furthermore, the lifetime risk of developing hypertension is ~90% for men and women ages 55–65.²

Diet and lifestyle have been reported to have a substantial impact on hypertension,³ and increased consumption of vegetables and fruits, such as the DASH diet, which contains high quantities of flavonols, flavanones, flavan-3-ols, β -carotene, β -cryptoxanthin, lycopene, lutein plus zeaxanthin, and phytosterols have been recommended.^{4,5} Bazzano et al⁶ reported that persons who consume more fruits and vegetables often have lower prevalence of important risk factors for cardiovascular disease (CVD), such as hypertension, obesity, and type 2 diabetes mellitus, and Liu⁷ proposed that the potent antioxidant activities of phytochemicals in fruit and vegetables result from synergy of the phytochemicals in fruits and vegetables. Numerous studies suggest a strong link between dietary intake of phytochemicals and reduced risk of cardiovascular disease.^{8,9}

Conventional medicine concedes that causes of hypertension are unknown in 90% of patients, and treatment is focused on treating symptoms of the disease: β -blockers, diuretics, and other high blood pressure medications are prescribed to artificially lower blood pressure without correcting the primary underlying problem—spasm of the blood vessel wall. According to Rath,¹⁰ the main cause of hypertension is a chronic deficiency of essential nutrients in arterial wall cells, including “relaxing factors” (nitric oxide), which decrease vascular wall tension and maintain normal blood pressure. Nutrients such as arginine, magnesium, vitamin C, and coenzyme Q₁₀ ensure optimum availability of these artery wall-relaxing factors.¹⁰ Deficiency of these nutrients can result in spasm and thickening of blood vessel walls with eventual elevation of blood pressure. A pilot study¹⁰ on 15 patients with severe hypertension ranging in age from 32 to 67 years found that treatment with a mixture of these nutrients over a period of 32 weeks resulted in improvement in all patients with an average decrease in systolic blood pressure of 16% and in diastolic pressure of 15%.

Currently angiotensin II is being regarded as the leading mediator of clinical systemic hypertension. Pharmaceutical compounds are being developed and routinely employed to reduce the symptoms of systemic hypertension by blocking angiotensin II formation from angiotensin I, that is, angiotensin-converting enzyme (ACE) inhibitors, or blocking its signaling cascade by specific inhibitors of type I angiotensin II receptors.¹¹ In addition, it has been reported in clinical and experimental research studies that angiotensin II plays a role in

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the restructuring of arterial walls in both atherosclerosis and hypertension. Therefore, blockage of angiotensin has been suggested as an important therapeutic consideration in the prevention and treatment of atherosclerotic disease.¹¹

Our previous *in vitro* studies^{12,13} demonstrated the antiatherogenic effects of the nutrient mixture of ascorbic acid, green tea polyphenols, lysine, proline, arginine, and N-acetyl cysteine using the model of cultured aortic smooth muscle cell (ASMC); the nutrient combination inhibited induced ASMC matrix invasion and migration, proliferation, and secretion of inflammatory mediators.

In this study we tested the effects of various bioflavonoids on the angiotensin II–induced contractile activity of cultured human ASMC embedded in a type I collagen matrix. We chose the collagen gel matrix model because it has been used to study the effect of test compounds on angiotensin-mediated contraction of various smooth muscle cells.^{14–16}

MATERIALS AND METHODS

Materials

Tissue culture plastics were obtained from Becton Dickinson, USA. Tissue culture supplies (growth media, antibiotics, and trypsin-EDTA) were obtained from Life Technologies, USA. Fetal bovine serum (FBS) was from BioWhittaker (Walkersville, MD). L-Ascorbic acid, bovine serum albumin (fraction V) (BSA), and other chemicals were from Sigma-Aldrich, USA.

Bioflavonoid extracts were provided as follows: citrus bioflavonoid extract from Brewster Foods, Reseda, CA; grape seed extract by Dry Creek Nutrition, Modesto, CA; pine bark extract by Natural Health Science, Hillside, NJ; green tea extract by Renaissance Herbs, Chatsworth, CA. Total bioflavonoid content, expressed as gallic acid equivalents, was evaluated based on manufacturer's specification.

Cell Culture

Human aortic smooth muscle cells (obtained from Clonetics) were cultured in DMEM (Dulbecco modified Eagle medium) supplemented with 10% fetal bovine serum, penicillin (100 $\mu\text{g}/\text{mL}$), and streptomycin (100 $\mu\text{g}/\text{mL}$) at 37°C in a humidified atmosphere containing 5% CO₂ and were split 1:3 to 1:5 on reaching confluence. ASMC at passages 6–8 were used in experiments.

Collagen Contraction

Collagen gel preparation was as described by Bogatkevich et al.¹⁴ Collagen lattices were prepared using type I collagen from rat tail tendon (BD Bioscience, Bedford, MA). ASMC (2.5×10^5 cells/mL final concentration) suspension was mixed with an equal volume of collagen solution (1.25 mg/mL of collagen final concentration) and aliquoted by 300 μL into 24-well plates pretreated with 2 mg/mL bovine serum albumin and dried before use. Collagen lattices were polymerized for 45 minutes in a humidified 10% CO₂ atmosphere at 37°C. To initiate collagen gel contraction, tested compounds in serum-free DMEM were added to the wells, and the gels were set afloat by gentle tapping. Digital photographs were taken of the gels after a 24-hour period of incubation at 37°C. The degree

of collagen gel contraction was determined after 24 hours by measuring the area of contracted gel using Scion Image software (compliments of Scion Corporation). The results are expressed as a percentage of the control gel area (DMEM or angiotensin II sample, as indicated) and presented as mean \pm SD from 3 or more replicates. Gel with embedded ASMC contracts spontaneously in DMEM in the absence of additional stimuli.

Because the absolute values of SMC-collagen gel contraction in controls and supplemented samples varied significantly from experiment to experiment, depending greatly on cell concentration, cell batch, batch of collagen reagent, incubation time, and other undefined conditions, the experimental results are presented as percentages to control samples, either nonsupplemented or angiotensin II–supplemented, depending on the design of particular experiment. This has reduced data variability. Because of high interexperimental variability, all comparisons between different tested compounds were made within the same experiment. The results presented in the figures are from the most illustrative experiment chosen out of at least 3 similar ones.

Gelatinase Zymography

MMP expression in conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% Novex precast SDS-polyacrylamide gel (Invitrogen Corporation) in the presence of 0.1% gelatin under nonreduced conditions. Culture media (20 μL) mixed with sample buffer was loaded, and SDS-PAGE was performed with Tris glycine SDS buffer as described by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 minutes at room temperature to remove SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM CaCl₂ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes and then destained. Protein standards were run concurrently, and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

RESULTS

Angiotensin-Induced Collagen Gel Contraction

Dose-Dependent Angiotensin-Induced Collagen Gel Contraction

Collagen gels did not contract in the absence of ASMC (Fig. 1). Incorporation of ASMC into a 3-dimensional collagen gel structure initiated spontaneous gel contraction. Angiotensin II (ATII) stimulated spontaneous contraction of collagen I gel in a dose-dependent manner, driven by embedded human aortic smooth muscle cells ($P = 0.053$ per ANOVA trend analysis of ATII data) (Fig. 1). Gel contraction-stimulating effects of angiotensin I (ATI) were less pronounced and required higher concentrations.

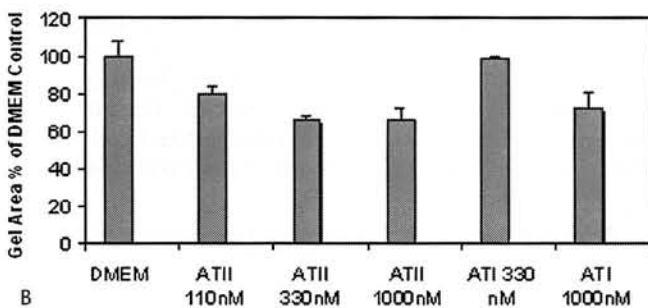
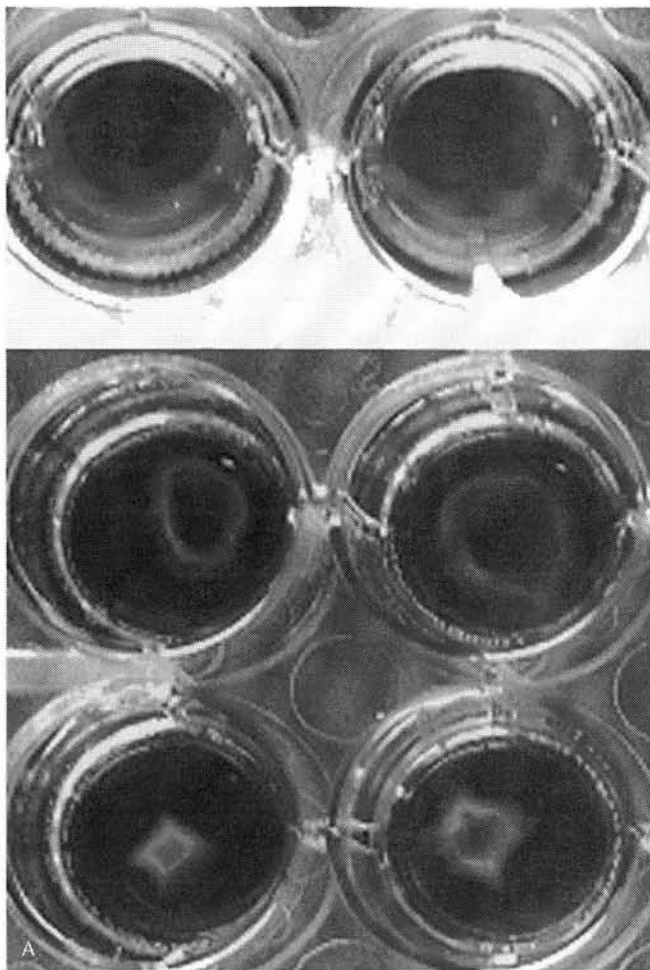


FIGURE 1. Effect of angiotensin (ATI) and II (ATII) on ASMC-collagen gel contraction, measured by the gel area, is represented as percentage of the control gel area.

Effect of Different Classes of Bioflavonoids on Angiotensin-Dependent Gel Contraction

Different classes of bioflavonoids showed variable efficiency in inhibiting angiotensin II-dependent collagen gel contraction by SMCs. The flavonol quercetin showed the most potent relaxation effect on the collagen gel (220% of control, $P = 0.007$) followed by the green tea polyphenol epigallocatechin gallate (EGCG, 197% of control, $P = 0.007$). Resveratrol, a grape stilbene (115%, $P = 0.04$) and genistein, a soy

isoflavone (125%, $P = 0.0003$), demonstrated only slight relaxation effects over the control (Fig. 2). The difference between quercetin and EGCG did not reach statistical significance ($P = 0.4$).

Effect of Glycosylation of Quercetin on Angiotensin-Mediated Collagen Contraction

In contrast to quercetin, its glycoside form rutin did not demonstrate significant inhibition of angiotensin II-dependent collagen gel contraction by SMC (Fig. 3); gel area with quercetin was 220% of control ($P = 0.007$) in contrast to rutin (105% of control, $P = 0.05$). The difference of 115% in gel area between quercetin and rutin was statistically significant ($P = 0.008$).

Effect of Green Tea Polyphenols on Angiotensin-Mediated Collagen Contraction

An increase in the number of gallate groups per catechin molecule was associated with increased inhibition of angiotensin II-dependent collagen gel contraction by SMC (Fig. 4). Epigallocatechin gallate (EGCG) was the most potent gel relaxant (263% of the control, $P = 0.01$); epicatechin gallate (ECG), with 1 less gallate group, demonstrated 233% of the control ($P = 0.007$), followed by epicatechin (EpiCat) with gel area of 145% of control ($P = 0.2$) and catechin 157% ($P = 0.02$). The difference between EGCG and epicatechin (118%, $P = 0.05$) and catechin (107%, $P = 0.04$) reached statistical

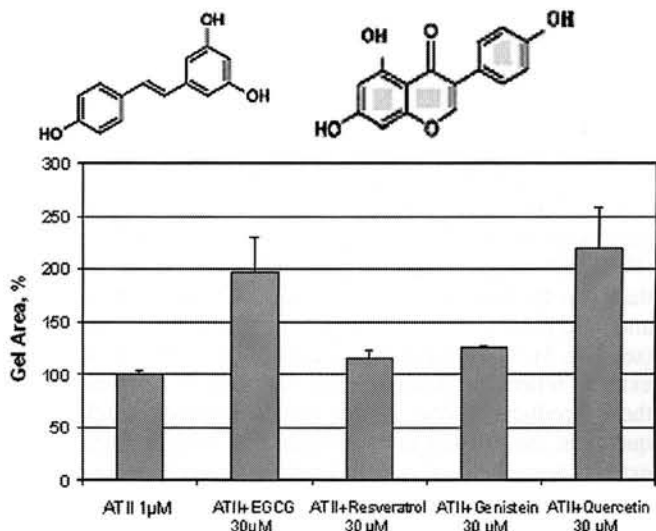
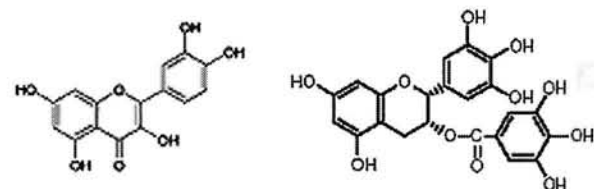


FIGURE 2. Effect of purified bioflavonoids on angiotensin II-dependent collagen I gel contraction by SMC.

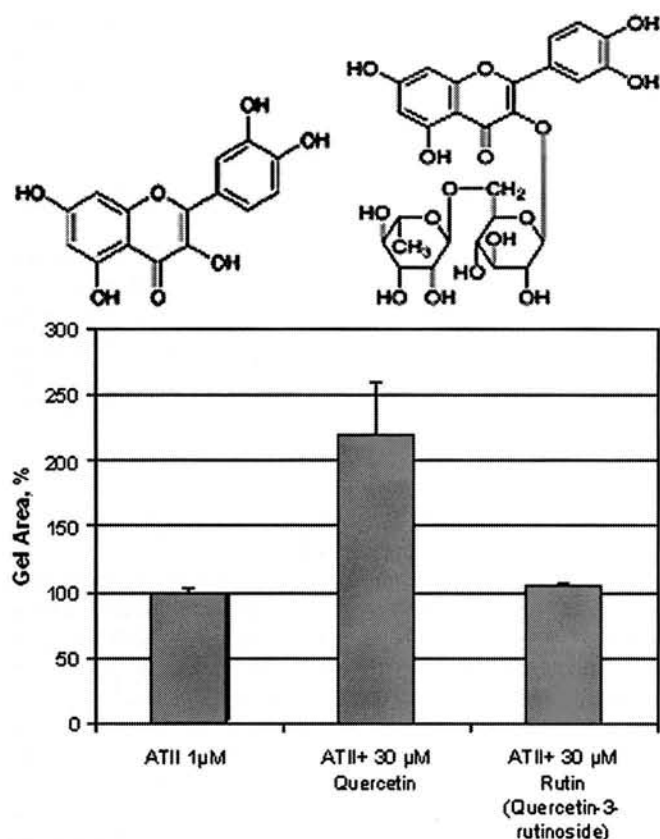


FIGURE 3. Effect of glycosylation of the quercetin molecule on its inhibitory effect on angiotensin II–dependent collagen I gel contraction by SMC.

significance. The difference between EGCG and ECG (30%, $P = 0.5$) did not reach statistical significance.

Effect of Phenolic-Enriched Plant-Derived Extracts on Angiotensin-Induced Gel Contraction

Phenolic-enriched plant-derived extracts (30 µg phenolics/mL) had an inhibitory effect on ATII-dependent collagen gel contraction by SMCs; the potency increased from citrus bioflavonoid extract (94%) to grape seed extract (263%) to pine bark extract (326%), and to green tea extract (455%). Grape seed extract inhibition was observed to be 169% greater than that by citrus bioflavonoid extract (CBE) ($P = 0.0016$); pine bark extract inhibition was 231% greater than that of CBE ($P = 0.0006$); green tea extract inhibition was 361% greater than that by CBE ($P = 0.007$). The difference between GTE and PBE (130%, $P = 0.1$) did not reach statistical significance (see Fig. 5). The differences in the effects of the various plant extracts reflect the differences in the phenolic composition of these products. Citrus bioflavonoids mainly include rutin, quercetin, hesperidin, and naringin. Pine bark and grape seed extracts are composed mainly of proanthocyanidins; grape seed (92%–95%) is considered more potent than pine bark (80%–85%) and contains gallic esters of proanthocyanidins, the most active free radical scavengers. Green tea extract (80% polyphenol) is mainly composed of catechins (~60%) and

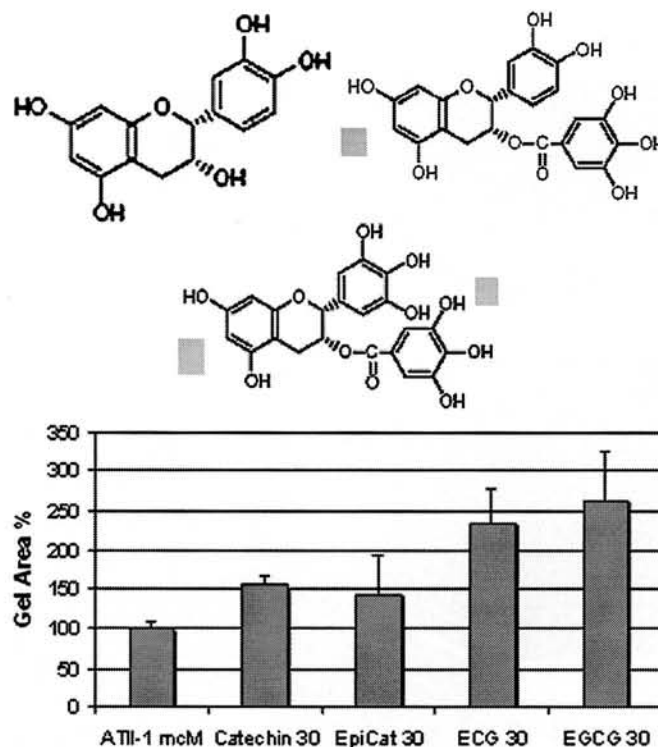


FIGURE 4. Effect of gallate residues on capacity of catechins to inhibit angiotensin II–stimulated contraction of collagen I gel by SMC.

proanthocyanidins; EGCG (55%) is considered the most active catechin.

Effect of Antioxidants Ascorbic Acid and N-Acetylcysteine on Angiotensin-Mediated Gel Contraction

N-Acetylcysteine (NAC) (Fig. 6A) and ascorbic acid (AsA) (Fig. 6B) did not demonstrate significant inhibition of ATII-induced SMC collagen gel contraction. Thus, the inhibitory effect of bioflavonoids on ATII-dependent collagen gel contraction does not depend on antioxidant properties.

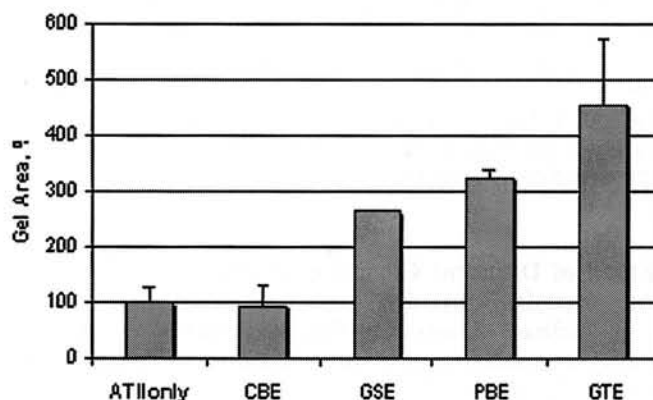


FIGURE 5. Effect of plant extracts (30 µg phenolics/mL) on SMC-gel contraction induced by 1 µM angiotensin II (ATII): citrus bioflavonoid (CBE); grape seed extract (GSE); pine bark extract (PBE); green tea extract (GTE).

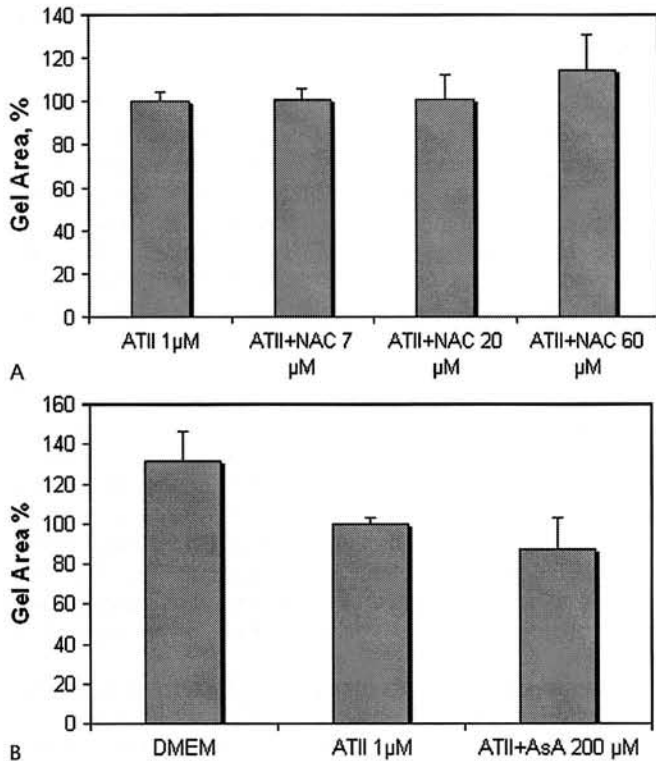


FIGURE 6. A, Effect of N-acetylcysteine (NAC) on SMC gel contraction stimulated by angiotensin II (ATII). B, Effect of ascorbic acid (AsA) on SMC gel contraction by angiotensin II (ATII).

Effect of Bioflavonoids on ASMC MMP-2 Expression

Doxycycline (DOX), a potent MMP inhibitor (Fig. 7B), demonstrated potent inhibition of gel contraction (Fig. 7A); DOX treatment resulted in gel area increase of 650% of the control ($P < 0.0001$). Green tea extract inhibition of collagen gel contraction by SMC correlated with inhibition of matrix metalloproteinase-2 expression (Fig. 7C).

DISCUSSION

Angiotensin (ATII greater than ATI) stimulated spontaneous dose-dependent contraction of collagen I gel driven by embedded human ASMC. The bioflavonoids tested had

variable inhibitory activity on the angiotensin II-induced collagen gel contraction. The degree of relaxation differed among classes of bioflavonoids; the flavonol quercetin had the most potent relaxing effect on angiotensin-induced ASMC collagen gel contraction, closely followed by the green tea polyphenol epigallocatechin gallate. Furthermore, structural changes, such as glycosylation of quercetin and the number of gallate groups per catechin molecule in green tea polyphenols, affected the degree of inhibition of angiotensin-induced ASMC gel contraction.

Quercetin is one of the most common flavonoids in the diet, and its biological activities have been extensively investigated.^{17,18} However, quercetin mainly occurs in its glycosylated derivatives both in plants and in humans. These derivatives can have different physicochemical and biological properties than their aglycone form. This prompted us to compare SMC-collagen gel-relaxing properties of quercetin to those of its common plant-derived glycoside, rutin (quercetin-3-rutinoside). As observed in this study, aglycone quercetin was significantly more active than its 3-O-glycoside rutin (Fig. 3). This is consistent with previously reported differences in such activities as protection of lipoproteins from oxidation¹⁹ and inhibition of cell growth.²⁰

A trend was observed between increasing gallic acid residues per catechin molecule and collagen gel relaxation activity in catechins. Epigallocatechin gallate (EGCG) was the most potent gel relaxant (263% of the control, $P = 0.01$). Epicatechin gallate (ECG), with 1 less gallate group, demonstrated 233% of the control ($P = 0.007$), followed by epicatechin (EpiCat), with gel area of 145% of control ($P = 0.2$), and catechin. There was no significant difference between catechin and epicatechin. Previous studies reported an apparent importance in gallic acid residues and gallyl moiety in the B-ring of catechin molecule for various catechin-dependent biological activities. An increase in number of gallic acid residues correlated with an increase in catechin antioxidant activity in direct quenching of superoxide anion²¹ and in regenerating oxidized tocopherol.²² The same order of activity was reported for catechin-dependent protection of erythrocytes and hepatocytes from oxidative stress.^{23,24} In our study, gel relaxation activity of catechin apparently was not dependent on their antioxidant activity because ascorbate did not have any effect in this system. Similarly, catechins inhibited P-glycoprotein function in an apparently redox-independent action following same order of biological activity.²⁵ In contrast to these data, catechin

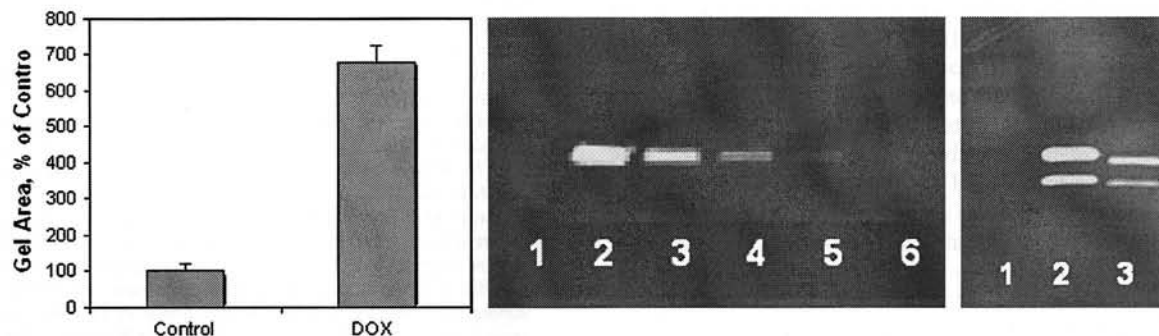


FIGURE 7. Left, Effect of MMP inhibitor doxycycline. Middle, MMP-2 expression by green tea extract. Right, Inhibition of SMC.

activity in inhibition of erythrocyte sodium/hydrogen exchanger showed an increase with decreased number of gallic acid residues.²⁶

Phenol-enriched plant-derived extracts also had an inhibitory effect on ATII-dependent collagen gel contraction by SMCs; potency significantly increased from citrus bioflavonoid extract to the proanthocyanidin-enriched grape seed extract and pine bark extract to mostly catechin-containing green tea extract.

Polyphenols and plant extracts have been shown to prevent hypertension in many studies. Red wine polyphenols alone or in association with ethanol prevent hypertension, cardiac hypertrophy, and production of reactive oxygen species in the insulin-resistant fructose-fed rat.²⁷ Both black and green tea polyphenols were shown to attenuate blood pressure increases in stroke-prone spontaneously hypertensive rats through their antioxidant properties. Western blot of proteins in the aorta demonstrated significantly increased catalase expression with green tea consumption and decreased phosphorylated myosin light chain with consumption of both black and green tea.²⁸ Phenols in green tea (epigallocatechin gallate, epigallocatechin, and epicatechin gallate) and black tea (theoflavin and thearrubigins) contribute to antioxidant activity of tea. The phenol antioxidant index of teas was significantly higher than of grape juices and wines.²⁹ Tea polyphenols, catechins, and flavonols scavenge ROS and chelate transition metal ions in a structure-dependent manner.³⁰ Flavonoids found in tea scavenge nitric oxide and peroxynitrite produced from superoxide radicals and effectively reduce bioavailability of endothelium-derived nitric oxide.^{31,32} Green tea polyphenols were shown to up-regulate genes coding for LDL receptor and PPAR- α and to down-regulate genes coding for PPAR- γ at the transcriptional level and thus have the inherent capacity to inhibit the development of atherosclerotic lesions.³³

SMC contraction occurs secondary to actomyosin filament sliding, facilitated by myosin light-chain phosphorylation and regulated by myosin light-chain kinase. When studying the role of extracellular matrix (ECM) in pulmonary hypertension, Lee et al³⁴ noted that cytoskeleton stiffness closely paralleled alterations in gel contraction and relaxation and found that cytoskeleton stiffness increased as the number of ECM contacts is raised and cell spreading is promoted.

The gel contraction of ASMC was not affected by ascorbic acid or N-acetylcysteine, suggesting that the antioxidant activity of bioflavonoids was not the mechanism of gel contraction. In this model, endothelial cells are absent, and, thus, the gel-relaxing effects are not mediated through endothelial nitric oxide synthase (eNOS). Ascorbic acid effects on vascular pressure were reported in various clinical studies. Oral doses of ascorbic acid as low as 500 mg per day^{35,36} were found to slightly lower systolic and mean arterial pressure. One month of treatment with 500 mg oral intake of ascorbic acid per day was also found to lower arterial blood pressure and improve arterial stiffness in patients with type 2 diabetes.³⁷ Grossman et al³⁸ found that when ascorbic acid, the main water-soluble antioxidant in human plasma, was infused in the dorsal hand veins of 23 healthy male nonsmokers, the ascorbic acid produced dose-dependent vasodilation in phenylephrine- and prostaglandin-precontracted veins. Ascorbate

anticontractile properties seem to be mediated through the endothelial layer and depend both on its antioxidant properties and on eNOS regulation.^{39,40}

Doxycycline, a nonspecific inhibitor of MMPs, effectively prevented ASMC-driven collagen gel contraction. This indicates the importance of ECM composition and cell-matrix interaction on the ability of ASMC to contract. Green tea extract was also shown to have extracellular matrix-remodeling effects by inhibiting ASMC MMP-2 secretion. Oak et al⁴¹ observed inhibition of MMP-2 expression and secretion by vascular SMCs by red wine polyphenolic compounds. This indicates a possible mechanism of action by plant phenolics. Cells in normal and diseased blood vessel walls produce and activate MMPs in a multistep fashion driven in part by soluble cytokines and cell-cell interactions. MMP activation contributes to intimal growth and vessel wall remodeling in response to injury, most notably by promoting migration of vascular smooth muscle cells. A higher level of MMP activation, especially associated with inflammation, could contribute to pathological matrix destruction and plaque rupture. Inhibiting activity of specific MMPs or preventing their up-regulation could ameliorate intimal thickening and prevent myocardial infarction.⁴²

In conclusion, bioflavonoids participate in the regulation of SMC-mediated contraction and have a strong potential in counteracting pathophysiological effects of angiotensin II. Bioflavonoid activity strongly depends on structural characteristics and can be related to extracellular matrix integrity

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