Original Article

Caffeic Acid Inhibits Vascular Smooth Muscle Cell Proliferation Induced by Angiotensin II in Stroke-Prone Spontaneously Hypertensive Rats

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Epidemiological studies have linked the consumption of phenolic acids with reduced risk of cardiovascular diseases. In the present study, we sought to investigate whether caffeic acid, a phenolic acid which is abundant in normal diet, can antagonize angiotensin II (Ang II)-induced vascular smooth muscle cell (VSMC) proliferation in stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar-Kyoto (WKY) rats, and if so, to elucidate the underlying cell signaling mechanisms. We exposed VSMCs to Ang II and caffeic acid and found that caffeic acid significantly inhibited intracellular superoxide anion generation (decreased from 127±6.3% to 100.3±6.6% of the control cells) and the cell proliferation induced by Ang II. Furthermore, caffeic acid significantly abolished the tyrosine phosphorylation of JAK2 (decreased from 7.4±0.6-fold to 2.4±0.6-fold at 2 min) and STAT1 (decreased from 1.8±0.2-fold to 0.5±0.1-fold at 2 min) and the phosphorylation of ERK1/2 (decreased from 99.2±10.2-fold to 49.8±10.9-fold at 2 min) that were induced by Ang II. These effects of caffeic acid were consistent with the inhibition of the proliferation of VSMCs by DPI, an NADPH oxidase inhibitor, and by AG-490, a JAK2 inhibitor. In conclusion, our findings suggest that caffeic acid attenuates the proliferative reaction of VSMCs to Ang II stimulation in both SHRSP and WKY rats by inhibiting the generation of reactive oxygen species and then partially blocking the JAK/STAT signaling cascade and the Ras/Raf-1/ERK1/2 cascade. (*Hypertens Res* 2005; 28: 369–377)

***Key Words*: caffeic acid, angiotensin II, vascular smooth muscle cells, proliferation, stroke-prone spontaneously hypertensive rats**

Introduction

Proliferation of vascular smooth muscle cells (VSMCs) is a crucial event in the development of hypertension and is activated by various growth stimulants and cytokines. It has been shown that angiotensin II (Ang II) acts not only as a vasoactive peptide but also as a growth factor (*1*, *2*). In particular, Ang II has been shown to stimulate proliferative and hypertrophic growth in vascular smooth muscle cells *via* angiotensin type 1 (AT1) receptor binding (*3*). In this regard, many studies have emphasized the important role played by the Janus kinase (JAK)/signal transducer and activators of transcription (STAT) and the Ras/Raf-1/mitogen-activated protein kinase (MAPK) cascades in mediating VSMC proliferation in response to G protein-coupled AT1 receptors, and found that the inhibition of these individual signaling molecules prevented VSMC proliferation (*4*, *5*). In addition,

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VSMC growth is redox-sensitive, and oxidative stress might be responsible for triggering the proliferation of VSMCs through activation of NADPH oxidase (*6*). For example, the stimulation of the JAK/STAT cascade by Ang II requires superoxide anions (O^{2-}) generated by the NADPH oxidase system (*7*, *8*), and antioxidants, such as diphenylene iodonium (DPI), blocked the mitogenic effect induced by the increase in reactive oxygen species (ROS) production in some studies (*9–11*), suggesting that antioxidant treatment may represent a potential therapy for vascular diseases.

Phenolic acids represent a large body of natural antioxidants that are abundant in plant foods, and caffeic acid is one of the most frequently encountered phenolic acids in normal diet, occurring in foods mainly as an ester with quinic acid called chlorogenic acid (5-caffeoylquinic acid), and both can be absorbed into circulation by rats and humans (*12*, *13*). Although many studies have shown the antioxidant activity and the cardiovascular disease-protective properties of caffeic acid, the mechanisms implicated are far from being clarified (*14–16*). The purpose of the current investigation was to determine the role of caffeic acid on Ang II-induced proliferative cellular reactions in aorta VSMC from stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar-Kyoto (WKY) rats and to elucidate the underlying cellular signaling transduction events in the effects of caffeic acid.

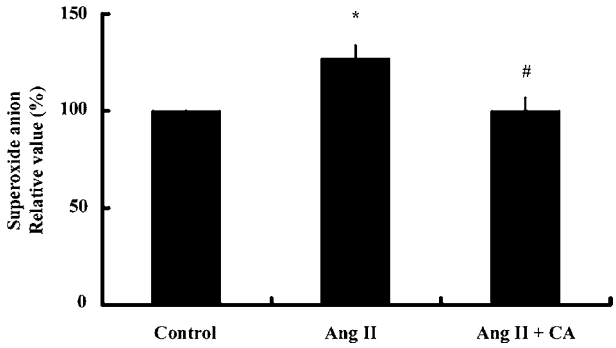

Fig. 1. *CA inhibited Ang II-induced intracellular superoxide anion production in VSMCs from SHRSP. Cells were treated with serum-free DMEM (control), 100 nmol/l Ang II, or 100 nmol/l Ang II plus 100 μmol/l CA for 24 h and then incubated with 0.5 mg/ml NBT solution for 20 min at 37°C. The reductions in NBT were quantified by measuring the absorbance at 510 nm. The value of the reduction in NBT in each sample is shown relative to that of the control group as 100%. Data are expressed as the mean±SEM (*n = 5*). *p < 0.05 vs. the control; #p < 0.05 vs. the Ang II group. Ang II, angiotensin II; CA, caffeic acid.*

Methods

Materials

Rabbit anti-STAT1, anti-JAK2, and anti-phospho-JAK2 (Y1007, Y1008) antibodies were from Upstate Biotechnology (Lake Placid, USA). Phospho-Stat1 (Tyr701) rabbit antibody and phosphor-p44/42 MAP kinase (Thr202/Tyr204) antibody were from Cell Signaling Technology (Beverly, USA). Anti-rabbit IgG peroxidase-linked species-specific whole antibody and anti-mouse IgG peroxidase-linked species-specific whole antibody were from Amersham Biosciences Corp. (Piscataway, USA). The Cell Counting Kit was from Dojindo Laboratories (Kumamoto, Japan). Caffeic acid, DPI, AG-490 and nitroblue tetrazolium (NBT) were from Wako Pure Chemicals (Osaka, Japan). Angiotensin II was from Sigma (St. Louis, USA). Mouse anti-HSP90 antibody was from Santa Cruz Biotechnology (Santa Cruz, USA). Mouse anti-α-tubulin antibody was from Molecular Probes (Eugene, USA). Other reagents used were of the highest grade commercially available.

Cell Culture

Aortic VSMCs were isolated from 12-week-old male SHRSP and WKY rats and maintained in 10% fetal bovine serum/Dulbecco's modified Eagle's medium (DMEM) as described previously (*17*). VSMCs from passages 5 to 14 at 70–90% confluence in 100-mm dishes were growth arrested by incubation in serum free DMEM for 16–24 h prior to use.

Western Blotting

Serum-starved VSMC in 100-mm dishes were stimulated with Ang II and caffeic acid for the indicated time and the whole cell extracts were prepared by lysing the cells in extraction buffer (RIPA's buffer) containing 50 mmol/l tris(hydroxymethyl)aminomethane (Tris)/HCl, pH 8.0, 150 mmol/l NaCl, 1% Nonidet-P40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mmol/l dithiothreitol (DTT), 0.05 mmol/l phenylmethyl-sufonyl-fluoride (PMSF), 0.002 mg/ml aprotinin, 0.002 mg/ml leupeptin, and 1 mmol/l $NaVO_3$ after stimulation. The protein concentration was quantified with BIO-RAD Dc protein assay reagent (Bio-Rad, Hercules, USA). Equal amounts of protein were mixed with SDS sample buffer and incubated for 5 min at 100°C before loading. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunological blotting were performed according to the method of Amersham Biosciences. Immunoreactive bands were detected by means of an ECL plus Western Blotting Detection System (Amersham Biosciences, Little Chalford, UK) as described previously (*18*). The chemiluminescent signals were scanned from films (Nippon Polaroid K.K., Tokyo, Japan), and imported into Adobe Photoshop (Adobe, San Jose, USA). Quantitative analysis was performed by NIH ImageJ 1.32j software.

Detection of Superoxide Anion by NBT Reduction

VSMCs were incubated with NBT in order to allow superox-

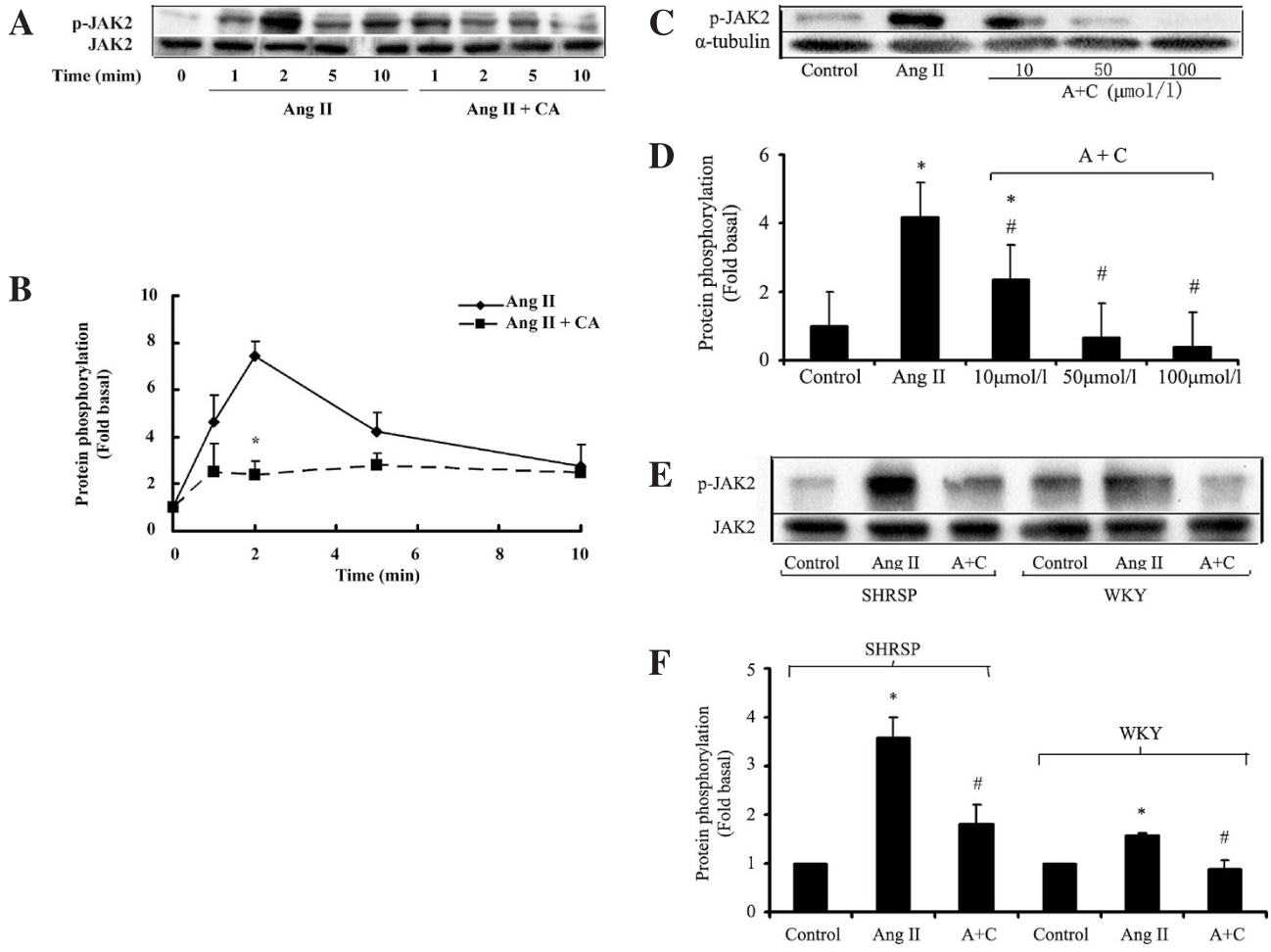

Fig. 2. *CA repressed Ang II-induced tyrosine phosphorylation of JAK2 in VSMCs from both SHRSP and normotensive WKY rats. A: VSMCs from SHRSP were growth-arrested in serum-free DMEM for 16–24 h and then treated with different condition mediums (serum-free DMEM, 100 nmol/l Ang II, and 100 nmol/l Ang II plus 100 μmol/l CA, respectively) for the indicated times following either no pretreatment (the Ang II group) or pretreatment with CA (the CA group) for 30 min as shown in lanes 1 to 9. Cells were lysed in RIPA buffer and then separated by SDS-PAGE and analyzed by Western blot (WB) using specific anti-phosphotyrosine JAK2 antibody. B: Densitometric analysis of the time course of the tyrosine phosphorylation of JAK2. C: VSMCs from SHRSP were growth-arrested in serum-free DMEM for 16–24 h and then treated with different condition mediums (serum-free DMEM, 100 nmol/l Ang II, and 100 nmol/l Ang II plus different concentrations of CA, respectively) for 2 min following either no pretreatment or pretreatment with CA for 30 min as shown in lanes 1 to 5. Cells were lysed in RIPA buffer and then separated by SDS-PAGE and analyzed by WB using specific anti-phosphotyrosine JAK2 antibody. D: Densitometric analysis of the dose-responsive tyrosine phosphorylation of JAK2. E: VSMCs from either SHRSP or WKY rats were growth-arrested in serum-free DMEM for 16–24 h and then treated with different condition mediums (serum-free DMEM, 100 nmol/l Ang II, and 100 nmol/l Ang II plus 100 μmol/l CA, respectively) for 2 min following either no pretreatment or pretreatment with CA for 30 min as shown in lanes 1 to 6. Cells were lysed in RIPA buffer and then separated by SDS-PAGE and analyzed by WB using specific anti-phosphotyrosine JAK2 antibody. F: Densitometric analysis of the tyrosine phosphorylation of JAK2 in the comparative investigation. The data in each panel are representative of three independent experiments. Each of the three separate experiments yielded similar results. Data are expressed as the mean ± SEM (*n* = 3).* *p < 0.05 vs. the control; # p < 0.05 vs. the Ang II group. Ang II, angiotensin II; CA, caffeic acid; A+C, angiotensin II+caffeic acid.*

ide anion generated by the cells to reduce the NBT to blue formazan (*19*, *20*). The amount of the superoxide anion generated by the VSMCs could be quantified by measuring the absorbance of blue formazan extracted from the cells. Cells were placed in 5 ml PBS buffer containing NBT (0.1%) for 20 min. They were then homogenized in a mixture of 0.1 mol/l NaOH and 0.1% SDS in water containing 40 mg/l of diethylenetriaminepentaacetic acid. The mixture was centri-

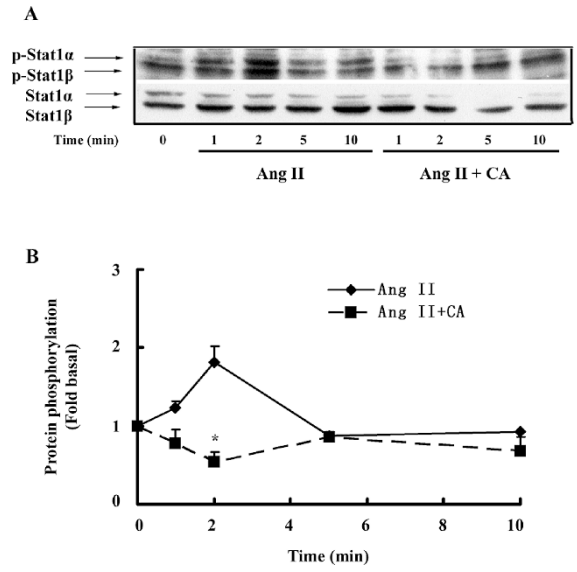

Fig. 3. *CA repressed Ang II-induced tyrosine phosphorylation of STAT1 in VSMCs from SHRSP. A: VSMCs from SHRSP were growth-arrested in serum-free DMEM for 16–24 h and then treated with different condition mediums (serum-free DMEM, 100 nmol/l Ang II, and 100 nmol/l Ang II plus 100 μmol/l CA, respectively) for the indicated times following either no pretreatment or pretreatment with CA for 30 min as shown in lanes 1 to 9. Cells were lysed in RIPA buffer and then separated by SDS-PAGE and analyzed by Western blot using specific anti-phosphotyrosine STAT1 antibody. B: Densitometric analysis of the tyrosine phosphorylation of STAT1. The results shown are representative of three experiments. Similar results were obtained in the three separate experiments. Data are expressed as the mean±SEM (*n*=3).* *$p<0.05$ vs. *the control;* #$p<0.05$ vs. *the Ang II group. Ang II, angiotensin II; CA, caffeic acid.*

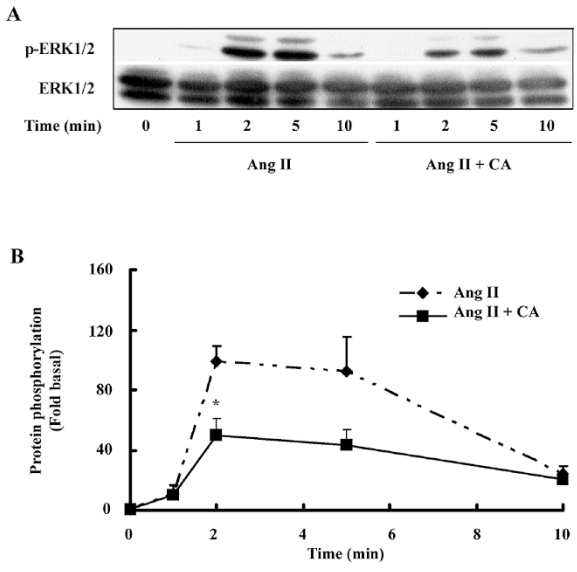

Fig. 4. *CA abolished Ang II-induced ERK1/2 phosphorylation in VSMCs from SHRSP. A: VSMCs from SHRSP were growth-arrested in serum-free DMEM for 16–24 h and then treated with different condition mediums (serum-free DMEM, 100 nmol/l Ang II, and 100 nmol/l Ang II plus 100 μmol/l CA, respectively) for the indicated times following either no pretreatment or pretreatment with CA for 30 min as shown in lanes 1 to 9. Cells were lysed in RIPA buffer and then separated by SDS-PAGE and analyzed by Western blot using specific anti-phospho-ERK1/2 antibody. B: Densitometric analysis of the phosphorylation of ERK1/2. The results shown are from one of three independent experiments that yielded similar results. Data are expressed as the mean±SEM (*n*=3).* *$p<0.05$ vs. *the control;* #$p<0.05$ vs. *the Ang II group. Ang II, angiotensin II; CA, caffeic acid.*

fuged at 14,000×*g* for 30 min. The resultant pellet was resuspended in 1.0 ml of pyridine during heating at 100°C for 10 min to extract blue formazan. The mixtures were subjected to a second centrifugation at 10,000×*g* for 10 min as previously described (*18*). The absorbance of blue formazan was determined spectrophotometrically at 510 nm. The extinction coefficient of blue formazan is 0.72 l $mmol^{-1}$ mm^{-1}. The quantity of blue formazan was calculated as follows: NBT reduction = $A \cdot V/(T \cdot N \cdot e \cdot l)$, where A is the absorbance of blue formazan at 510 nm, V is the volume of the solution, T is the time period during which cells were incubated with NBT, N is the number of the VSMCs, e is the extinction coefficient, and l is the length of the light path. Results are reported as pmol/min/10^6 cells.

Cell Proliferation Assay

The mitogenic responses of VSMCs to different treatments were simultaneously assessed by a Sample Measurement Unit (Sysmex F-520P; Sysmex, Kobe, Japan) to directly count the cell number and a cell counting kit to colorimetrically determine the cell number. In the direct cell counting analysis, cells were plated onto 6-multiwell plates at a density of 1×10^4 cells per well in DMEM supplemented with 10% FBS. After 24 h, cells were rinsed with PBS and the medium was changed to serum-free DMEM supplemented with agonists or the appropriate vehicle. Following 48-h incubation in serum-free medium (control), Ang II at 100 nmol/l (Ang II), or Ang II at 100 nmol/l plus caffeic acid at 100 μmol/l (Ang II + CA), cells (6 wells for each condition) were trypsinized and directly counted with the Sample Measurement Unit. Data were expressed as cell number per well. Additionally, in another assay, 10 μmol/l AG-490, a JAK2 inhibitor, and 50 μmol/l DPI, an NADPH oxidase inhibitor, were used instead of caffeic acid to investigate the effects of abolishing the Ang II-induced cell proliferation.

Finally, to ensure the validity of the direct cell counting assay, an independent comparative investigation of the differences between SHRSP and WKY rats in terms of the mitogenic reactions in VSMCs upon stimulation with Ang II and caffeic acid treatment was also carried out using a colorimetric cell counting kit according to the protocols of the manufacturer.

Data Analysis

All numerical data are presented as the mean±SEM. For parametric data, comparisons among treatment groups were performed with one-way analysis of variance and an appropriate *post hoc* comparison. Statistical significance was accepted if the null hypothesis was rejected at the level of $p<0.05$.

Results

Caffeic Acid Inhibited Ang II-Induced Intracellular Superoxide Anion Production in VSMCs from SHRSP

As shown in Fig. 1, VSMCs from SHRSP pretreated with caffeic acid showed a significant decrease in the production of superoxide anion stimulated by 100 nmol/l Ang II. The reductions in NBT in each sample are shown relative to the control value (100%). Stimulation with 100 nmol/l Ang II significantly increased the production of superoxide anion from 100% in the controls to 127±6.3% ($p<0.05$), and the level decreased to 100.3±6.6% ($p<0.05$ *vs.* Ang II stimulation group) with caffeic acid treatment.

Caffeic Acid Inhibited Ang II-Induced Tyrosine Phosphorylation of JAK2 in VSMCs from Both SHRSP and WKY Rats

As shown in Fig. 2A, following stimulation with 100 nmol/l of Ang II, JAK2 was rapidly phosphorylated within 1 min, peaked at 2 min and was sustained for 10 min in VSMCs from SHRSP. The maximal increase in the tyrosine phosphorylation of JAK2 in response to Ang II was 7.4±0.6-fold (Fig. 2B) at 2 min. However, the tyrosine phosphorylation of JAK2 stimulated by Ang II was partially abolished by simultaneous treatment with 100 μmol/l of caffeic acid, and the densitometric analysis shows that JAK2 phosphorylation was significantly inhibited by caffeic acid at 2 min (2.4±0.6-fold increase, $p<0.05$ *vs.* Ang II stimulation group) (Fig. 2B).

To investigate the effects of different doses of caffeic acid on Ang II-induced JAK2 phosphorylation, VSMCs from SHRSP were treated simultaneously with 100 nmol/l of Ang II and different concentrations of caffeic acid for 2 min. As shown in Fig. 2C, the phosphorylation of JAK2 induced by Ang II was dose-dependently inhibited by caffeic acid. The densitometric analysis shows that although caffeic acid began to show its inhibition effect on Ang II-induced JAK2 phosphorylation at concentrations as low as 10 μmol/l, the optimal inhibition was achieved at concentrations greater than 50 μmol/l (Fig. 2D).

Subsequently, we investigated the phosphorylation of JAK2 in normotensive WKY rats and compared it with that in SHRSP. As shown in Fig. 2E, caffeic acid at 100 μmol/l significantly repressed the tyrosine phosphorylation of JAK2 induced by 100 nmol/l of Ang II at 2 min in VSMCs from both SHRSP and WKY rats. However, the intensity of the phosphorylation of JAK2 induced by Ang II in SHRSP was much more intense than that in the normotensive WKY rats (3.58-fold *vs.* 1.59-fold, respectively) (Fig. 2E, F) when the same amount of lysate protein was loaded in the Western blot assay.

Caffeic Acid Repressed Ang II-Induced Tyrosine Phosphorylation of STAT1 in VSMCs from SHRSP

Ang II-induced STAT1 tyrosine phosphorylation is mediated by JAK2 (*4*). In the present study, we observed that both STAT1α and STAT1β were rapidly tyrosine-phosphorylated after stimulation with 100 nmol/l of Ang II (Fig. 3A). The increase in tyrosine phosphorylation of STAT1α/β in response to Ang II was maximal at 2 min (1.8±0.2-fold increase). Densitometric analysis shows that, when VSMCs were treated simultaneously with 100 nmol/l of Ang II and 100 μmol/l of caffeic acid, the tyrosine phosphorylation of STAT1α/β stimulated with Ang II decreased and was significantly lower than that of the cells stimulated with Ang II alone at 2 min (0.5±0.1-fold increase, $p<0.05$ *vs.* Ang II stimulation group) (Fig. 3B).

Caffeic Acid Significantly Abolished Ang II-Induced ERK1/2 Phosphorylation in VSMCs from SHRSP

The extracellular signal regulated kinase (ERK) cascade is the best-characterized component of the Ras/Raf-1/ MAPK pathway (*21*). As shown in Fig. 4, Ang II rapidly phosphorylated both ERK1 and ERK2 in VSMCs from SHRSP (Fig. 4A). The phosphorylation of ERK1/2 was maximal from 2 min to 5 min after Ang II stimulation (99.2±10.2-fold increase at 2 min) (Fig. 4B). However, when cells were pre-incubated with 100 μmol/l of caffeic acid, the phosphorylation of ERK1/2 decreased markedly. Densitometric analysis shows that the enhanced phosphorylation of ERK1/2 decreased significantly at 2 min when the medium contained caffeic acid (49.8±10.9-fold increase, $p<0.05$ *vs.* Ang II stimulation group) (Fig. 4B).

Caffeic Acid Inhibited Ang II-Induced HSP90 Protein Expression in VSMCs from SHRSP

The 90 kDa heat shock protein, HSP90, responds to oxidative

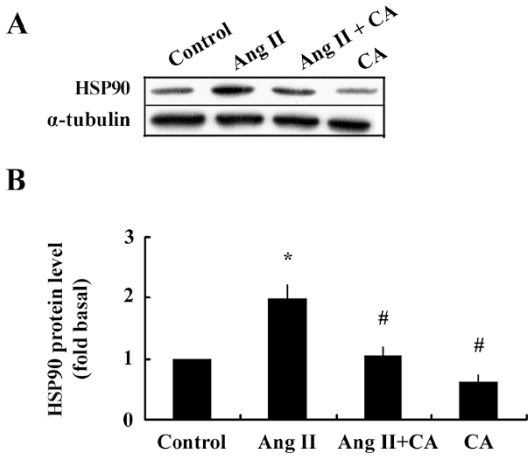

Fig. 5. *CA inhibited Ang II-induced HSP90 protein expression in VSMCs from SHRSP. A: VSMCs from SHRSP were growth-arrested in DMEM containing 0.1% FBS for 16–24 h and then treated with different condition mediums (serum-free DMEM, 100 nmol/l Ang II, 100 nmol/l Ang II plus 100 μmol/l of CA, 100 μmol/l CA, respectively) for 24 h as shown in lanes 1 to 4. Cells were lysed in RIPA buffer and then separated by SDS-PAGE and analyzed by Western blot using specific anti-HSP90 antibody. B: Densitometric analysis of HSP90 protein. The results shown are representative of three independent experiments that yielded similar results. Data are expressed as the mean±SEM (*n=3). *$p<0.05$ vs. *the control;* #$p<0.05$ vs. *the Ang II group. Ang II, angiotensin II; CA, caffeic acid.*

stress and is involved in cell proliferation. For instance, its occurrence has been associated with many components and regulators of the Ras/Raf-1/ERK pathway (*22*). Therefore, the expression of HSP90 protein after Ang II and caffeic acid treatment in VSMCs from SHRSP was examined. As shown in Fig. 5, HSP90 protein accumulated significantly after the cells were incubated with Ang II for 24 h (1.99±0.23-fold increase, $p<0.05$ *vs.* the control cells). However, when 100 μmol/l of caffeic acid was added to the medium, caffeic acid clearly antagonized the effect of Ang II; the level of HSP90 decreased significantly as compared with that in the Ang II group (1.05±0.15-fold increase, $p<0.05$). In addition, when the cells were incubated with caffeic acid alone, the level of HSP90 decreased slightly (0.63±0.12-fold increase), which may have been mainly due to the decreased oxidative stress.

Ang II-Induced VSMC Proliferation Was Inhibited by Caffeic Acid in Both SHRSP and WKY Rats, Consistent with the Effects of DPI and AG-490

As shown in Fig. 6A, 48 h after Ang II stimulation, the number of VSMCs from SHRSP increased significantly ($9.6\pm0.9\times10^4$ cells/well *vs.* $6.4\pm0.5\times10^4$ cells/well in the control, $n=6$, $p<0.05$). However, when cells were incubated with caffeic acid and Ang II simultaneously for the same time period, the cell number did not increase and was significantly lower than that of the Ang II group ($5.3\pm0.5\times10^4$ cells/well, $n=6$, $p<0.05$) (Fig. 6A).

To investigate whether the cell proliferation of VSMCs was associated with the generation of ROS and the activation of the JAK/STAT pathway as well as the ERK1/2 pathway, in addition to using DPI, a NADPH oxidase inhibitor, as a substitute for caffeic acid in the cell number counting assay, AG-490, a JAK2 inhibitor, was also used in place of caffeic acid in the direct cell number counting assay, since JAK2 plays a key role in the activation of both pathways (*3*, *4*). As shown in Fig. 6B, Ang II-induced proliferation of VSMCs was significantly inhibited by both 50 μmol/l DPI ($6.7\pm0.9\times10^4$ cells/well *vs.* $13.0\pm0.6\times10^4$ cells/well in the Ang II group, $p<0.05$) and 10 μmol/l AG-490 ($7.8\pm0.3\times10^4$ cells/well, $p<0.05$), which showed the roles of JAK2 and NADPH oxidase in the process of VSMC proliferation in SHRSP.

In an independent trial, we confirmed the inhibition effect of caffeic acid on Ang II-induced cell proliferation and compared the differences in the mitogenic response in VSMCs from SHRSP and WKY rats upon stimulation with 100 nmol/l Ang II and 100 μmol/l caffeic acid by using a colorimetric cell counting kit. As shown in Fig. 6C, consistent with the reactions observed in the JAK2 phosphorylation, the proliferation of VSMCs from SHRSP upon stimulation with Ang II was much greater than that of the VSMCs from normotensive WKY rats (130.24% *vs.* 112.52% as compared with the sham-treated controls), although both kinds of VSMCs showed some degree of increase in proliferation. Thus, caffeic acid has a much more obvious inhibitory effect on proliferation in SHRSP than in normotensive WKY rats.

Discussion

Caffeic acid has been shown to be beneficial in cardiovascular diseases and to have a hypotensive effect in spontaneously hypertensive rats (*14–16*). We previously reported that caffeic acid reduces the Rac1 GTPase protein and activity level, followed by a down-regulation of NADPH oxidase activity (*23*). In this study we examined the effects of caffeic acid on the proliferative cellular events induced by Ang II in cultured VSMCs from SHRSP and WKY rats. We found that caffeic acid inhibited both the JAK/STAT pathway (Figs. 2, 3) and the ERK1/2 pathway (Fig. 4) as well as the cell proliferation (Fig. 6) induced by Ang II in cultured VSMCs.

Proliferation of VSMCs is a crucial event in the formation of hypertensive tissues and is regulated by growth factors. Thus, the inhibition of VSMC proliferation may have a beneficial effect in retarding the development of hypertension. It is now considered that the JAK/STAT and the Ras/Raf-1/MAPK are the two major intermediate signal transduction pathways in response to growth factors or other stimuli that lead to cellular proliferation or hypertrophy (*4*, *5*). When these pathways are activated they transfer the mitogenic sig-

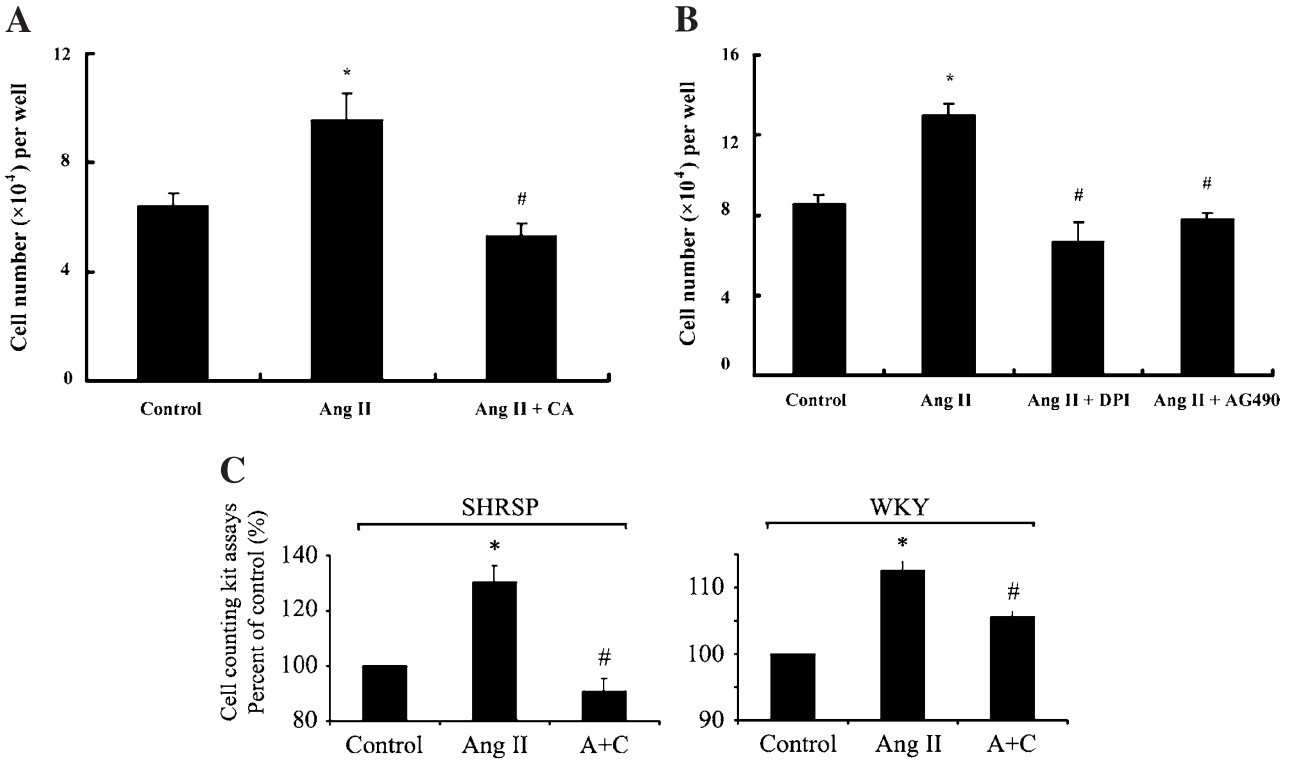

Fig. 6. *Ang II-induced cell proliferation was inhibited by CA, DPI and AG-490 in VSMCs. A: In the sham-operated control, VSMCs from SHRSP were incubated in serum-free DMEM for 48 h. Other groups were treated for 48 h with either 100 nmol/l Ang II or 100 nmol/l Ang II plus 100 μmol/l CA, respectively. Cells were trypsinized, and directly counted with a Sample Measurement Unit. B: In the sham-operated control, VSMCs from SHRSP were incubated in serum-free DMEM for 48 h. Other groups were treated for 48 h with either 100 nmol/l Ang II, 100 nmol/l Ang II plus 50 μmol/l DPI, or 100 nmol/l Ang II plus 10 μmol/l AG-490, respectively. Cells in A and B were trypsinized, and counted with a direct counter. The cell numbers were compared among the different groups. Each group contained 6 different plates. C: VSMCs from either SHRSP or WKY rats were plated onto two 96-well plates at a density of 10^4 cells/well. After cells adhered to the wall of the wells, the medium was changed to 100 μl of serum-free DMEM (control) or 100 μl of serum-free DMEM containing either 100 nmol/l Ang II or 100 nmol/l Ang II plus 100 μmol/l CA. After 48 h of incubation in the conditioned medium, 10 μl of a mixture of Reagent A and Reagent B was added to the medium. The color was allowed to develop for 3 h and the absorbance at 450 nm was read. The comparison of cell number among different groups was done with the absorbance of the control as 100%. Each group contains 6 different samples in triplicate. Data are expressed as the mean±SEM (*n = 6). *$p < 0.05$ vs. *the control;* #$p < 0.05$ vs. *the Ang II group. Ang II, angiotensin II; CA, caffeic acid; A+C, angiotensin II+caffeic acid.*

nal to the nucleus, resulting in the expression of early growth response genes and either accumulation of protein inside the cells (hypertrophy) or mitogenesis of the cells (hyperplasia). Moreover, ROS have also been implicated in the pathogenesis of atherosclerosis and hypertension (*24*), and oxidative stress has been recognized as an important stimulus for the vessel wall which mediates the cellular proliferation in VSMCs (*6–10*). In this regard, it has been reported that growth factors such as platelet-derived growth factor PDGF-BB and G protein-coupled receptor agonists such as Ang II and thrombin stimulate VSMC growth through the production of ROS (*6*, *11*, *25*).

Among various extracellular growth stimuli, Ang II is of special importance. It is not only a vasoactive hormone but also a mitogen that mediates the proliferative action of VSMCs (*26*). Interestingly, some forms of hypertension, notably those associated with high circulating levels of Ang II, are accompanied by and a consequence of the production of superoxide anion (O^{2-}) (*7*, *27*, *28*). Some studies indicate that Ang II directly activates an NADPH oxidase in cultured VSMCs, inducing oxidative stress and promoting VSMC proliferation through the induction of ROS (*10*, *28–31*).

It has been shown that the intracellular release of ROS in response to ligand stimulation acts as a second messenger in signal transduction, playing important roles in both the JAK2/STATs pathway (*8*) and the ERK1/2 pathway (*32–34*) in inducing VSMC proliferation. For instance, stimulation of the JAK/STAT cascade by Ang II requires O^{2-} anions generated

by the NADPH oxidase system (*8*). Superoxide anion or hydrogen peroxide are thought to be upstream of phosphorylation of JAK2 and ERK1/2 (*34*); importantly, inhibition of these enzymatic pathways by antioxidants such as DPI, a nonspecific NADPH oxidase inhibitor, or by antisense transfection of $p22^{phox}$, a critical component of the NADPH oxidase, blocks the Ang II-induced activation of kinases and cell proliferation (*10*, *29*, *34*). Therefore, it is possible for exogenous antioxidants to block the cellular proliferative processes in response to Ang II in VSMCs in hypertension and other cardiovascular diseases. Our findings that caffeic acid inhibited the intracellular superoxide anion generation (Fig. 1), the JAK2/STAT1 pathway (Figs. 2, 3), and the ERK1/2 pathway (Fig. 4) as well as the cell proliferation (Fig. 6A) in cultured VSMCs are in agreement with published data on the role of antioxidants in cellular signal transduction and VSMC proliferation. Although caffeic acid has previously been demonstrated to be beneficial in cardiovascular diseases (*14–16*), the mechanisms underlying its beneficial effects are mostly not understood. Our present findings at least partially explain these effects.

Our findings that caffeic acid decreased the intracellular superoxide anion level (Fig. 1) and cell proliferation (Fig. 6) induced by Ang II are in agreement with the effect of DPI (Fig. 6B) (*29*, *34*). Both caffeic acid and DPI inhibited the generation of intracellular superoxide anion; as a result, the proliferation response ceased. Because AG-490 also abolished the proliferation of VSMCs in culture (Fig. 6B), it is reasonable to speculate that the effect of caffeic acid was associated with the inhibition of the phosphorylation of JAK2, STAT1 and ERK1/2, considering that the activations of both STAT1 and ERK1/2 are mediated by JAK2 (*35*).

Heat shock proteins (HSPs) are a family of cellular protective proteins characterized by up-regulation in response to stresses, including oxidative stress (*36*). Accumulation of HSPs has been reported in cardiac tissue during ischemia and reperfusion, conditions known to produce ROS (*37*) and which may contribute to VSMC proliferation leading to the onset of vascular diseases such as hypertension (*22*). It should be mentioned that HSPs were initially described as chaperones that facilitate the folding of other proteins. However, recent studies indicate that HSPs also play a role in signal transduction and regulate stress-responsive signaling pathways. For instance, it was reported that oxidative stress stimulates VSMCs to release protein factors, including HSP90 and several unidentified proteins that activate ERK1/2 (*22*), and that HSP90 is linked with both the JAK2/STAT pathway and the ERK1/2 pathway and plays a role in Ang II-induced cell proliferation in VSMCs (*35*, *38*).

In this study, treatment of VSMCs with Ang II for 24 h led to an accumulation of HSP90 protein (Fig. 6). However, treatment of VSMC with 100 μmol/l of caffeic acid significantly abolished the Ang II-induced increase in HSP90 steady-state protein level (Fig. 6). In light of the previous finding that HSP90 expression was induced in VSMCs *via* activation of the JAK/STAT pathway (*35*) and the present result that JAK2 was inhibited by caffeic acid, we conjecture that the increased levels of HSP90 abated in response to the inhibition of JAK2 tyrosine phosphorylation by caffeic acid on the one hand, and to the decreased intracellular oxidative stress on the other (Fig. 6). Caffeic acid, by itself, decreased the level of HSP90 protein only slightly. This may have been mainly attributable to the decreased intracellular oxidative stress.

Finally, regarding the difference in responses to Ang II stimulation between SHRSP and WKY rats, our findings on JAK2 phosphorylation (Fig. 2E and F) and VSMC proliferation (Fig. 6C) indicate that, although both kinds of VSMCs show a proliferative response to Ang II stimulation, the VSMCs derived from SHRSP, which exhibit higher oxidative stress than normotensive WKY rats (*39*), are more sensitive to mitogenic stimulation of Ang II and have a much higher inclination to proliferate than WKY rats, consistent with previous studies (*40*).

In summary, the data presented here indicate that caffeic acid inhibits Ang II-induced cell proliferation in VSMCs from both SHRSP and WKY rats, possibly through scavenging of the ROS induced by Ang II and then partially abolishing the signaling transduction events which are mediated by both the JAK/STAT pathway and the Ras/Raf-1/ERK pathway. Our findings should help to clarify the effect of antioxidant caffeic acid on Ang II-mediated mitogenic signaling and may therefore be beneficial in the prevention and alleviation of hypertension and other vascular diseases.

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