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Endothelial nitric oxide synthase is a molecular vascular target for the Chinese herb Danshen in hypertension

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Kim DD, Sánchez FA, Durán RG, Kanetaka T, Durán WN. Endothelial nitric oxide synthase is a molecular vascular target for the Chinese herb Danshen in hypertension. *Am J Physiol Heart Circ Physiol* 292: H2131–H2137, 2007. First published December 15, 2006; doi:10.1152/ajpheart.01027.2006.—Danshen, a Chinese herb, reduces hypertension in Oriental medicine. We hypothesized that Danshen acts partially through endothelial nitric oxide synthase (eNOS) signaling mechanisms. We tested the hypothesis using tanshinone II_A, an active ingredient of Danshen, and the two-kidney, one-clip renovascular hypertension model in hamsters. Oral tanshinone (50 μg/100 g body wt) reduced mean arterial pressure (MAP) from 161.2 ± 6.9 to 130.0 ± 7.8 mmHg (mean $\pm$ SE; $P < 0.05$) in hypertensive hamsters. MAP in sham-operated hamsters was 114.3 ± 9.2 mmHg. Topical tanshinone at 1 μg/ml and 5 μg/ml increased normalized arteriolar diameter from 1.00 to 1.25 ± 0.08 and 1.57 ± 0.11, respectively, and increased periarteriolar nitric oxide concentration from 87.1 ± 11.3 to 146.9 ± 23.1 nM ($P < 0.05$) at 5 μg/ml in hamster cheek pouch. N^G-monomethyl-L-arginine inhibited tanshinone-induced vasodilation. Hypertension reduced eNOS protein relative to sham-operated control. Tanshinone prevented the hypertension-induced reduction of eNOS and increased eNOS expression to levels higher than sham-operated control in hamster cheek pouch. Topical tanshinone increased normalized arteriolar diameter from 1.0 to 1.47 ± 0.08 in the cremaster muscle of control mice and to 1.12 ± 0.13 in cremasters of eNOS knockout mice. In ECV-304 cells transfected with eNOS-green fluorescent protein, tanshinone increased eNOS protein expression 1.35 ± 0.05- and 1.85 ± 0.07-fold above control after 5-min and 1-h application, respectively. Tanshinone also increased eNOS phosphorylation 1.19 ± 0.07- and 1.72 ± 0.20-fold relative to control after 5-min and 1-h application. Our data provide a basis to understand the action of a Chinese herb used in alternative medicine. We conclude that eNOS stimulation is one mechanism by which tanshinone induces vasodilation and reduces blood pressure.

tanshinone II_A; Radix Salvia miltiorrhizae; complementary and alternative medicine

COMPLEMENTARY AND ALTERNATIVE medicine, which includes herbal medicine, is a current focus of interest for the general public and the medical profession. Medicinal herbs have been used in the treatment of cardiovascular disease for a long time. In particular, Chinese herbs have been used traditionally for the reduction of hypertension. However, this ancient practice has not been subjected systematically to scientific testing.

Hypertension affects millions of people in the world. It causes loss of lives and carries significant economic cost. In addition, some medical therapeutic regimens and medications available for treating hypertension have significant side effects.

Thus it is important to investigate the mechanisms of action responsible for the benefits of Chinese herbs in the treatment of hypertension. The scientific assessment of this alternative treatment modality may help to achieve greater success in curing or controlling this silent killer and to minimize the side effects of current available treatment methods.

The vascular regulatory site for the control of blood pressure by adjustment of total peripheral resistance resides mainly in arterioles (23, 29, 38). The pathogenesis of hypertension has been associated with the function of endothelial nitric oxide (NO) synthase (eNOS), an enzyme that plays a crucial role in the regulation of vessel diameter, which is an important determinant of blood flow and resistance (1, 13, 24). eNOS catalyzes the production of NO, which in turn mediates arteriolar vasodilation, reduces peripheral resistance, and lowers the blood pressure. Thus alterations in the function of this enzyme can lead to elevation of blood pressure. In fact, experimental animals lacking this enzyme demonstrate hypertension (12, 19, 33).

We hypothesized that Chinese herbs may reduce hypertension by activation of microvascular eNOS. Among the available Chinese herbs, Danshen (*Radix Salvia miltiorrhizae*) is used in Oriental medicine to promote blood flow and to treat cardiovascular disease (2, 43). Tanshinone II_A, one of the active lipophilic ingredients responsible for the beneficial actions of Danshen (14, 41), has become commercially available. Thus we used tanshinone II_A to investigate the hypothesis that Chinese herb Danshen induces its beneficial antihypertensive effects by activating eNOS in the microcirculation.

MATERIALS AND METHODS

The experimental protocols for hamsters and mice were approved by the University of Medicine and Dentistry of New Jersey-New Jersey Medical School Institutional Animal Care and Use Committee and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

Induction of hypertension. We applied the two-kidney, one-clip (2K,1C) protocol to induce renal hypertension in male golden Syrian hamsters, weighing 80–120 g. Animals were anesthetized with pentobarbital sodium (50 mg/kg ip). We used a retroperitoneal approach and placed a silver clip (3-mm width, 5-mm length, and 0.23-mm inner space) to constrict the right renal artery. The renal vein and ureter were uncompromised by the clip. After surgery, muscle layers and skin were sutured. Sham-operated hamsters underwent the same procedure, except for placement of a clip. Buprenorphine (0.1 mg/kg sc) was administered upon recovery and 24 h later to prevent pain.

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Hamsters were caged and maintained until their use in experiments 2 wk later.

Hamster cheek pouch preparation. Hamsters were anesthetized with pentobarbital sodium (50 mg/kg ip). A tracheotomy was performed to ensure a clear airway passage. The right jugular vein was cannulated for administration of supplemental doses of anesthetic. The right carotid artery was cannulated for monitoring blood pressure using a PowerLab Pressure Monitor (ADInstruments, Colorado Springs, CO). The left hamster cheek pouch was prepared for intravital microscopy as described previously (8, 9, 18). At the completion of the experiment, the hamster was killed by an anesthetic overdose of pentobarbital sodium (150 mg/kg iv) while under anesthesia.

Mouse cremaster muscle preparation. Male wild-type mice (C57BL/6J, Jackson Laboratory, Bar Harbor, MA) and eNOS knockout mice [eNOS$^{-/-}$, B6.129P2-Nos3^{tm1Unc} (C57BL/6J background), Jackson Laboratory], 8–12 wk old, 25–30 g in body weight, were anesthetized with sodium pentobarbital (50 mg/kg ip). The right cremaster muscle was prepared for intravital microscopy as published previously (4, 37). At the completion of the experiment, the mouse was killed by an anesthetic overdose of pentobarbital sodium (150 mg/kg iv) while under anesthesia.

Cell culture. The initial batch of transfected eNOS-green fluorescent protein ECV-304 cells (ECVeNOS-GFP) was kindly provided by Dr. William C. Sessa (Yale Univ. School of Medicine). ECV-304 cells are cells with mixed endothelial and epithelial properties derived from a human bladder tumor (6) and can be stably transfected to express eNOS-GFP at relatively high levels (34). We have used ECV-304 as mimics of endothelial cells and detected minimal expression of eNOS (31). Cells were cultured in Dulbecco's modified Eagle's medium containing 400 µg/ml G-418 (Invitrogen, Carlsbad, CA). Media were supplemented with 10% fetal bovine serum, L-glutamine (1 mM), penicillin (100 IU/ml), and streptomycin (100 µg/ml). When cells reached confluence, ECVeNOS-GFP cells were prepared for experiments in the absence or presence of tanshinone II$_A$ for 5 min or 1 h.

Western blotting analysis. We extracted total proteins from ECVeNOS-GFP cells [according to Breslin et al. (5) and Sánchez et al. (31)] and from hamster cheek pouch [according to Durán et al. (10) and Kim et al. (17)] to detect eNOS and eNOS phosphorylation. After extractions of proteins, equal amounts of protein for each sample were loaded into 7.5% polyacrylamide gels, separated by SDS-polyacrylamide electrophoresis, and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) by electrophoresis. The membranes were incubated with mouse anti-NOS antibodies (BD Transduction Laboratories, San Jose, CA) that recognized human eNOS or phosphorylated-eNOS (p-eNOS). Subsequently, the membranes were allowed to react with mouse IgG secondary antibodies (Sigma Chemicals, St. Louis, MO). Bands corresponding to eNOS or p-eNOS protein were visualized using the enhanced chemiluminescence system (ECL, Pierce, Rockford, IL) and analyzed with a gel documentation system (IS-1000 Digital Imaging System, Alpha Innotech, San Leandro, CA).

Microscopy. Observations were made with an Olympus BH microscope. The recording system comprises an Optronics TEC-470 microscope camera (Optronics, Goleta, CA), a Sony monitor, and a MetaMorph image system (Universal Imaging, Downingtown, PA) for computer recording directly from the microscope camera and for image processing.

Arteriolar diameter measurements. Arterial luminal diameter was measured as the width of the transilluminated blood column using the MetaMorph image system. The image system was calibrated with a slide micrometer. Three or four arterioles were studied per animal. Baseline diameters were normalized to a value of 1. For each vessel, the experimental diameter was expressed as the ratio of baseline diameter (relative luminal diameter). To compare diameters before and after each intervention, diameters were measured at the same place in the arterioles of interest.

Periarteriolar NO measurement. The NO concentration was measured with an NO-sensitive microelectrode (3, 17). The microelectrodes were polarized at +0.9 V, and the current generated was measured with an electrometer (model 6517A, Keithley, Cleveland, OH). Calibration, using a gas tonometer at 37°C, was performed for each experiment by measuring the microelectrode current generated by 0, 600, and 1,200 nM NO. Microelectrodes having a linear relationship of electrical current to NO concentration were used. After the microelectrode tip was properly located, the arteriole and microelectrode were allowed to stabilize for 30 min before experimental protocols were applied.

Experimental protocol. To test the hypothesis that tanshinone II$_A$ has an antihypertensive effect, hamsters received 50 µg of tanshinone II$_A$ per 100 g of body weight once a day by oral gavage administration for 2 wk. We selected the concentration of tanshinone II$_A$ on the basis of previous publications (2, 7, 39). As a reference for toxicity levels, the median lethal dose for peritoneal injection of Danshen in mice is 36.7 g/kg (2). After the 2-wk tanshinone II$_A$ treatment period, the right carotid artery was cannulated for the measurement of mean arterial blood pressure (MAP). To examine the microvascular actions of the Chinese herb ingredient, after baseline data collection, tanshinone II$_A$ was applied to the hamster cheek pouch or mouse cremaster for 5 min via a side port into the suffusate bicarbonate buffer line to achieve the final concentration of 1 µg/ml or 5 µg/ml. After tanshinone II$_A$ application, the experiment was continued for an additional 60-min period for the measurement of arteriolar diameter and periarteriolar NO.

Chemicals. Tanshinone II$_A$ was purchased from Ningbo Shuanglin Traditional Chinese Medicine (Ningbo, China). The purity of tanshinone II$_A$ was more than 95% (batch number 030606). Tanshinone II$_A$ was dissolved initially in dimethyl sulfoxide (DMSO) and subsequently diluted to the desired experimental concentration with a mixture of 1.5% bovine serum albumin and bicarbonate buffer as a vehicle. N^G-monomethyl-L-arginine (L-NMMA; Calbiochem, San Diego, CA) was dissolved initially in distilled water to a concentration of 10^{-1} M and subsequently diluted to 10^{-5} M with bicarbonate buffer solution.

Statistical analysis. All data are expressed as means ± SE. Statistical analysis was performed using a one-way analysis of variance. When significant values were obtained, the Student-Newman-Keuls test was applied to determine which measurement differed significantly from another. Differences were considered significant for values of $P < 0.05$. All statistical analyses were performed using the InStat package (GraphPad, San Diego, CA).

RESULTS

Tanshinone II$_A$ significantly reduces blood pressure in hamsters. Experimental constriction of the renal artery increased MAP to 161.2 ± 6.9 mmHg relative to 114.3 ± 9.2 mmHg in age-matched nontreated normotensive sham-operated hamsters. Treatment with 50 µg tanshinone II$_A$ per 100 g of body weight for 2 wk reduced MAP from 161.2 ± 6.9 to 130.0 ± 7.8 mmHg (Fig. 1). To investigate the effect of tanshinone on normotensive hamsters, we treated a sham-operated group with tanshinone. We found no significant differences in MAP between sham-operated hamsters with and without tanshinone treatment (112.1 ± 8.3 vs. 114.3 ± 9.2 mmHg). Because tanshinone II$_A$ was dissolved in 0.2% DMSO for oral administration, we administered 0.2% DMSO once a day by oral gavage for 2 wk to hypertensive hamsters. Administration of 0.2% DMSO did not change MAP relative to nontreated hypertensive hamsters (160.0 ± 7.6 vs. 161.2 ± 6.9 mmHg).
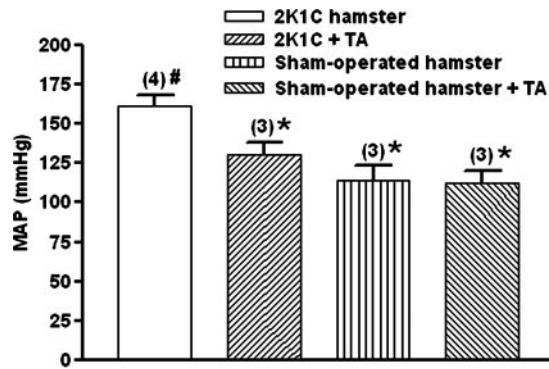
Fig. 1. Tanshinone II$_A$ (TA) reduces mean arterial blood pressure (MAP) in experimental renovascular hypertension. Bars represent means $\pm$ SE. The numbers in parentheses show the number of hamsters included in each group. *$P < 0.05$ compared with nontreated renovascular hypertensive [two-kidney, one-clip (2K,1C)] hamsters; #$P < 0.05$ compared with sham-operated hamsters.

Fig. 3. Blockade of nitric oxide (NO) synthase (NOS) inhibits tanshinone II$_A$-induced vasodilation. Graph shows maximal vasodilation responses to tanshinone II$_A$ in the presence and absence of N^G-monomethyl-L-arginine (L-NMMA) in hamster cheek pouch. Data represent means $\pm$ SE. The numbers in parentheses show the number of animals in each group. *$P < 0.05$ compared with vehicle; #$P < 0.05$ compared with 5 μg/ml tanshinone II$_A$.

Tanshinone II$_A$ produces vasodilation in the hamster cheek pouch microcirculation via NOS. Topical application of tanshinone II$_A$ at 1 μg/ml and 5 μg/ml caused significant dose-related vasodilation, which is indicated by the increased agent-to-control ratio of arteriolar diameters from 1.0 to 1.25 $\pm$ 0.08 and 1.57 $\pm$ 0.11, respectively, in the hamster cheek pouch (Fig. 2). The increase in arteriolar diameter ratio was significant relative to vehicle for each concentration as well as for comparison between the two concentrations of tanshinone II$_A$ ($P < 0.05$).

We used L-NMMA, an inhibitor of nitric oxide synthases, to initially test the hypothesis that activation of NOS is a key element in the biochemical-signaling pathway stimulated by tanshinone II$_A$. We applied L-NMMA topically at 10^{-5} M, a concentration that effectively blocks acetylcholine-induced vasodilation (30). Administration of 10^{-5} M L-NMMA produced a 75% reduction of the 5 μg/ml tanshinone-induced vasodilation, supporting our working hypothesis (Fig. 3).

Tanshinone II$_A$ increases periarteriolar NO concentration in hamster cheek pouch. If NOS plays a role as a signaling molecule, then the agonist must stimulate the production of NO. To test this required step of our hypothesis, we applied 5 μg/ml tanshinone II$_A$ topically and measured periarteriolar NO concentration with NO-sensitive microelectrodes. The mean

control diameter of the selected test arterioles was 40 $\pm$ 2 μm. Figure 4 shows that topical application of 5 μg/ml tanshinone II$_A$ significantly increased periarteriolar NO concentration from 87.1 $\pm$ 11.3 to 146.9 $\pm$ 23.1 nM ($P < 0.05$). The periarteriolar NO concentration reached its maximum value during the 5-min topical application and declined slowly after removal of tanshinone from the suffusate; baseline levels were achieved ~15–20 min later. The vehicle did not change periarteriolar NO concentration.

Tanshinone II$_A$ requires eNOS to induce vasodilation. Because pharmacological agents have intrinsic limitations (specificity, efficacy) and because NO may be produced by different isoforms of NOS, we opted to use eNOS$^{-/-}$ mice and their control eNOS$^{+/+}$ mice to more unequivocally and definitively test our hypothesis regarding the mechanisms of the beneficial actions of tanshinone II$_A$ on microvascular reactivity. Figure 5 shows the maximum vasodilation in response to 5 μg/ml tanshinone II$_A$ in the cremaster muscle of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice. A significant difference exists in responses in the cremasteric microvasculature of wild-type (eNOS$^{+/+}$) versus eNOS$^{-/-}$ mice. The arteriolar luminal diameter of eNOS$^{+/+}$ increased to a maximum of 1.47 $\pm$ 0.08 within 10 min and returned to control diameter 30 min after application of tanshinone II$_A$. In contrast, the luminal diameter of the cremasteric arterioles of eNOS$^{-/-}$ mice increased to a maxi-
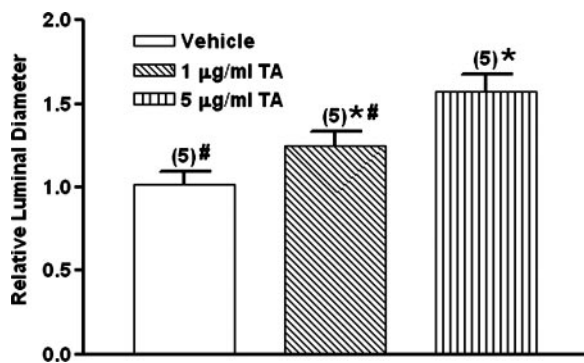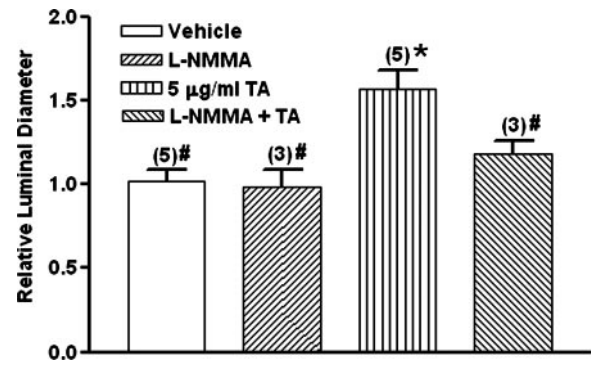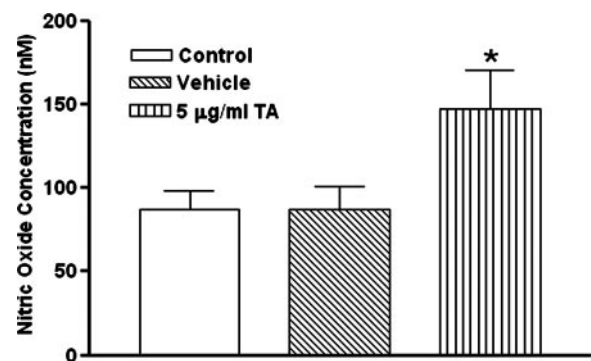

Fig. 2. Tanshinone II$_A$ increases arterial diameter. Bars show maximal vasodilation responses to tanshinone II$_A$ in hamster cheek pouch. Data represent means $\pm$ SE. The numbers in parentheses show the number of animals in each group. *$P < 0.05$ compared with vehicle; #$P < 0.05$ compared with 5 μg/ml tanshinone II$_A$.

Fig. 4. Tanshinone II$_A$ increases periarteriolar NO concentration. Bar graph shows maximal increments in response to tanshinone II$_A$ in hamster cheek pouch. Data represent means $\pm$ SE. Each group represents 3 hamsters. *$P < 0.05$ compared with control.
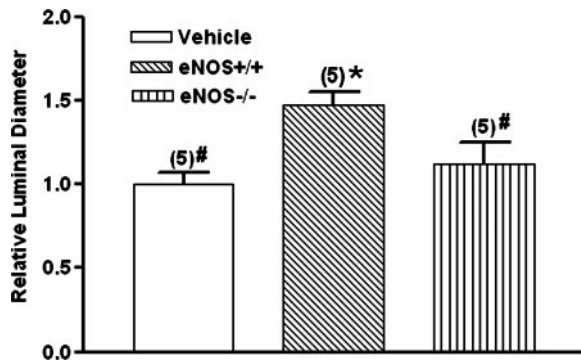
Fig. 5. Tanshinone II_A-induced vasodilation is mediated by endothelial NOS (eNOS)-generated NO in mouse cremaster muscle. *$P < 0.05$ compared with vehicle; #$P < 0.05$ compared with control eNOS$^{+/+}$ mice.

mum of 1.12 ± 0.13 ($P < 0.05$ compared with eNOS$^{+/+}$ mice) and returned to control diameter 20 min after application of tanshinone II_A.

Tanshinone II_A increased the levels of eNOS protein in hamster cheek pouch. After showing that tanshinone treatment has antihypertensive effects by enhancing eNOS activity, we tested the hypothesis that these beneficial effects are due to upregulation of eNOS. We determined the expression of eNOS protein in hamster cheek pouch by Western blotting. We detected eNOS in all four groups and measured their respective band intensity (Fig. 6). To standardize the analysis, we expressed the results as the ratio of the intensity of the experimental to the corresponding sham-operated control group (i.e., 2K,1C/sham). The results of the analysis of the net band intensities yielded 0.79 ± 0.03 (2K,1C hypertensive group), 1.15 ± 0.05 (treated 2K,1C group), and 1.01 ± 0.07 (treated sham-operated group) relative to the nontreated sham-operated control group in the hamster cheek pouch showing that *1*) hypertension reduced the expression of eNOS relative to the sham-operated group ($P < 0.05$) and *2*) tanshinone prevented the reduction of eNOS associated with hypertension and showed even higher eNOS protein expression than the sham-operated control in hamster cheek pouch ($P < 0.05$).

Tanshinone II_A increased the levels of eNOS and p-eNOS proteins in ECVeNOS-GFP cells. To test directly at a fundamental level the hypothesis that tanshinone activates eNOS, we determined the action of tanshinone on ECV-304 cells stably transfected with eNOS-green fluorescent protein expression plasmid (ECVeNOS-GFP). These transformed cells normally express low levels of eNOS and have been used as mimics of endothelial cells to assess trafficking and function of eNOS (25, 31, 34). Consequently, we examined the expression of eNOS protein and its phosphorylation in ECVeNOS-GFP cells by Western blotting, using the same method used in hamsters to standardize the eNOS band intensity (Fig. 7). The tanshinone-treated-to-nontreated control group (denominator) intensity ratios for eNOS were 1.35 ± 0.05 (5-min tanshinone application; $P < 0.05$ compared with control) and 1.85 ± 0.07 (1-h tanshinone application; $P < 0.05$ compared with both control and 5-min application). The respective values for p-eNOS were 1.19 ± 0.07 (5-min tanshinone application; $P < 0.05$ compared with control) and 1.72 ± 0.20 (1-h tanshinone application; $P < 0.05$ compared with both control and 5-min application).

To assess whether the increased levels of p-eNOS were due to enhanced mass of eNOS or to elevated phosphorylation, we calculated the ratio of p-eNOS to total eNOS for ECVeNOS-GFP cells. The p-eNOS-to-eNOS band intensity ratios of net intensity were 0.98 ± 0.02 (control), 0.87 ± 0.07 (5-min application), and 0.92 ± 0.11 (1-h application). There was no significant difference among the groups.

DISCUSSION

Our study demonstrates that tanshinone II_A *1*) reduces experimental renovascular hypertension in hamsters, *2*) increases production of NO and induces vasodilatation in hamster cheek pouch arterioles, and *3*) targets eNOS as a signaling element.

Tanshinone II_A is one of the active components of Danshen (*Radix Salvia miltiorrhizae*). Danshen, among other Chinese herbs, has been used traditionally for the treatment of hypertension in Asia (2, 43). Both aqueous and lipid soluble fractions of Danshen contain active components responsible for the observed clinical effects. The two active hydrophilic compo-
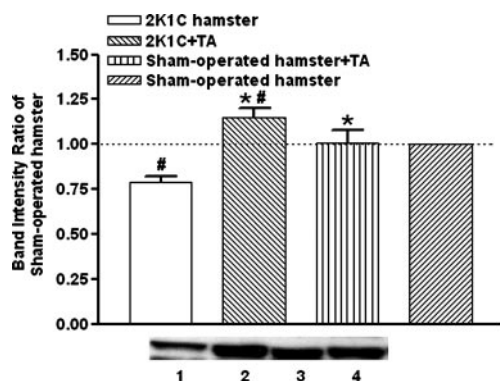

Fig. 6. Western blotting analysis of eNOS in hamster cheek pouch. The band intensities in the Western blots were divided by the band intensity of corresponding nontreated sham-operated hamster. Data represent means ± SE. Each group represents 3 hamsters. *$P < 0.05$ compared with nontreated 2K,1C hamsters; #$P < 0.05$ compared with sham-operated hamsters. *Bottom*: representative Western blot bands for each group: *1*, 2K,1C; *2*, 2K,1C + TA; *3*, sham-operated + TA; and *4*, sham-operated.
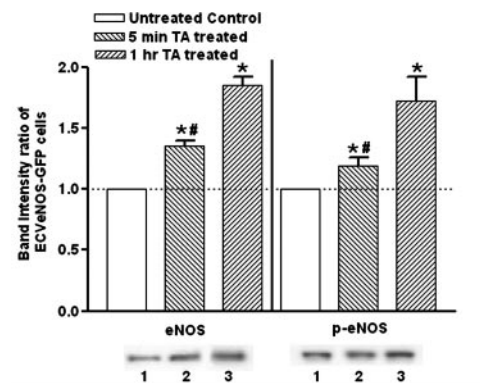

Fig. 7. Western blotting analysis of eNOS and phosphorylated eNOS (p-eNOS) proteins in green fluorescent protein ECV-304 cells (ECVeNOS-GFP) cells. Band intensities in the Western blots of treated cells were divided by the band intensity of corresponding nontreated control cells. *$P < 0.05$ compared with nontreated control; #$P < 0.05$ compared with 1-h tanshinone application. *Bottom*: representative Western blot bands for each group: *1*, control; *2*, 5-min tanshinone application; and *3*, 1-h tanshinone application.

nents of Danshen are danshensu and magnesium tanshinoate B, whereas cryptotanshinone and tanshinone II_A are the two lipophilic components (41). These four components are responsible for many of the actions of Danshen (2, 14, 43). Tanshinone II_A has antioxidant properties and protects against lipid peroxidation in vitro and in vivo, making it a potential antidote for free radical-based disorders (27, 39, 42). In addition, tanshinone II_A has neuroprotective effects in cerebral ischemia and attenuates angiotensin II-induced hypertrophy (21, 35, 40). Tanshinone II_A may also have a role as a therapeutic drug in the treatment of bone diseases such as osteoporosis (20). As a result, there is a great interest in the therapeutic potential of tanshinone II_A.

The mechanisms of action of tanshinone II_A or of Danshen in hypertension have not been fully investigated. Danshen reduces systolic blood pressure in rats partially through the inhibition of angiotensin-converting enzyme (15). Whether or not tanshinone is the active ingredient of Danshen responsible for the inhibition of angiotensin-converting enzyme remains to be elucidated; however, exploring that interesting possibility was beyond the scope of our inquiry. Our data demonstrate that tanshinone significantly reduced MAP in renal hypertensive hamsters, and we provide evidence that tanshinone-induced reduction in blood pressure is mediated, at least in part, by stimulation of eNOS.

It is worth noting that tanshinone II_A lowered blood pressure in experimental renovascular hypertensive hamsters but did not influence blood pressure in sham-operated hamsters. This observation is in agreement with a report that oral administration of the Chinese herb Folium Clerodendri Trichotomi reduced blood pressure in spontaneously hypertensive rats but not in normotensive control rats (26). The lack of hypotensive action of Chinese herbs in control normotensive animals remains an unexplained but reproducible observation.

We focused our attention on eNOS as a possible signaling target for tanshinone II_A because this enzyme is the major source of NO production in endothelial cells and an important regulator of vascular homeostasis. NO plays a crucial role in the state of blood vessel tone and hence blood pressure regulation (1, 13, 24). Our results in the microvasculature of hamsters and mice demonstrate that tanshinone II_A definitively has an impact on the activity of eNOS. In fact, tanshinone II_A produces arteriolar vasodilation that is directly associated with increased production of NO. The tanshinone II_A-induced vasodilation is blocked by NOS inhibition in hamsters and nearly abolished in mice lacking the gene encoding for eNOS. In addition, tanshinone II_A has a significant impact on the expression and activity of eNOS in hypertension. Hypertensive 2K,1C hamsters treated with tanshinone II_A showed higher levels of eNOS expression than did nontreated hypertensive 2K,1C hamsters.

Our results using tanshinone II_A in ECVeNOS-GFP cells support the report that magnesium tanshinoate B, a hydrophilic ingredient of Danshen, increases the level of eNOS protein in ECV-304 cells (16). However, some differences apparently exist between the cell lines used in the two studies. Karmin et al. (16) referred consistently to ECV-304 as endothelial cells [as characterized by Takahashi et al. (36)] and determined sizable levels of eNOS in these cells. We have used ECV-304 cells as mimics of endothelial cells and detected minimal expression of eNOS (31). It seems that ECV-304 cells *1*) are

derived from a human bladder tumor (6), but their phenotype partially overlaps with that of endothelial cells; *2*) demonstrate low or no expression of eNOS (28, 34); and *3*) can be stably transfected to uniformly express eNOS-GFP at relatively high levels (34). Thus these cells can serve as a useful model in which to study eNOS trafficking and functional biology (25, 28, 31, 34).

We demonstrate that tanshinone significantly increases total eNOS and p-eNOS in ECVeNOS-GFP cells after exposure of 5 min and 1 h. Overall, these data support, at a fundamental level, the concept that tanshinone II_A (and thus, Danshen) stimulates eNOS directly and utilizes the eNOS-related signaling pathways to induce vasodilation and decrease blood pressure. Phosphorylation of eNOS is recognized as a critical regulatory mechanism controlling eNOS activity in vitro and in vivo (10, 11). We indeed demonstrated a rapid increase in NO production in vivo, most likely subsequent to eNOS phosphorylation. However, our calculations showing that the ratio of p-eNOS to total eNOS remains constant for all the ECVeNOS-GFP cell groups support the conclusion that the increase in eNOS mass is probably the predominant factor responsible for the beneficial microvascular hypotensive results induced by tanshinone.

The increase in eNOS at 1-h corroborates the finding by Karmin et al. (16) using magnesium tanshinoate B; however, the increase in ECVeNOS-GFP cells after only 5 min of exposure is novel and surprising. According to textbooks of biochemistry, most de novo protein synthesis takes ~45 min as a minimum; thus it is unlikely that the increase in total eNOS after 5 min of exposure to tanshinone II_A represents de novo protein synthesis. We did not investigate the underlying biochemical mechanisms for this observation. We speculate that the rapid response induced by tanshinone II_A may represent a direct posttranscriptional effect on eNOS mRNA. A given pool of mRNA may stay in the cytosol for as long as 35 h (32). Thus synthesis of eNOS may be greatly accelerated by tanshinone stimulation of the appropriate mRNA pool, bypassing the requirement for gene transcription and translation.

We are not aware of studies relating Danshen directly to the eNOS pathway. However, Lam et al. (22) showed that the vasorelaxant actions of Danshen on rat isolated femoral artery were produced primarily by inhibition of Ca^{2+} influx and were mediated partially by the opening of K^+ channels. In addition, Zhou et al. (44) reported that cryptotanshinone induced a concentration-dependent increase eNOS expression without significantly changing neuronal NOS expression in human umbilical vein endothelial cells (44). From our studies, it is plausible that tanshinone may influence either eNOS directly or indirectly by modifications of signaling molecules upstream of eNOS, such as Akt, phosphatidylinositol 3-kinase, or G proteins. Our data, showing an increased expression of eNOS after tanshinone in hypertensive animals, favor a direct action on this enzyme, but the data do not rule out changes in the activity of associated elements in the eNOS signaling cascade.

We demonstrate that enhanced microvascular eNOS protein synthesis and its associated increase in phosphorylation and NO production are a potentially important mechanism by which the active ingredients of Chinese herbs decrease blood pressure in hypertension. In particular, our study focused on tanshinone II_A, an active lipophilic ingredient of Danshen. A better understanding of the microvascular mechanisms of ac-

tion of Chinese herbs used in the treatment of hypertension should contribute to bring closer together the practices of complementary and alternative medicine and allopathic medicine in the treatment of hypertensive patients.

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