Gene Therapy With Human Tissue Kallikrein Reduces Hypertension and Hyperinsulinemia in Fructose-Induced Hypertensive Rats

Chunxia Zhao, Peihua Wang, Xiao Xiao, Julie Chao, Lee Chao, Dao Wen Wang, Darryl C. Zeldin

Abstract—This study investigates gene therapy with human tissue kallikrein as a treatment for fructose-induced hypertension in rats. Hypertension was induced by addition of 10% fructose to drinking water. Fructose-fed rats also had increased serum insulin and triglycerides, decreased urine osmolarity, increased urine volume and endothelin-1, and increased aortic endothelin-1, endothelin-A receptor, and angiotensin II receptor type 1 mRNA levels. Fructose-induced hypertensive and control rats were injected intravenously with a construct containing the human tissue kallikrein cDNA. Two weeks after injection of hypertensive rats, systolic blood pressure and serum insulin levels normalized, urine osmolarity increased, urine endothelin-1 levels decreased, and aortic endothelin-1, endothelin-A receptor, and angiotensin II receptor type 1 mRNA levels decreased. In contrast, injection of the human tissue kallikrein cDNA had minimal effect on blood pressure or insulin levels in control rats. These results suggest that gene therapy with human tissue kallikrein may have potential as a treatment for hypertension and associated insulin resistance. Moreover, our data suggest that the beneficial effects of human tissue kallikrein on these parameters are associated with changes in endothelin-1, endothelin-A receptor, and angiotensin II receptor type 1 expression. (Hypertension. 2003;42:1026-1033.)

Key Words: kinins, diabetes mellitus, hypertension, renal, endothelin, receptors, endothelin, angiotensin II

Hypertension is a chronic disease of major public health importance. Hypertension affects ~50 million individuals in the United States and ~1 billion individuals worldwide. The relation between blood pressure and risk of cardiovascular events is continuous, consistent, and independent of other risk factors. The higher the blood pressure, the higher the chance of myocardial infarction, heart failure, stroke, and kidney disease.1

Tissue kallikrein is a serine protease that converts kininogen to the peptide hormone kinin. Kinin is a potent vasodilator that plays important roles in controlling vascular tone, local blood flow, electrolyte and glucose transport, pain, inflammation, and vascular permeability.2 Tissue kallikrein is significantly reduced in the urine of patients with hypertension3 and in kidneys and urine of hypertensive rats.4 In addition, transient hypotension can be induced by repeated administration of high doses of tissue kallikrein.3 These results suggest that tissue kallikrein may be involved in blood pressure homeostasis and chronic disease processes related to development and maintenance of systemic hypertension.

Genetic studies confirm the putative role of tissue kallikrein in hypertension and suggest that gene therapy might be feasible and efficacious. For example, mice lacking the kallikrein gene are hypertensive,5 and gene transfer can lower blood pressure in hypertensive animals.7–9 Drug therapy for hypertension can effectively reduce blood pressure; however, medication side effects are common and maintenance of normal blood pressure requires daily medication. Thus, additional studies of the efficacy and benefits of gene therapy in hypertensive rodent models are warranted.

This study tests gene delivery of tissue kallikrein as a therapeutic modality in a rat model of hypertension. Sprague-Dawley rats were fed water containing 10% fructose, which increased blood pressure, serum insulin, serum triglycerides and urine volume, decreased urine osmolarity, and increased urine endothelin-1 (ET-1) and aortic ET-1, endothelin-A receptor (ET_A-R), and angiotensin II receptor type 1 (AT(R1)) mRNA levels. Hypertensive and control rats were then injected intravenously with a construct containing the cDNA for human tissue kallikrein under control of the CMV promoter. Near normal physiology was restored in hypertensive animals, and no significant changes were observed in control rats.
Methods

Materials

Goat anti-rabbit horseradish peroxidase was obtained from Jackson Immunoresearch Laboratories, enhanced chemiluminescence reagent from Pierce, pre-stained molecular weight standards from Bio-Rad, PVDF membranes from Schleicher and Schuell, and fructose from Promega. Anti-human tissue kallikrein antibody and human tissue kallikrein cDNA were gifts from Dr. Lee Chao (Medical University of South Carolina). All other chemicals/reagents were from Sigma-Aldrich unless otherwise specified.

Animals

Male Sprague-Dawley rats weighing 180 to 200 g were from the Experimental Animal Center of Shanghai. All animals were housed at 25°C with 12-hour light/dark cycles and allowed free access to normal rat chow and water ad libitum. Experimental protocols complied with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by The Academy of Sciences of China. Animals were randomized to treatment groups.

Preparation of pcDNA3.1-HK

The human tissue kallikrein cDNA was cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen) using NotI and XhoI restriction sites (Figure 1). The resulting plasmid (pcDNA3.1-HK) was purified with the use of a kit from Qiagen.

Fructose Feeding Protocol

Rats were monitored for 1 week to establish baseline blood pressures. Control rats (n=18) were fed normal chow and given normal water; experimental animals (n=22) were fed normal chow and given water containing 10% fructose. Blood pressure was measured at baseline and weekly for 2 weeks before initiating gene delivery experiments.

Intravenous Injection of pcDNA3.1 or pcDNA3.1-HK

Control rats either received injection with empty pcDNA3.1 vector (N+pcDNA3.1), pcDNA3.1-HK (N+pcDNA-HK), or 0.9% NaCl (normal) (n=6 per group). Fructose rats either received injection with empty pcDNA3.1 vector (F+pcDNA3.1) or pcDNA3.1-HK (F+pcDNA-HK) (n=11 per group). Animals were anesthetized with ether before injection. Purified plasmid DNA was dissolved in 0.9% NaCl at 1 mg/mL and injected into the sublingual vein at a dose of 2.5 mg/kg body weight.

Figure 1. Schematic diagram of pcDNA3.1-HK vector. Human tissue kallikrein cDNA was cloned downstream of the CMV promoter. Bovine growth hormone (BGH) polyA tail is present to enhance mRNA stability.

Blood Pressure Measurements

Systolic blood pressure was measured in awake rats with a manometer-tachometer (Rat Tail NIBP System, ADI Instruments) by means of the tail-cuff method.22 Rats were allowed to acclimate, and 5 to 10 blood pressure readings were taken per animal per day. These values were averaged and group means were calculated for each measurement day. All measurements were made between 9:00 AM and noon.

Serum and Urine Analysis

Blood samples (~1 mL) were taken from the tail vein at baseline, immediately before injection of plasmid DNA or vehicle (ie, 2 weeks after beginning fructose or water administration), 2 weeks after injection of plasmid DNA or vehicle, and at the end of the experiment (terminal bleed through cardiac puncture). Animals were anesthetized with ether, and blood samples were collected between 8:30 and 10:30 AM after an overnight fast. Serum was prepared and stored at −80°C. Twenty-four-hour urine samples were collected from 8:00 AM to 8:00 AM the following day in brown bottles containing dimethylamine. Serum glucose, cholesterol, and triglyceride and urine magnesium were measured in duplicate on an AEROSET Clinical Chemistry System (Abbott Laboratories). Serum insulin was measured with the use of a magnetic solid phase enzyme immunoassay kit from BioChem ImmunoSystems. Insulin resistance was calculated by means of the homeostasis model assessment (HOMA) method.21 Urine osmolarity was measured on a model 3D3 osmometer (Advanced Instruments, Inc.). Serum and urine ET-1 concentrations were determined by ELISA with a kit from Cayman Chemical Company.

Western Blot

Two weeks after injection with DNA or vehicle, 3 rats from each group were killed by lethal intraperitoneal injection of pentobarbital (100 mg/kg). After animals were exsanguinated with 0.9% NaCl, hearts, kidneys, lungs, livers, and spleens were excised, frozen, and stored at −80°C. Proteins were extracted through the use of TRIZOL reagent (Invitrogen Life Technologies) and protein concentrations were estimated by the Bradford method.23 Protein samples (20 μg per lane) were separated by 10% SDS/PAGE, electrophoretically transferred onto PVDF membranes, and the membranes were incubated for 2 hours at room temperature with 5% nonfat dried milk in 10 mmol/L Tris-Cl (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween-20 (TBS-T). The membranes were incubated overnight at 4°C with rabbit anti-human tissue kallikrein polyclonal antibody (1:2000), followed by goat anti-rabbit IgG conjugated to horseradish peroxidase for 2 hours at room temperature. After each incubation, the blot was washed 4 times in TBS-T at room temperature and then developed with enhanced chemiluminescence.

Human Tissue Kallikrein ELISA

Human tissue kallikrein was measured in rat sera and urine by ELISA by using anti-human tissue kallikrein IgG coupled to biotin as described.8 Plates were read at 405 nm with a Universal Microplate Reader (Bio-Tek Instruments).

RNA Extraction and RT-PCR Analysis of ET-1, ETA-R and ATR1

RNA was extracted from frozen rat aortas through the use of TRIZOL reagent. The following oligonucleotide primers were used for amplification of the ET-1, ETA-R and ATR1 cDNAs from reverse-transcribed aortic RNA: ET-1(foward), 5′-TCCCGTATCT-TCCTCTCCGTC-3′; ET-1(reverse), 5′-GGCTCTGATGATCAGGTG-3′; ETA-R(forward), 5′-GGCCACTTCCTCTAAAGAATC-3′; ETA-R(reverse), 5′-ATCCAAAGGACCACCACTG-3′; ATR1(forward), 5′-GAGGGTACCACAAGGGACAG-3′; ATR1(reverse), 5′-GACAGATCCGTATACGTTAGTGA-3′. After incubation with Moloney murine leukemia virus reverse transcriptase at 42°C for 15 minutes, amplifications were performed with reagents from Applied Biosystems under the following conditions: denaturation at 94°C, annealing at 50°C, elongation at 72°C, 30 cycles. PCR...
Fructose Drinking Induces Hypertension and Changes in Plasma Insulin and Urine Osmolarity

Sprague-Dawley rats were either fed normal chow and water or normal chow and water without fructose (control group). Systolic blood pressure, body weight, urine volume, osmolality and magnesium, and serum glucose, insulin, cholesterol, and triglycerides were measured at baseline and again 2 weeks after beginning fructose or water administration. There were no significant differences between the 2 groups in any of these parameters at baseline (data not shown). Two weeks after beginning fructose or water administration, fructose rats had a significant increase in systolic blood pressure compared with control rats (134.7 ± 0.4 versus 120.2 ± 0.4 mm Hg, respectively, \( P < 0.001 \)) (Table 1). In addition, there was a significant increase in serum insulin, serum triglyceride and urine volume, a significant decrease in urine osmolality, and a significant increase in urinary excretion of magnesium in fructose rats (Table 1). There were no significant changes in plasma glucose, cholesterol or body weight. Insulin resistance (HOMA) was significantly increased in fructose rats. These data indicate that fructose administration induces hypertension associated with hyperinsulinemia, insulin resistance, hypertriglyceridemia, and hypo-osmolar diuresis with increased urinary magnesium loss.

### Results

**Physiological Parameters in Rats Injected With pcDNA3.1-HK**

Physiological parameters were measured in serum and urine of injected rats; the results are shown in Table 2. In fructose-induced hypertensive rats injected with empty pcDNA3.1 vector, serum insulin remained elevated (14.00 ± 1.55mIU/L, \( P < 0.05 \) versus non–fructose-fed groups). Interestingly, injection with pcDNA3.1-HK reduced serum insulin to 9.64 ± 1.00 mIU/L (\( P < 0.05 \) versus fructose-fed rats receiving empty pcDNA3.1 vector). In contrast, no significant changes in serum insulin were observed in non–fructose-fed animals injected with either pcDNA3.1-HK, pcDNA3.1 empty vector,
Expression of Human Tissue Kallikrein in Rats Injected With pcDNA3.1-HK

Two weeks after injection, immunoblot analysis was carried out with the use of an antibody to tissue kallikrein that reacts with both human and rat isoforms. Compared to rats injected with empty pcDNA3.1 vector, tissue kallikrein expression increased in liver, kidney, heart, lung, and spleen after single injection of pcDNA3.1-HK (Figure 3). These results were independently confirmed by ELISA. Tissue kallikrein was detected in serum and urine of rats injected with pcDNA3.1-HK but not in control rats injected with empty pcDNA3.1 vector (Figure 4). Levels of tissue kallikrein in serum and urine were lower at 3 weeks after injection than at 2 weeks after injection, consistent with the transient nature of target gene expression with the pcDNA3.1 vector. These data confirm that rats injected with pcDNA3.1-HK overexpress tissue kallikrein.

**ET-1, ET₄₅-R, and ATR1 Expression**

Aortic expression of ET-1, ET₄₅-R, and ATR1 transcripts was determined by RT-PCR analysis to examine effects of fructose feeding and human tissue kallikrein gene delivery on these pathways. Fructose administration resulted in small but significant increases in aortic levels of ET-1, ET₄₅-R, and ATR1 mRNAs in rats injected with empty pcDNA3.1 vector (Figures 5A through 5F). In contrast, these changes were attenuated in rats injected with pcDNA3.1-HK. Thus, ET-1, ET₄₅-R, and ATR1 transcript levels were 1.8-, 2.6- and 1.7-fold lower in fructose-fed pcDNA3.1-HK injected rats compared with fructose-fed empty pcDNA3.1 vector injected rats, respectively (P<0.05). Injection with pcDNA3.1-HK or empty pcDNA3.1 vector had no effect on ET-1, ET₄₅-R, and ATR1 transcript levels in non-fructose-fed animals. There were no significant differences in serum ET-1 concentrations between the groups (Figure 6A). Fructose administration

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<th>TABLE 2. Physiological Parameters in Rats Injected With Human Kallikrein cDNA</th>
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Values are mean±SEM. Groups N+pcDNA3.1, N+pcDNA-HK and Normal were fed water without fructose, and groups F+pcDNA3.1 and F+pcDNA-HK were fed water containing 10% fructose. Groups N+pcDNA3.1 and F+pcDNA3.1 were injected with empty pcDNA3.1 vector. These data confirm that rats injected with pcDNA3.1-HK overexpress tissue kallikrein.

Figure 3. Expression of tissue kallikrein by immunoblot analysis in fructose-induced hypertensive rats 2 weeks after injection with either pcDNA3.1-HK or empty pcDNA3.1 vector. Protein was prepared from liver (Li), kidney (K), heart (H), lung (L), and spleen (Sp) of injected rats. Experiment was repeated 4 times. Tissue kallikrein was detected with rabbit polyclonal anti-human kallikrein antibody that cross-reacts with rat kallikrein.
Discussion

This study demonstrates that gene therapy has beneficial effects on elevated blood pressure in fructose-induced hypertensive rats. Human tissue kallikrein expression was achieved with the pcDNA3.1 eukaryotic expression vector. A single injection of human tissue kallikrein cDNA under control of CMV promoter caused significant reductions in blood pressure and associated hyperinsulinemia in fructose-induced hypertensive rats, an effect that persisted for at least 3 weeks. In contrast, injection of the kallikrein cDNA did not affect blood pressure or circulating insulin levels in control rats. These results suggest that kallikrein gene therapy might have potential as an alternative to drug therapy for hypertension in humans. Hypertension is a major public health problem with significant associated morbidity and mortality rates from cardiovascular disease, stroke, and renal disease. Drug therapy is not always suitable or efficacious in patients with hypertension; thus, a successful therapeutic approach based on gene delivery could potentially reduce the public health burden of this disease.

Previous studies have demonstrated that hypertension develops in rats when a high concentration of fructose is present in their drinking water or added to their food. Dai and coworkers used 5%, 10%, or 20% fructose in drinking water to determine the most suitable concentration/duration of fructose treatment for inducing hypertension in Wistar rats. They concluded that treatment with 10% fructose in drinking water (which is equivalent to a diet containing 48% to 57% fructose) for >1 week is appropriate for the rapid production of fructose-induced hypertension. The precise pathophysiological mechanism(s) responsible for the elevated blood pressure in fructose-fed animals are poorly understood, but several possible causes have been suggested. For example, fructose-induced hypertension in rats may result from an increase in sympathetic nervous system activity, impaired endothelium-dependent vasodilation, reduced capillary permeability, and/or elevated vascular expression of ET-1 and ET\(_A\)-R genes. In this regard, we demonstrated that vascular levels of ET-1 and ET\(_A\)-R transcripts were increased and urinary ET-1 levels were elevated after fructose feeding. Together, these data are consistent with a role for ET-1 and ET\(_A\)-R in the pathogenesis of fructose-induced hypertension.

The hypotensive effect of pcDNA3.1-HK injection "wore off" after 2 weeks and was associated with reduced plasma and urine concentrations of tissue kallikrein. Moreover, the effect of pcDNA3.1-HK on urine osmolarity and magnesium was also attenuated 3 weeks after injection. The data suggest that the temporary effect of pcDNA3.1-HK injection observed in the current study is not due to tachyphylaxis but rather due to the use of a transient expression system that drives target gene expression in vivo for \(\approx\)3 to 4 weeks, with maximal expression at \(\approx\)2 weeks after injection. Indeed, this transient expression pattern is similar to that observed by other groups who used similar expression vectors in other model systems. For example, Wang and coworkers reported that maximal effect on blood pressure occurred 2 to 3 weeks after a single injection of human kallikrein plasmid DNA in an SHR model of hypertension. In that study, human kallikrein levels in sera and urine were highest at 1 to 2 weeks after injection. In another study by the same investigators, a single intravenous injection of eNOS plasmid DNA caused a temporary reduction of blood pressure in SHR.

Recently, we used a recombinant adeno-associated viral (rAAV) vector to deliver the human kallikrein gene to SHR animals. Our preliminary results demonstrate that a single rAAV · HK injection induced a long-term (6 to 8 months) reduction in blood pressure in this model. Future work will determine if human kallikrein gene delivery through the rAAV system will lead to sustained reductions in blood pressure and amelioration of metabolic abnormalities in the fructose-induced hypertension model.

The mechanisms by which kallikrein mediates reduced blood pressure in fructose-induced hypertensive rats are not known. In a previous study involving kallikrein gene therapy in rats with spontaneous hypertension, the hypotensive effect of kallikrein was mediated by the bradykinin B\(_1\) receptor. Thus, it seems likely that the hypotensive effect of kallikrein is mediated by kinin through a NOx-cGMP signal transduction pathway. Kinin binds to the bradykinin B\(_1\) receptor, which initiates release of nitric oxide. Nitric oxide signaling can lead to increased vasodilation, enhanced blood flow, and
increased glucose transport in skeletal muscle. However, increased kinin does not alter blood pressure in control mice, suggesting that blood pressure homeostasis is also regulated by other factors. In this regard, we showed that vascular levels of ET-1 and ETA-R transcripts and urinary ET-1 levels were significantly lower in fructose-fed rats injected with pcDNA3.1-HK than in fructose-fed rats injected with empty pcDNA3.1 vector. This suggests that changes in the endothelin pathway may mediate some of the beneficial effects of human tissue kallikrein gene therapy on blood pressure in this model.

Magnesium deficiency may play a role in inducing metabolic and/or blood pressure abnormalities in fructose-fed rats, as reported by Balon and coworkers. We found that fructose drinking significantly increased urinary magnesium concentrations, suggesting renal loss of this electrolyte. Interest-

Figure 5. RT-PCR analysis showing expression of ET-1 (A), ETA-R (C), and ATR1 (E) transcripts in aortic tissue from normal rats injected with saline (lane 1), fructose-fed rats injected with empty pcDNA3.1 vector (lane 2), fructose-fed rats injected with pcDNA3.1-HK (lane 3), normal rats injected with empty pcDNA3.1 (lane 4), and normal rats injected with pcDNA3.1-HK (lane 5). Expression of GAPDH is also shown (lower bands). B, D, and F, Densitometry data normalized to GAPDH values. Values are mean±SEM of 3 independent experiments. *P<0.05 vs normal group; **P<0.05 vs fructose-fed group receiving injection with empty pcDNA3.1 vector.

Figure 6. Serum (A) and urine (B) ET-1 concentrations in normal control rats, fructose-fed rats injected with empty pcDNA3.1 vector, and fructose-fed rats injected with pcDNA3.1-HK. Values are mean±SEM. *P<0.05 vs normal control group; **P<0.05 vs fructose-fed group receiving injection with empty pcDNA3.1 vector.
ingly, human kallikrein gene delivery significantly attenuated the urinary magnesium loss. Although the mechanism for this effect is unknown, we speculate that renal tubules may be damaged, resulting in elevated urinary magnesium excretion in the fructose-induced hypertensive rats and that human kallikrein gene therapy may prevent this renal tubular injury and hence attenuate magnesium loss. Whether attenuation of magnesium loss played a significant role in the hypotensive and beneficial metabolic effects of human kallikrein gene delivery remain to be determined.

Previous studies demonstrate that alterations in the renin-angiotensin system occur in the fructose hypertension model. Indeed, elevation of plasma angiotensin II and ATR1 density have been proposed to play important roles in fructose-induced hypertension and related pathophysiology. We examined the effects of human kallikrein gene delivery on changes in expression of ATR1, which mediates the hypertensive actions of angiotensin II, in fructose-induced hypertensive rats. We found that ATR1 was upregulated in fructose-induced hypertensive rats compared with normal water drinking control rats. Importantly, human kallikrein gene delivery markedly attenuated the elevated ATR1 mRNA levels. The data suggest that one potential mechanism for the beneficial effects of human kallikrein gene therapy in the fructose-induced hypertension model involves limitation of angiotensin II actions.

Taurine lowers blood pressure and insulin resistance in fructose-fed rats, and it elevates kallikrein levels. Additionally, taurine can amplify kallikrein and reduce blood pressure in salt-induced hypertensive Dahl rats. From these reports, we can speculate that the main mechanism of taurine action is to enhance or amplify kallikrein actions in vivo.

The results presented herein also confirm that hyperinsulinemia and insulin resistance develop in fructose-induced hypertensive rats. It is worth noting that a similar association is observed in humans. Hypertension and hyperinsulinemia may be caused by related physiological processes, because blood pressure does not increase in rats fed high fructose water if hyperinsulinemia is prevented by infusion of a somatostatin analogue. However, a direct relation between hypertension and hyperinsulinemia has not yet been conclusively demonstrated. The mechanisms by which kallikrein attenuates insulin resistance remain to be elucidated. Maximum glucose uptake, insulin sensitivity index, and insulin clearance were reduced in kininogen-deficient rats, and chronic in vivo treatment with bradykinin significantly improved whole-body glucose tolerance. These investigations are consistent with the attenuation of hyperinsulinemia observed in the current study after human tissue kallikrein gene therapy.

We observed that rats consuming water with fructose have a higher urine volume and lower urine osmolarity than control animals. A study by Iyer and Katovich found that there were no differences in sodium, potassium, or urine excretion and no differences in hematocrit, plasma volume, or extracellular fluid volume between control and fructose-fed rats. They concluded that elevation of blood pressure in fructose-fed rats did not occur directly through sodium retention or an increase in fluid volume. One important difference between their study and ours was that they used food containing 60% fructose, whereas we fed regular laboratory chow and added 10% fructose to drinking water. We did not adjust the water for level of sweetness in our study, as this would have introduced additional experimental variables that could complicate interpretation of the data. We found that water intake in the fructose-drinking rats was ~1.8-fold higher that in control rats. Indeed, 10% fructose in water may encourage rats to drink more and therefore have increased urine volumes. In contrast, addition of fructose to food may not have this effect on drinking behavior. We believe that this difference accounts, at least in part, for the increased urine volume in the fructose animals in our study. Interestingly, we found that human kallikrein gene therapy reduced fructose water intake by ~20%. Urine osmolarity was increased in both control and fructose-treated animals after human kallikrein gene delivery. We do not know the exact mechanisms for this effect but speculate that human kallikrein gene delivery may enhance renal tubular function, which leads to increased urinary concentrating capacity.

In summary, this study shows that gene therapy with human tissue kallikrein has beneficial effects in fructose-induced hypertensive rats. Additional experiments are needed to determine if a similar approach can be developed for treatment of hypertension and hyperinsulinemia in human patients.

Acknowledgments

This work was supported by funds from Wuhan City International Collaboration grant (No. 997005135G), National “863” Plan Project (No. 2001AA217121), and the National Institute of Environmental Health Sciences, Division of Intramural Research. The authors wish to thank Drs Hong Wang and Yun Zhao for helpful comments during preparation of the manuscript. We also wish to thank Miriam Sander for editorial assistance.

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Hypertension. 2003;42:1026-1033; originally published online October 20, 2003; doi: 10.1161/01.HYP.0000097603.55404.35

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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