Central and peripheral renin-angiotensin systems in ouabain-induced hypertension

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Submitted 31 October 2005; accepted in final form 24 February 2006


First published March 24, 2006; doi:10.1152/ajpheart.01148.2005.—Chronic subcutaneous infusion of ouabain causes hypertension via central pathways involving angiotensin type 1 (AT1) receptor stimulation. The present study assessed plasma and tissue ANG I and II levels as well as AT1 receptor and angiotensin-converting enzyme (ACE) mRNA levels and binding densities by real-time PCR and in vitro autoradiography in relevant brain nuclei and peripheral tissues (heart and kidney) in rats at 1 and/or 2 wk after start of ouabain infusion at 50 μg/day. After 2 wk (but not after 1 wk), blood pressures significantly increased (+15 mmHg). At 2 wk, plasma ANG I and II levels were markedly suppressed by ouabain. In contrast, in the heart and kidneys, ANG I levels were not affected, and ANG II levels tended to decrease, whereas in the hypothalamus ANG II content clearly increased. At 1 wk, no changes in ACE or AT1 receptor densities were seen. After 2 wk, there were significant decreases in AT1 receptor mRNA and densities in the organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO), and paraventricular nucleus (PVN). ACE densities decreased only in the OVLT and SFO, but ACE mRNA showed more variable responses (decrease in OVLT vs. increase in PVN). In the kidneys, at 2 wk both AT1 receptor and ACE densities were decreased, but mRNA abundance did not change. The heart showed no significant changes. The increase in hypothalamic ANG II content and associated decreases in central AT1 receptor and ACE densities support the involvement of the brain renin-angiotensin system in the central hypertensive mechanism of action of ouabain.

angiotensin II; angiotensin type 1 receptors; angiotensin-converting enzyme; brain; kidneys

MATERIALS AND METHODS

Animals

Male Wistar rats (Charles River, Montreal, Canada) weighing 150–200 g were housed on a 12:12-h light-dark cycle at 24°C, fed regular rodent chow, and allowed tap water ad libitum for 5 days to acclimatize before entering the study. All experimental procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Ottawa Animal Care Committee for the use and care of laboratory animals.

Treatment

Rats received a chronic subcutaneous infusion of either 0.9% saline or 50 μg of ouabain/day via osmotic minipump for 1 wk (2 experiments) or for 2 wk (4 experiments). Ouabain infused subcutaneously at a dose of 50 μg/day causes mild hypertension in Wistar rats (26). An osmotic minipump (model 2002, Alza, Palo Alto, CA), with an infusion rate of 12 μl/day and filled with either saline or ouabain dissolved in saline (4.17 mg/ml), was implanted subcutaneously on the back of the rat under halothane-oxygen anesthesia. First, after both 1 and 2 wk of infusion, two separate experiments were performed, one for measurement of blood pressure and one for binding densities to

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AT₁ receptor, angiotensin type 1 receptor; ACE, angiotensin-converting enzyme; PGK, phosphoglycerate kinase.

exclude beguiling influences of surgical stress on brain AT₁ receptor and ACE densities. Subsequently, at 2 wk, two additional experiments were done, one for measurement of plasma and tissue angiotensins and one for mRNA measurements.

Blood Pressure

Resting blood pressure was determined as described previously (26). Briefly, at the end of the treatment period, while the animals were under halothane-oxygen anesthesia, a polyethylene catheter (PE-50) was inserted into the left carotid artery, tunneled subcutaneously to the nape, and secured to the skin. After a 17-h recovery period, mean arterial pressure and heart rate were recorded in conscious, unrestrained animals. In one 2-wk experiment, an arterial blood sample was then obtained, and the heart, kidneys, and brain were rapidly excised as previously described (11).

Quantitative In Vitro AT₁ Receptor and ACE Autoradiography

Rats were killed by decapitation, and the brains, hearts, and kidneys were removed and quickly frozen in 2-methylbutane at −40°C and stored at −80°C. Standard autoradiography for quantitative analysis of ACE and AT₁ receptor binding was carried out as described previously (27). To investigate AT₁ receptor binding, 20-μm sections were preincubated in 10 mM sodium phosphate buffer, pH 7.4, containing 5 mM Na₂EDTA and 0.2% BSA for 15 min to remove endogenous angiotensins. After this, sections were incubated in phosphate buffer containing 0.3 μCi/ml ¹²⁵I-labeled [Sar¹Ile⁸]ANG II (2,176 Ci/mmol, Washington State Univ. Peptide Radiodination Service Center, Pullman, WA) and an angiotensin type 2 receptor antagonist, PD-123319 (10⁻⁵ M, Sigma-Aldrich, St. Louis, MO) for 1 h. Nonspecific binding was determined in the presence of 1 μmol unlabeled ANG II and was less than 2–5%. Sections for the analysis of ACE densities were also preincubated for 15 min in 10 mM sodium phosphate buffer, pH 7.4, containing 0.2% BSA and then incubated in phosphate buffer containing 0.3 μCi/ml (30 pM) ¹²⁵I-labeled 351A (¹²⁵I-351A, a derivative of lisinopril, iodinated by the chloramine T method) for 1 h. Nonspecific binding was detected in the presence of 100 mM EDTA, which completely abolished the ¹²⁵I-351A binding signal. All incubations were done at room temperature. After four successive 1-min washes in their respective ice-cold wash buffer without BSA, slides for AT₁ receptor and ACE binding were allowed to dry and then exposed for 24–48 h to Kodak Biomax MR film (Eastman Kodak, Rochester, NY), along with a set of methylacrylate ¹²⁵I standards (Washington State Univ. Peptide Radiodination Service Center). The ¹²⁵I-ANG II and ¹²⁵I-351A binding densities were measured in the heart and kidney, and in specific hypothalamic brain nuclei defined according to the rat brain atlas of Paxinos and Watson (15). Quantification was done by using a computer-assisted image analysis system, AIS/C (Imaging Research, St. Catharines, Ontario, Canada), and conversion (to fmol/mg and fmol/g wet weight of tissue, respectively) was done by comparing them with the calibrated relative optical density of the ¹²⁵I-standards.

RT-PCR

RNA isolation and reverse transcription reaction. Rats were perfused with chilled diethyl pyrocarbonate-treated PBS (pH 7.4) under pentobarbital sodium anesthesia. The brains, hearts and kidneys were removed, immediately frozen in isopentane, and placed on dry ice and then stored at −80°C until use. Renal cortex and medulla as well as the left ventricle (LV) and right ventricle (RV) of the heart were homogenized in TRIzol Reagent buffer (Invitrogen, Burlington, ON, Canada) by using a Polytron. Serial 80-μm-thick coronal brain slices were cryosectioned, and specific nuclei (OVLT, MnPO, SFO, and PVN) (15) were punched out by using prechilled 25-μl Drummond Microdispensers (Drummond Scientific). The tissue pellet was homogenized in 0.2 ml TRIzol Reagent by using a pestle (Bel-Art-Products, Pequannock, NJ) driven by a Pellet Pestle Motor, and another 0.3 ml TRIzol was added thereafter. Total RNA was isolated from tissues according to the manufacturer’s instructions. To eliminate potential genomic DNA contamination, samples were treated

Table 1. Primer sequences for detection of AT₁ receptor, ACE, and PGK mRNA transcripts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Amplicon Length, bp</th>
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<tbody>
<tr>
<td>AT₁ receptor</td>
<td>Sense</td>
<td>385</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>385</td>
</tr>
<tr>
<td>ACE</td>
<td>Sense</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>191</td>
</tr>
<tr>
<td>PGK</td>
<td>Sense</td>
<td>263</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>263</td>
</tr>
</tbody>
</table>

Fig. 1. Representative autoradiographs of angiotensin type 1 (AT₁) and angiotensin-converting enzyme (ACE) binding in the organum vasculosum of the lamina terminalis (OVLT) and paraventricular nucleus (PVN) in rats treated with vehicle vs. ouabain subcutaneously for 2 wk.
with DNase I (Ambion, Austin, TX) before reverse transcription reaction. cDNA was synthesized using Superscript II RNase H-Reverse Transcriptase (Invitrogen) at 42°C for 50 min.

**Real-time PCR.** Real-time PCR amplifications were performed with a Roche Light Cycler by using Fast Start DNA Master SYBR Green I (Roche Diagnostics, Penzberg, Germany). Two microliters of the RT product from each sample was used as a template. The specific AT1 receptor, ACE, and phosphoglycerate kinase (PGK) primer sequences used are listed in Table 1. The real-time PCR conditions were as follows: an initial step of 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 5 s, and annealing of primers to the target for 5 s at 60°C for the AT1 receptor primers, or 62°C for the ACE and PGK primers. The extension step was performed at 72°C, and the extension time was determined by the formula: amplicon length/25 s. Real-time PCR efficiencies (E) for AT1 receptor, ACE, and PGK amplification were 1.96, 1.84, and 1.88, respectively, and were calculated according to the equation: $E = 10^{-1/slope}$. The specificity of real-time PCR products was documented with a melting curve analysis. In addition, high-resolution gel electrophoresis was performed to confirm the amplification of a single product of the appropriate size for each gene (Table 1). Real-time RT-PCR analysis was performed in duplicate.

Serial 10-fold dilutions of RT product (initially 5 μg of RNA) were used to generate relative external standards for each gene. Expression was normalized with PGK levels as an endogenous reference, dividing the amount of the target gene by the PGK quantity.

**ANG I and II and Ouabain Assays**

Procedures for the sample collection, processing of samples, as well as the actual assays have been described in detail (9, 11, 17). Plasma and tissue angiotensins were assessed by radioimmunoassay (RIA) after separation on HPLC. PRA was determined by RIA for ANG I, generated by incubation of plasma for 30 min at 37°C and pH 7.5. Plasma ouabain levels were measured by ELISA.

**Statistical Analysis**

Data represent means ± SE. Differences between groups were compared by unpaired t-test. The level of statistical significance was set at $P < 0.05$. 

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**Fig. 2.** Effects of subcutaneous infusion of ouabain for 1 wk (A) and 2 wk (B) at 50 μg/day on AT1 receptor densities in the OVLT, subfornical organ (SFO), median preoptic nucleus (MnPO), and PVN of the hypothalamus. Values are means ± SE (n = 6–8). *P < 0.05 vs. vehicle. BBB, blood-brain barrier.

**Fig. 3.** Effects of subcutaneous infusion of ouabain for 1 wk (A) and 2 wk (B) at 50 μg/day on ACE densities in the OVLT, SFO, MnPO, and PVN of the hypothalamus. Values are means ± SE (n = 6–8). *P < 0.05 vs. vehicle.
RESULTS

Mean Arterial Pressure, Heart Rate, and Plasma Ouabain Levels

Chronic subcutaneous infusion of ouabain over 2 wk significantly increased resting mean arterial pressure (118 ± 2 vs. 103 ± 2 mmHg, P < 0.05 in one experiment, and 121 ± 2 vs. 104 ± 3 mmHg, P < 0.05 in the second experiment) but not yet significantly after 1 wk (116 ± 3 vs. 109 ± 4 mmHg), compared with controls. Resting heart rate showed minor increases by 10–40 beats/min (significant in one 2-wk experiment). Body weights did not differ between ouabain- vs. vehicle-treated animals. After 2-wk infusion of ouabain, plasma ouabain levels were 8.7 ± 1.1 ng/ml compared with 2.1 ± 0.4 ng/ml (P < 0.001) in control rats, the latter reflecting endogenous “ouabain.”

AT1 Receptor and ACE Densities

Brain. Representative autoradiographs for AT1 receptor and ACE binding densities in the OVLT and PVN are shown in Fig. 1. Subcutaneous ouabain infusion for 1 wk caused small, nonsignificant decreases in AT1 receptor densities in the brain areas studied (Fig. 2A). After 2 wk of ouabain treatment, AT1 receptor densities had significantly decreased in the OVLT, SFO, and PVN (by 14–18%) and not significantly in the MnPO (Fig. 2B). Ouabain infused subcutaneously for 1 wk had no effect on ACE densities in the brain areas studied (Fig. 3A). However, ACE densities were decreased after 2 wk of ouabain treatment but only significantly in brain nuclei outside the BBB (Fig. 3B). ACE densities decreased by 48% in the OVLT and by 26% in the SFO vs. 14% (not significant) in the PVN.

Kidney and heart. In the kidney, AT1 receptor binding is localized to the cortex and medulla, while ACE binding is present in the proximal convoluted tubule. Subcutaneous infusion of ouabain for 1 wk caused small, nonsignificant decreases in renal AT1 receptor and ACE densities (Fig. 4). In contrast, both AT1 receptor and ACE densities decreased significantly after 2 wk of ouabain treatment (Fig. 4). AT1 receptor densities decreased by 34% in the cortex and by 29% in the medulla, whereas ACE densities in the proximal convoluted tubule decreased by 14%.

In contrast to the brain and the kidney, subcutaneous infusion of ouabain caused only small, nonsignificant decreases in AT1 receptor or ACE densities in the LV of the heart (Fig. 4).

AT1 Receptor and ACE mRNA Levels

Brain. Subcutaneous infusion of ouabain for 2 wk caused significant decreases in AT1 receptor mRNA levels in the OVLT and PVN (by ∼50%) compared with the control group and tended (NS) to decrease levels in the MnPO and SFO (Fig. 5A). ACE mRNA levels were significantly decreased by 15% in the OVLT but increased by 90% in the PVN after 2-wk subcutaneous infusion of ouabain (Fig. 5A). The MnPO and SFO showed minor nonsignificant increases in ACE mRNA abundance (Fig. 5B).

Kidney. Infusion of ouabain for 2 wk caused only minor (NS) changes in AT1 receptor and ACE mRNA abundance in both the cortex and the medulla (data not shown).

Fig. 4. Effects of subcutaneous infusion of ouabain for 1 and 2 wk at 50 μg/day on AT1 receptor and ACE densities in the kidney and heart. Values are means ± SE (n = 6–8). *P < 0.05 vs. vehicle.
both ACE and AT1 receptors showed clear decreases in bind-

The present study demonstrates that the development of ouabain-induced hypertension is associated with a different pattern of changes in the brain vs. the renal and circulatory RAS. In the hypothalamus, ANG II levels did not change, but ANG II levels increased severalfold (Table 2).

**DISCUSSION**

The present study demonstrates that the development of ouabain-induced hypertension is associated with a different pattern of changes in the brain vs. the renal and circulatory RAS. In the hypothalamus, ANG II levels did not change, but ANG II levels increased severalfold (Table 2).

**Brain RAS**

Ouabain for 2 wk increased hypothalamic ANG II levels severalfold with no change in ANG I levels. Because plasma renin, ANG I, and ANG II levels markedly decreased, it is likely that these increased ANG II levels in the hypothalamus reflect locally produced ANG II. The higher hypothalamic ANG II levels likely do not only reflect increased ANG II stores but also increased release of ANG II from nerve terminals because the ouabain-induced hypertension depends on AT1 receptor stimulation in the central nervous system (CNS) (7). These higher ANG II levels were associated with significant decreases in AT1 receptor densities in several nuclei. Autoradiography assesses the number of receptors on the plasma membrane. Several in vitro studies demonstrated that ANG II causes a reduction of AT1 receptor numbers at the cell surface (16, 24). Considering that ouabain can increase local release of ANG II by increasing ANG II release from nerve terminals (18), it appears reasonable to conclude that an increase in local ANG II release by ouabain leads to enhanced internalization of the ligand-receptor complex and thereby less AT1 receptors at the cell surface. More chronically, an actual decrease in production of receptors by ANG II also may contribute (1). In the present study, after 2 wk of ouabain both mRNA and densities for AT1 receptors were largely in parallel found decreased in the brain nuclei studied. This decrease in AT1 receptors production may compensate for enhanced ANG II release, if AT1 receptor numbers on the neuronal membrane are rate limiting, i.e., not present in excess relative to the local ANG II levels. If so, this likely only in part offsets the impact of higher ANG II release because the chronic sympathoexcitatory and pressor responses to ouabain are mediated through central AT1 receptors stimulation (7).

ACE densities in the brain decreased as well, but interestingly only clearly in the two nuclei outside the BBB (i.e., the OVLT and the SFO), and little in the two nuclei inside the BBB (i.e., PVN and MnPO). ACE mRNA abundance showed a small decrease in the OVLT, minor increases in MnPO and SFO, and a marked increase in the PVN. The CVOs, OVLT and SFO, do not express angiotensinogen (4) and respond to circulating ANG II. Ouabain markedly decreased plasma ANG II levels and presumably therefore also the levels in these two CVOs. It is not known whether ACE in these areas responds to

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Ouabain</th>
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<tr>
<td>Plasma</td>
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</tr>
<tr>
<td>PRA, ng/ml</td>
<td>11±2</td>
</tr>
<tr>
<td>ANG I, pg/ml</td>
<td>344±75</td>
</tr>
<tr>
<td>ANG II, pg/ml</td>
<td>15±4</td>
</tr>
<tr>
<td>Left ventricle</td>
<td></td>
</tr>
<tr>
<td>ANG I, pg/g</td>
<td>42±8</td>
</tr>
<tr>
<td>ANG II, pg/g</td>
<td>15±4</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
</tr>
<tr>
<td>ANG I</td>
<td>1,886±372</td>
</tr>
<tr>
<td>ANG II</td>
<td>94±19</td>
</tr>
<tr>
<td>Medulla</td>
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</tr>
<tr>
<td>ANG I</td>
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</tr>
<tr>
<td>ANG II</td>
<td>145±63</td>
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<tr>
<td>ANG I</td>
<td>406±54</td>
</tr>
<tr>
<td>ANG II</td>
<td>3±2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5–6 for vehicle group and n = 7–8 for ouabain group. PRA, plasma renin activity; RAS, renin-angiotensin system. *P < 0.05, †P < 0.01 vs. vehicle.

Fig. 5. Effects of subcutaneous infusion of ouabain for 2 wk at 50 μg/day on AT1 receptor mRNA (A) and ACE mRNA levels (B) in the OVLT, SFO, MnPO, and PVN of the hypothalamus. The control group served as calibrator and values for the ouabain group are expressed as percentage changes. Values are means ± SE (n = 6–8). *P < 0.05 vs. vehicle.
ANG II in a manner similar to lung ACE, i.e., downregulates (19), or renal ACE, i.e., upregulates (6). The mRNA abundance changed somewhat differently in the two CVOs: a small decrease in the OVLT vs. small increase in the SFO despite similar decreases in ACE densities. The different mRNA response in OVLT vs. SFO may be a chance finding or may have biological relevance. Either way, most of the effect of ouabain on ACE binding densities appears to be posttranscriptional. Interestingly, the PVN showed a distinctly different pattern with a clear increase in mRNA abundance but a small (nonsignificant) decrease in ACE densities in response to 2-wk infusion of ouabain. Considering that hypothalamic ANG II levels significantly increase and this may reflect local production, we speculate that this dissociation may indicate increased turnover of membrane-bound ACE to the cytoplasmic compartment, and ACE transcription increased to compensate. Possible mechanisms contributing may involve ANG II or direct effects of ouabain or Na\(^+\)-K\(^+\)-ATPase inhibition on different aspects of regulation of ACE or on other regulatory mechanisms in the CNS, such as aldosterone or endothelin.

**Comparison with Responses to Endogenous “Ouabain”**

The responses of ACE and AT\(_1\) receptors in the hypothalamus to the endogenous equivalent of ouabain, i.e., “ouabain” or ouabain-like compound, show a distinctly different pattern. In rats with congestive heart failure after myocardial infarction, AT\(_1\) receptor and ACE densities show clear increases in the SFO, OVLT, MnPO, and PVN, and most of the increases are secondary to “ouabain” in the CNS because central infusions of Fab fragments binding endogenous “ouabain” prevent the increases (22). Similarly, we reported that in Dahl salt-sensitive rats on high salt intake, ACE mRNA and enzymatic activity markedly increase in the hypothalamus, which could be fully prevented by blockade of central “ouabain” by Fab fragments (29). Several factors may contribute to this apparent discrepancy between effects of exogenous ouabain and endogenous “ouabain” on regulation of ACE and AT\(_1\) receptors in the hypothalamus. The most important difference may be that systemic infusion of ouabain will likely result in a global distribution of ouabain across the CNS and therefore exert global effects, whereas the release and action of endogenous “ouabain” is likely more localized, e.g., in the MnPO (3), and therefore causes specific, localized effects. Downstream, such nucleus specific effects may cause opposite regulation of ACE and AT\(_1\) receptors than the global effects of exogenous ouabain, which are also confounded potentially by the peripheral effects of subcutaneous infused ouabain.

**Circulatory and Renal RAS**

Plasma renin and plasma ANG I and II levels were markedly suppressed by chronic infusion of ouabain. This finding is consistent with previous studies showing decreases in renin release from the kidneys by ouabain both in vitro (13) and in vivo after acute intrarenal infusion (10). Other studies did not report changes in PRA (12) and plasma ANG II levels (23), perhaps reflecting the lower rates of infusion in these studies. ANG I and II levels in the heart and kidneys showed more limited responses to ouabain with modest decreases mainly in the renal medulla. This differential pattern of changes compared with the circulatory RAS likely reflects the active regulation of the tissue ANG I and II levels in these two organs (11, 30). On the other hand, the kidneys showed significant decreases in ACE and AT\(_1\) receptor densities without a change in mRNA abundance. Increases in ANG II levels by chronic infusion decrease renal AT\(_1\) receptor densities (6, 20) without downregulation of mRNA or protein levels (5, 20), consistent with posttranscriptional regulation by, e.g., increased receptor internalization after binding of ANG II to its receptor (24). It appears unlikely, therefore, that a decrease in renal ANG II levels, as caused by ouabain, would have caused the decrease in renal densities. However, the ANG II levels measured reflect tissue steady-state levels, and one cannot exclude that these were decreased because of higher local release by ouabain (14, 21). Besides renin and ANG II release, ouabain affects several other mechanisms, such as endothelin (28), aldosterone (21), or renal sympathetic activity (7, 8), which may change AT\(_1\) receptor densities. In the kidneys, ouabain also decreased ACE densities without a concomitant change in mRNA abundance. ACE is generally considered to be downregulated by ANG II (19). However, in the kidneys, chronic infusion of ANG II caused a clear (35%) increase in ACE binding densities in the proximal convoluted tubules (6), in contrast to the decrease in AT\(_1\) receptor densities (6). In this study (6), ACE mRNA was not measured. If ouabain indeed lowered intrarenal renin release and ANG I and II formation, lower ANG II levels may therefore have decreased ACE binding densities through posttranscriptional regulation because mRNA abundance did not change. However, we measured mRNA levels in the whole cortex and medulla and, e.g., in situ hybridization is needed to assess whether local changes occurred in proximal convoluted tubules.

In conclusion, the present study indicates that chronic subcutaneous infusion of ouabain at 50 µg/day is associated in the CNS with a severalfold increase in ANG II content in the hypothalamus and decreases in AT\(_1\) receptor and ACE densities in nuclei involved in cardiovascular control. In contrast, the circulatory and renal RAS were suppressed. Together with previous functional studies showing that sympathoexcitatory and pressor responses to ouabain are mediated through AT\(_1\) receptor stimulation in the CNS, these studies suggest that ouabain specifically activates the brain RAS.

**ACKNOWLEDGMENTS**

The derivate of lisinopril, 351A, was kindly donated by Dr. Y. Sun, Div. of Cardiovascular Diseases, Dept. of Medicine, Univ. of Tennessee Health Science Center, Memphis, TN 38163.

**GRANTS**

This research was supported by operating grant MOP-11897 from the Canadian Institutes of Health Research. W. J. Cheung was supported by Summer Studentship Awards from The Canadian Hypertension Society and The Heart and Stroke Foundation of Ontario. E. El-Shahat was supported by Program Grant PRG-5275 from the Heart and Stroke Foundation of Ontario. F. H. H. Leenen is Pfizer Chair in Hypertension Research, an endowed chair supported by Pfizer Canada, University of Ottawa Heart Institute Foundation, and Canadian Institutes of Health Research.

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