

# Hypertension

JOURNAL OF THE AMERICAN HEART ASSOCIATION



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**Late Breaking Abstracts From the 58th Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research in Association with the Council on Kidney in Cardiovascular Disease**

*Hypertension* 2004;44;554-564

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

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# Late Breaking Abstracts

## Characterization of Brain ACE2: Evidence for a Secreted Form of the Enzyme

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Angiotensin converting enzyme 2 (ACE2) is a homologue of ACE that preferentially forms angiotensin-(1-7) [Ang-(1-7)] from Ang II and is not blocked by ACE inhibitors. We previously identified ACE2 mRNA and protein in the brains from both neonatal and adult Sprague-Dawley rats as well as in astrocytes isolated from the medulla oblongata and cerebellum of neonatal rat brain. The distribution of ACE2 was examined in the dorsal and ventral medulla oblongata of adult rat brain using an affinity-purified antibody. ACE2 reaction product was localized to the nucleus of the tractus solitarius (nTS), dorsal motor nucleus of the vagus (dmnX) and hypoglossal nuclei. In cultured neonatal rat astrocytes, ACE2 immunoreactivity was observed throughout the perinuclear, vesicular, and nuclear regions of the cells with little or no immunoreactive ACE2 in the plasma membrane. The immunoreactive staining was blocked by the peptide used to generate the ACE2 antibody. Moreover, the cellular staining pattern was characteristic of secreted proteins, suggesting that ACE2 may be packaged in vesicles and secreted from astrocytes. Western blot hybridization of astrocyte homogenates showed a prominent band at about 70 kDa as well as a faint band at 90 kDa. This 70 kDa protein was also found in concentrated conditioned media from the astrocytes, further indicating the presence of a secreted form of ACE2. Incubation of Ang II with concentrated conditioned media from the astrocytes resulted in the formation of Ang-(1-7) as detected by HPLC. Addition of either EDTA or the specific ACE2 inhibitor ML00106791 (Millennium Corporation) completely blocked the reaction, confirming the identity of the 70 kDa protein as ACE2. The attenuation of ACE2 activity by the inhibitor was dose-dependent with an  $IC_{50}$  of  $1.5 \times 10^{-7}$  M. Consistent with secretion of ACE2 in astrocytes, we detected the 70 kDa immunoreactive ACE2 in cerebrospinal fluid from male Sprague-Dawley rats. Our studies are the first to show endogenous production of an active, secreted form of ACE2 and suggest that astrocytes constitute a novel paracrine system to balance the extracellular forms of Ang II and Ang-(1-7) in the brain.

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## Oral BNP: A Newly Developed Conjugated Form of Human BNP Lowers Blood Pressure and Activates cGMP in Normal Conscious Dogs

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**Background:** The objective of the current study was to address the feasibility and the biological activities of oral administered human BNP (hBNP). Proprietary technology (Nobex, Durham NC) has developed amphiphilic oligomers covalently attached to peptides, which protect peptides from proteolysis. These conjugated peptides have improved pharmacokinetic profile enabling oral administration. We hypothesized that a novel conjugated oral hBNP increases plasma hBNP and cGMP, with a reduction in mean arterial pressure (MAP) thus demonstrating biological activity. **Methods:** In this crossover designed study we tested for the first time the bioavailability and efficacy of oral conjugated hBNP compared to vehicle randomly administered in 6 normal dogs. Baseline measurements of MAP and blood draw for canine atrial natriuretic peptide (cANP) and BNP (cBNP), hBNP and cGMP were made. MAP and blood sampling was repeated at 10, 30, 60, 120, 180, and 240 min after oral administration. **Results:** hBNP was not detectable in canine plasma at baseline, but was present and increased after oral BNP (peak concentration  $2509 \pm 370$  pg/ml,  $p < 0.01$ ) throughout the time course of the study in each of 6 dogs. hBNP was not detectable after vehicle administration ( $p < 0.0008$  between groups). Plasma cGMP increased after oral BNP for 60 min (from baseline  $10.8 \pm 3$  to  $36.8 \pm 26$  pmol/ml,  $p < 0.05$ ), and decreased in the vehicle group ( $p < 0.0001$  between groups). MAP decreased at 10 minutes and remained decreased for 60 minutes after oral BNP (from  $113 \pm 8$ ; to  $101 \pm 12$  after 10 min to  $97.5 \pm 10$  after 30 min; to  $99 \pm 13$  mmHg after 60 min), while remaining unchanged in the vehicle group ( $p < 0.05$  between groups). Plasma cANP and cBNP were also unchanged compared to baseline throughout the protocol. **Conclusions:** This study reports for the first time that a novel conjugated oral BNP activates cGMP and induces a significant reduction of MAP. These data advance a new concept for chronic BNP therapy.

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## C-REACTIVE PROTEIN (CRP) IS VASOACTIVE

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CRP is an acute-phase response protein within the pentraxin family. CRP is used as a marker for cardiovascular inflammation and disease with normal plasma levels around .8 mg/L, and over 10 mg/L under conditions of inflammation in the human. Biological activities of CRP outside of inflammatory functions are poorly understood. We hypothesized that CRP was vasoactive, and examined arterial reactivity to CRP in isometric tissue baths and changes in blood pressure in chronically-instrumented, conscious rats. In SDS-polyacrylamide gels, commercially available human CRP migrated as one band at the predicted molecular weight (23 kDa). In rat thoracic aorta and mesenteric resistance arteries, CRP ( $10^{-5}$  to  $1$  mg/L) caused a concentration-dependent relaxation in phenylephrine (PE)-contracted tissues [-log EC50 (mg/L), aorta =  $3.06 \pm .04$ , mesenteric resistance artery =  $2.31 \pm .13$ ]. In aorta, potency of CRP relaxation was similar when tissues were contracted with 5-HT (-log EC50 =  $3.03 \pm .02$  mg/L) and KCl (-log EC50 =  $2.98 \pm .06$  mg/L). Downloaded from <http://ahajournals.org/> by on December 2, 2009

relaxation was unaffected by endothelial cell removal, the nitric oxide synthase inhibitor Nitro-L-arginine (100  $\mu$ M), the K<sup>+</sup> channel inhibitors barium (30  $\mu$ M) and tetraethylammonium (100  $\mu$ M). By contrast, cobra venom factor (CVF) (1.5 mg/L), a peptide inhibitor of complement, reduced CRP potency by 3-fold. ODQ (10  $\mu$ M), an inhibitor of guanylate cyclase, abolished CRP-induced aortic relaxation, while 0.5  $\mu$ M ODQ reduced the potency of CRP by 30-fold. Importantly, CRP (3  $\mu$ g/kg to 300  $\mu$ g/kg) reduced blood pressure in conscious rats (max =  $-25.44 \pm 6.89$  mmHg). Thus we have revealed the ability of CRP to stimulate arterial relaxation via guanylate cyclase and complement in an endothelial- and NO- independent manner, suggesting an unappreciated mechanism by which CRP affects vascular function. These are the first data demonstrating that CRP is a vasodepressor. CRP should be viewed as a substance with the potential to modify blood pressure in pro-inflammatory diseases such as hypertension.

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## Paracrine Role of Adventitial Fibroblasts and Macrophages in Angiotensin II-Induced Reactive Oxygen Species and Hypertrophy of the Mouse Carotid Artery

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We previously have examined whether transfection of carotid arteries (CCA) with Ad-PDGF-gp91ds-eGFP (adenovirus expressing NAD(P)H oxidase inhibitor and marker protein-eGFP targeted to fibroblasts) would reduce medial hypertrophy in response to AngII. Ad-CMV-eGFP served as control. eGFP staining in oxidase inhibitor-treated CCA appeared mainly in fibroblasts and in some macrophages of the adventitia, but not medial cells. The oxidase inhibitor significantly attenuated AngII-induced reactive oxygen species (ROS) and medial hypertrophy, suggesting fibroblasts play a role in this response. Since the inhibitor was expressed in some macrophages which are enriched in NAD(P)H oxidase, we postulated that inhibition of macrophages participated in this response. To examine the relative contribution of macrophages, we applied inhibitor and control vectors to the CCA adventitia in wild-type (WT) and CCR2 -/- mice (deficient in the MCP-1 receptor and macrophage infiltration) and AngII (750  $\mu$ g/kg/d s.c.) infusion began 2 days later. A third group of each strain received vehicle. We observed a significant and comparable rise in blood pressure with AngII (treated with either vector) vs. vehicle in both strains. In WT, AngII induced a significant rise in CCA medial cross-sectional area which was significantly attenuated by the oxidase inhibitor ( $p < 0.05$ ). In contrast, in CCR2 -/- mice AngII did not increase medial area and the oxidase inhibitor had no effect (vehicle =  $18.4 \pm 1.5$ , AngII+control =  $18.6 \pm 1.2$ , AngII + inhibitor =  $22.0 \pm 2.0 \times 1000 \mu\text{m}^2$ ). In WT, ROS (measured by 4-hydroxynonenal expression) was significantly elevated with AngII and suppressed by the oxidase inhibitor (vehicle =  $1.6 \pm 0.2$ , AngII+ control =  $2.9 \pm 0.1$ , AngII + inhibitor =  $1.7 \pm 0.2$  arbitrary units). In CCR2 -/- mice, no increase was observed comparing vehicle and AngII groups; yet there was a decrease in basal ROS (AngII+control =  $1.9 \pm 0.2$ , AngII+inh. =  $1.3 \pm 0.1$ ). These data suggest that in the absence of macrophages, fibroblasts still contribute to overall ROS. However, the data underscore the critical importance of macrophages in vascular hypertrophy and suggest an important permissive effect between adventitial macrophages and fibroblasts in mediation of ROS-induced vascular hypertrophy.

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## Angiotensin II Type 2 Receptor Attenuates RhoA/Rho Kinase Signaling and Induces Vasodilation in Resistance Arteries from Stroke Prone Spontaneously Hypertensive Rats

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## Angiotensin II Type 2 Receptor Attenuates RhoA/Rho Kinase Signaling and Induces Vasodilation in Resistance Arteries from Stroke Prone Spontaneously Hypertensive Rats.

We previously demonstrated that Ang II inhibits RhoA-dependent phosphorylation of myosin light chain (p-MLC) via AT<sub>2</sub>R in vascular smooth muscle cells (VSMC) and that AT<sub>2</sub>R-mediated RhoA effects are involved in Ang II-induced vasodilation. Here we investigated mechanisms of AT<sub>2</sub>R-induced inhibition of RhoA/Rho kinase (ROK) and the role of NO and NADPH oxidase-derived  $\bullet\text{O}_2^-$  in AT<sub>2</sub>R-induced vascular actions in arteries from stroke-prone spontaneously hypertensive rats (SHRSP). SHRSP and WKY rats were treated with the AT<sub>1</sub>R blocker valsartan (30mg/Kg/day, VAL, 14 days). Dilatory responses to Ang II ( $10^{-9}$ - $10^{-6}$  M) were performed in norepinephrine pre-contracted vessels in the presence of VAL ( $10^{-6}$  M)  $\pm$  the AT<sub>2</sub>R antagonist PD123319 ( $10^{-6}$  M, PD), the nitric oxide synthase (NOS) inhibitor L-NAME ( $10^{-4}$  M) or fasudil ( $10^{-6}$  M, FAS), selective ROK inhibitor. AT<sub>2</sub>R, eNOS, cGMP-dependent protein kinase (cGK) expression, Rho translocation and p-MLC were assessed by immunoblotting. NO bioavailability and NADPH oxidase activity were evaluated by chemiluminescence. Ang II elicited vasodilation in arteries from VAL-treated SHRSP (max dilation  $40 \pm 7$  %). PD and L-NAME inhibited this effect, indicating AT<sub>2</sub>R and NO-mediated actions. FAS increased 2-fold AngII-induced relaxation in SHRSP but not in WKY vessels. AT<sub>2</sub>R and eNOS expression was increased (2-fold) by VAL only in SHRSP. NO concentration was increased in VAL-treated SHRSP. Expression of cGK, which inactivates RhoA, was reduced in SHRSP and restored after VAL treatment. RhoA translocation was increased only in untreated SHRSP compared to WKY ( $p < 0.05$ ) and was reduced after VAL treatment ( $p < 0.05$ ). These changes were associated with reduced p-MLC. NADPH oxidase activity was increased  $\approx$ 3-fold in SHRSP vs WKY ( $p < 0.05$ ) and reduced after VAL treatment. In conclusion, Ang II induces vasodilation via AT<sub>2</sub>R in SHRSP through downregulation of the RhoA/ROK system. This effect is due, partially, to activation of the NO/cGMP/cGK cascade and is mediated through increased bioavailability of NO and reduced scavenging by reactive oxygen

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### Exaggerated Hypertensive Target Organ Damage in Mice Lacking Peripheral Serotonin

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Serotonin is not only a prominent neurotransmitter but also a hormone active in the cardiovascular system. We have recently shown that mice lacking tryptophan hydroxylase (TPH) 1 lack serotonin in the blood but exhibit normal levels of the hormone in the central nervous system due to the presence of the previously unknown neuronal isoenzyme, TPH2. These mice are ideal models to study the peripheral functions of serotonin. In first experiments we could show that TPH1 knockout (KO) mice have an impaired hemostasis due to attenuated secretion of von-Willebrand factor from platelets. In the present study we have induced hypertension in these mice by the DOCA/salt regimen. TPH1-KO mice on C57Bl/6 background and controls (WT) did not differ in their basic blood pressure values measured by telemetry ( $111.8 \pm 0.8$  mmHg in TPH1-KO vs  $108.1 \pm 3.0$  mmHg in WT) and developed equally increased mean arterial pressures after three weeks of DOCA/salt treatment ( $134.8 \pm 1.1$  mmHg in TPH1-KO,  $p < 0.0001$ , vs  $132.3 \pm 2.4$  mmHg in WT,  $p < 0.005$ ). However, cardiac hypertrophy induced by DOCA/salt treatment was with 143% of basal cardiac weight (basic relative left ventricular weight:  $3.19 \pm 0.10$  mg/g vs after DOCA:  $4.55 \pm 0.18$  mg/g,  $p < 0.00005$ ) significantly ( $p < 0.005$ ) more pronounced in the mice lacking peripheral serotonin than in WT mice with 123% (basal:  $3.00 \pm 0.05$  mg/g vs after DOCA:  $3.67 \pm 0.07$  mg/g,  $p < 0.0002$ ). Furthermore, kidney damage assessed by histology and albumin excretion into the urine (urinary albumine:  $1200.1 \pm 152.8$   $\mu$ g/24h in TPH1-KO vs  $174.4 \pm 103.4$   $\mu$ g/24h in WT,  $p < 0.01$ ) was markedly exaggerated in these mice. In conclusion, serotonin in the circulation is not involved in the development of hypertension in the DOCA/salt model but protects animals from target organ damage induced by the treatment. Immunomodulatory actions of peripheral serotonin, for which we also collected evidence in TPH1-KO mice, may be involved in these effects.

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### Human heme oxygenase gene transfer into newborn rats prevents hyperglycemia-mediated superoxide production and attenuates endothelial cell sloughing

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Heme oxygenase-1 (HO-1) is a stress protein, which has been implicated as a key defense mechanism against oxidative injury in the vascular endothelium. Hyperglycemia has been directly linked to increased oxidative stress, leading to endothelial dysfunction, delayed cell replication and apoptosis. We examined the effect of HO-1 overexpression and underexpression on the vascular endothelium in Type I diabetic rats by measuring the level of oxidants and endothelial cell sloughing. The effect of streptozotocin (STZ)-induced hyperglycemia on aortic HO activity and heme content, the number of circulating endothelial cells, and urinary 8-epi-isoprostane was measured in control rats (LXSN), transgenic rats overexpressing HO-1 (LSN-human-HO-1) and transgenic rats underexpressing HO-1 (LSN-rat-HO-1-AS). HO-1 protein expression was unchanged, but HO activity was decreased in hyperglycemic rats compared to control rats ( $p < 0.05$ ). Hyperglycemia increased urinary 8-epi-isoprostane in control rats ( $n=5$ ;  $159 \pm 16$  pg/min). The increase in urinary 8-epi-isoprostane was significantly augmented ( $p < 0.05$ ) in diabetic rats underexpressing HO-1, LSN-rat-HO-1-AS rats ( $n=5$ ;  $321 \pm 24$  pg/min), but was attenuated in rats overexpressing human HO-1 (LSN-human-HO-1). The number of differentiated sloughed endothelial cells in blood was increased in hyperglycemic rats and was magnified in rats underexpressing HO-1, but was diminished in rats overexpressing HO-1. The number of circulating endothelial cells in control rats, control hyperglycemic rats, hyperglycemic rats overexpressing human HO-1 (LSN-human-HO-1), and hyperglycemic rats underexpressing rat HO-1 (LSN-rat-HO-1-AS) was  $2.5 \pm 1.5$ ,  $16.0 \pm 4.3$ ,  $7.0 \pm 3.5$ , and  $29.5 \pm 5.5$  per ml of whole blood, respectively. Taken together, these data demonstrate that delivery of HO-1 gene to the vascular system during development brings about a reduction in 8-epi-isoprostane and decreased endothelial cell sloughing in diabetes. Upregulation of HO-1 may provide a pharmacological means to decrease oxidants, endothelial cell shedding, and hyperglycemia-induced cardiovascular complications.

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### Regulation of Connexin Expression After Balloon Injury: Mechanism of Antiproliferative Effect of Statins?

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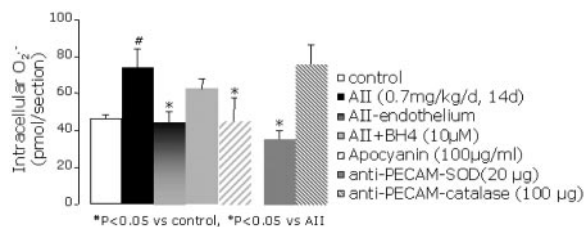
Statins, the HMG-CoA reductase inhibitors, have been shown to inhibit the migration of macrophages and smooth muscle cell proliferation leading to an antiproliferative effect. Although this beneficial effect of statins has been suggested to be independent of lipid lowering properties, the possible mechanisms responsible for this action is largely unknown. Gap junctions, which serve as channels for direct intercellular exchange of ions, secondary messengers, and small signaling molecules, play an important role in tissue homeostasis and regulation of growth, differentiation, and development. This study was designed to test the hypothesis that the expression of the component proteins of gap junctions, connexins 40 and 43 (Cx40 and Cx43), is up-regulated in arteries subjected to balloon injury, which can be suppressed by statin therapy. Male New Zealand white rabbits were subjected to injury in which angioplasty catheter was introduced into the right iliac artery from the femoral artery under fluoroscopic guidance. Five groups of rabbits ( $n=7$ ) were treated for 2 weeks with: balloon injury (BL), BL+lovastatin (BL+L, 10 mg/kg/day), BL+fluvastatin (BL+F, 10 mg/kg/day), sham operation (Sham), and control (Con). Immunohistochemistry studies showed that Cx40 and Cx43 were expressed in normal smooth muscle cells (SMCs) throughout the media. RT-PCR and Western blot analysis showed that Cx40 and Cx43 mRNA and protein expression were elevated after injury ( $p < 0.001$  for both proteins and both assays) and these elevations were suppressed by lovastatin and fluvastatin to a similar degree ( $p < 0.05$  for both drugs and both assays). Consistently, immunostaining of Cx40 and Cx43 was enhanced in the neointimal area after injury and lovastatin and fluvastatin reduced staining of these proteins in the lessened neointimal layer. Transmission electron microscopy revealed that there were abundant gap junctions between neointimal SMCs and fewer and smaller gap junctions after statin treatment. Therefore, balloon injury causes up-regulation of Cx40 and Cx43 in neointimal SMCs. Lovastatin and fluvastatin reduce neointimal proliferation and suppress upregulated Cx40 and Cx43 expression, suggesting that Cx40 and Cx43 play a role in statin-induced

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### Endothelial immunotargeting of superoxide dismutase corrects endothelial dysfunction in angiotensin II-induced hypertension

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Angiotensin II (AII)-induced hypertension increases vascular superoxide ( $O_2^{\cdot-}$ ) production and causes endothelial dysfunction due to inactivation of NO by  $O_2^{\cdot-}$ . It remains unclear whether increased  $O_2^{\cdot-}$  production is predominantly in the endothelium or in the vascular smooth muscle. To examine this question and to target therapy more precisely, we measured aortic  $O_2^{\cdot-}$  in mice with AII-induced hypertension via HPLC detection of oxy-ethidium, the reaction product of dihydroethidium and  $O_2^{\cdot-}$ . To evaluate specifically the role of  $O_2^{\cdot-}$ , we conjugated superoxide dismutase (SOD) or catalase to PECAM antibody to target the endothelium. AII-infusion increased aortic  $O_2^{\cdot-}$  production and this was abolished by endothelial denudation (figure), suggesting that the endothelium is the source of  $O_2^{\cdot-}$ . Tetrahydrobiopterin (BH4) and to a further extent apocynin (NADPH oxidase inhibitor) decreased AII-induced  $O_2^{\cdot-}$  production, suggesting that both the NADPH oxidase and uncoupled NO synthase were sources of  $O_2^{\cdot-}$ . AII-induced hypertension reduced endothelium-dependent vasodilatation, and this was corrected by IV anti-PECAM/SOD (maximal relaxation to Ach:  $97 \pm 0\%$  vs  $72 \pm 4\%$  in untreated AII,  $P < 0.05$ ). Anti-PECAM/SOD also completely normalized aortic  $O_2^{\cdot-}$  production. In contrast, anti-PECAM/catalase had no effects on vasoreactivity and  $O_2^{\cdot-}$  production. We conclude that chronic AII stimulates endothelial  $O_2^{\cdot-}$  production from both NADPH oxidases and uncoupled eNOS. The subsequent endothelial dysfunction can be prevented by immunotargeting of SOD to endothelial cells. Immunotargeting with anti-PECAM/SOD represents a promising approach for treatment of vascular oxidative stress.



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### Induction of Renal 20-Hydroxycyclooctaenoic Acid (20-HETE) Prevents Angiotensin II (Ang-II) Dependent Hypertension in Mice

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Recently, we found that the activity of CYP enzymes that produce 20-HETE are similar in the liver of mice and rats but that the activity in the

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### Attenuated Mean Arterial Pressure in Interleukin-6 Knockout Mice during Angiotensin II hypertension and a high-salt diet

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Previous reports show a positive correlation between plasma levels of interleukin-6 (IL-6) and increased blood pressure. Angiotensin II (AngII) has been shown to stimulate IL-6 production from various cell types. The goal of this study was to test the role of IL-6 in mediating AngII-Salt hypertension. We hypothesized that the hypertensive response to AngII would be decreased in IL-6 knockout (KO) mice when compared to wild-type (WT) mice. Male C57BL6 and IL-6 KO mice were implanted with biotelemetry devices and placed in metabolic cages to continuously monitor mean arterial pressure (MAP), heart rate (HR), motor activity, water, food intake and urinary sodium excretion. Baseline MAP during the control period averaged  $119 \pm 2$  and  $113 \pm 1$  mmHg for WT and IL-6 KO mice, respectively. Baseline urinary sodium excretion averaged  $0.50 \pm 0.1$  and  $0.52 \pm 0.1$  mEq/day, respectively, for WT and IL-6 KO mice and increased to  $2.42 \pm 0.4$  and  $2.87 \pm 0.4$  mEq/day on high salt (4% NaCl) diet. MAP, however, did not change significantly on HS, averaging  $116 \pm 1$  and  $114 \pm 1$  mmHg in the WT and KO mice, respectively. AngII was administered via osmotic minipump (90 ng/min, sc.) and caused a rapid increase in MAP in both groups, averaging  $138 \pm 4$  and  $142 \pm 4$  mmHg on day 2 in WT and IL-6 KO mice, respectively. However, there was a continued increase in the WT mice over the first week of AngII that did not occur in the KO mice, with the average MAP on days 2–6 averaging  $148 \pm 3$  and  $139 \pm 1$  in the respective groups. Interestingly, urinary sodium excretion was significantly less in the WT mice over that time period, averaging  $1.86 \pm 0.33$  mEq/day compared to  $2.42 \pm 0.13$  mEq/day in the KO mice, with no difference in sodium intake. MAP over the last 4 days of AngII averaged  $167 \pm 6$  and  $135 \pm 2$  in the WT and IL-6 KO mice. Urinary albumin excretion was increased in both groups by day 14 of AngII, but was still at control levels in both groups on day 4 of AngII. These data suggest that IL-6 plays an important role in mediating AngII-Salt hypertension. However, these results also suggest that the divergence of MAP in WT versus IL-6 KO mice occurs before significant renal injury as manifested in increased urinary albumin excretion.

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### Physiological significance of Heme oxygenase (HO)-1 preconditioning in protection of endothelial cell function in spontaneously hypertensive rats (SHR)

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Heme oxygenase-1 (HO-1) represents a key defense mechanism against oxidative injury. We investigated the hypothesis that HO-1 reduces vascular damage in a rat model of hypertension. The aim of this study was to determine whether HO-1-mediated increase in carbon monoxide (CO), an anti-apoptotic, and bilirubin, an antioxidant, may play a role in restoring endothelial cell function and normalizing blood pressure (BP) in SHR. We measured circulating endothelial cells (CEC), superoxide ( $O_2^-$ ), CO formation, HO-1 protein expression and acetylcholine-induced relaxation in mesenteric small arteries (100–280  $\mu$ m diameter). Two groups of rats (SHR and WKY, 8-week-old) were treated with an inducer of HO-1, cobalt protoporphyrin (CoPP) at a dose of  $50 \mu$ mol/100 gm body wt, once a week for 4 weeks, s.c. Preconditioning with HO-1 decreased BP from  $179 \pm 21$  mmHg to  $141 \pm 14$  mmHg, ( $p < 0.05$ ). Untreated SHR aorta  $O_2^-$  levels ( $3.87 \pm 0.41$   $\mu$ mol/mg protein) were significantly higher than SHR preconditioned with HO-1 ( $1.32 \pm 0.36$   $\mu$ mol/mg protein,  $p < 0.01$ ). CoPP increased HO-1 protein in aorta, arterioles and renal tissues by 3-, 12- and 4-fold, respectively, compared to control rats. Mesenteric arterioles relaxation to acetylcholine ( $10^{-7}$  to  $10^{-9}$  M) were increased in SHR preconditioned with HO-1 compared to control SHR ( $p < 0.01$ ). CEC (not progenitor's cells) were increased in SHR ( $78 \pm 19$  CEC/ml of blood) at 12 weeks compared to CEC in control SHR ( $17 \pm 6$  CEC/ml of blood) at 8 weeks. In WKY, CEC did not significantly change between 8–12 weeks of study. CoPP reversed the increase in CEC in SHR seen at 12 weeks. Because the number of CEC represents a surrogate marker for endothelial dysfunction, it may be used as an index of the level of oxidative stress in SHR. In conclusion, HO-1 upregulation decreases hypertension associated with a reduction in  $O_2^-$  production and a decrease in circulating endothelial cells in SHR, suggest that HO-1 preconditioning for ameliorating blood pressure elevation and improving vascular function in experimental hypertensive rats.

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### Aldosterone Stimulates Reactive Oxygen Spices Production through the Activation of NADPH Oxidase in Rat Mesangial Cells

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We recently demonstrated that glomerular mesangial injury is associated with increases in reactive oxygen species (ROS) levels in aldosterone-infused hypertensive rats. It was also observed that treatment with the superoxide mimetic, tempol, prevented the elevation of ROS levels and ameliorated glomerular injury in these animals. The aim of the present study was to determine the mechanisms responsible for aldosterone-induced ROS generation in cultured rat mesangial cells (RMCs). The oxidative fluorescent dihydroethidium was used to evaluate intracellular production of superoxide in intact cells. The lucigenin-derived chemiluminescence assay was used to determine NADPH oxidase activity in cell homogenates. Membranous translocation of p47phox and p67phox was determined by immunoblotting. mRNA expression of NADPH oxidase membrane components (p22phox and Nox-4) were quantitatively analyzed by real-time transcription-polymerase chain reaction. Aldosterone (1–100 nmol/L) dose-dependently increased the staining of dihydroethidium and NADPH oxidase activity with a peak at 3 hours in RMCs ( $n = 3-6$ , each). These were associated with increased intracellular

translocation from cytoplasm to membrane, as assessed by immunoblotting. Aldosterone (100 nmol/L) increased the abundance of p47phox and p67phox in the membrane fraction by approximately 70 and 60% ( $n = 4$ , respectively). On the other hand, aldosterone did not change protein levels of p47phox and p67phox in total lysates ( $n = 4$ , respectively). In addition, aldosterone (100 nmol/L) treatment for 1–48 hours did not alter mRNA expression of p22phox and Nox-4 ( $n = 6$ , respectively). These results demonstrate for the first time that aldosterone-induced ROS generation is associated with NADPH oxidase activation through the membranous translocation of p47phox and p67phox. These direct cellular actions of aldosterone may participate in the pathogenesis of glomerular mesangial injury.

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### Transduction of CYP4A1 In Vivo Impairs Endothelial Function in Renal Arteries

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Renal interlobar arteries (RA) express cytochrome P450–4A enzymes (CYP4A) which catalyze synthesis of 20-hydroxyecosatetraenoic acid (20-HETE). Overexpression of CYP4A enhances the sensitivity of RA to constrictor stimuli. This study examines the hypothesis that CYP4A overexpression interferes with endothelium-dependent vasodilation. Experiments were conducted in rats 7 days after injection with adenoviral constructs (200  $\mu$ l of  $10^{12}$  pfu/ml, iv) expressing CYP4A2 cDNA (Adv-CYP4A2) or enhanced green fluorescent protein cDNA (Adv-EGFP). Adv-CYP4A2 increased ( $P < 0.05$ ) systolic blood pressure ( $118 \pm 1$  vs  $131 \pm 2$  mmHg) whereas Adv-EGFP did not ( $117 \pm 3$  vs  $120 \pm 2$  mmHg). Relaxation to acetylcholine (ACH,  $10^{-9}$ – $10^{-4}$  M) and NONOate ( $10^{-9}$ – $5 \times 10^{-5}$  M) were examined in RA mounted on a wire-myograph, bathed in Krebs buffer containing  $10^{-5}$  M indomethacin, and preconstricted with phenylephrine to 70% of maximal response. RA were relaxed in a concentration-dependent manner by both ACH and NONOate. At the maximally effective concentration of relaxing agonist, the response to ACH in RA of Adv-CYP4A2-transduced rats ( $54 \pm 4\%$  relaxation) was exceeded ( $P < 0.05$ ) by that in RA of Adv-EGFP-transduced rats ( $87 \pm 2\%$  relaxation). After NO synthesis inhibition with 1 mM L-NAME, the residual (NO-independent) relaxing effect of ACH was similar in RA of rats transduced with Adv-CYP4A2 ( $30 \pm 5\%$  relaxation) or Adv-EGFP ( $39 \pm 5\%$  relaxation). DDMS (30  $\mu$ M), a CYP4A inhibitor, enhanced ( $P < 0.05$ ) ACH-induced relaxation in RA of rats transduced with Adv-CYP4A2 (from  $54 \pm 4$  to  $74 \pm 8\%$  relaxation) but not with Adv-EGFP ( $87 \pm 2$  to  $92 \pm 4\%$  relaxation). 20-HETE (10  $\mu$ M) reduced ( $P < 0.02$ ) the response to ACH in DDMS-treated RA of rats transduced with Adv-CYP4A2 (from  $74 \pm 8$  to  $47 \pm 2\%$  relaxation) or Adv-EGFP (from  $92 \pm 4$  to  $47 \pm 9\%$  relaxation). Relaxing responses to NONOate were comparable in RA of rats treated with Adv-CYP4A2 or Adv-EGFP. These data suggest that vascular overexpression of CYP4A2 attenuates ACH-induced dilation of RA, presumably via a mechanism involving interference by 20-HETE with the NO-dependent component of the vasorelaxing action of ACH.

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### The T-786C and Glu298Asp Polymorphisms of the Endothelial Nitric Oxide Synthase Gene are Associated with Postural Tachycardia Syndrome

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Patients with postural tachycardia syndrome (POTS) represent a heterogeneous population characterized by orthostatic symptoms, an increase in heart rate (HR)  $\geq 30$  beats per minute (bpm) and an excessive rise in plasma norepinephrine (NE) upon standing. Some cases of POTS have an underlying peripheral pathophysiology whereas hyperadrenergic POTS likely originates in the brain. We previously reported the sole genetic link identified thus far for POTS, an extremely rare A457P mutation in the NE transporter gene (SLC6A2). Nitric oxide (NO) is synthesized via three isoforms of NO synthase (NOS). In addition to its role in NO-mediated vasodilation, endothelial NOS (eNOS) contributes to NO production in the brain. NO inhibits central sympathetic output, and over expression of eNOS in the *nucleus tractus solitarius* causes hypotension and bradycardia. An aberration in central NO-dependent mechanisms could underlie the hyperadrenergic state of POTS patients. We conducted a case-control study to test the hypothesis that eNOS genotypes are associated with POTS. We studied two functional polymorphisms of eNOS (T-786C and Glu298Asp) that reduce eNOS activity. DNAs from 56 controls (27 females) and 85 POTS patients (76 females), all Caucasian, were genotyped for the T-786C and Glu298Asp polymorphisms. Genotype frequencies for T-786C ( $P = 0.001$ ) and Glu298Asp ( $P = 0.037$ ) differed significantly between controls and cases (for controls vs POTS, T-786C: TT=33.3 vs 32.9%, TC=35.2 vs 57.6%, CC=31.5 vs 9.4%; Glu298Asp: GG=50.0 vs 40.5%, GT=30.4 vs 50.0%, TT=19.9 vs 9.5%). In the POTS population, the T-786C genotype had a significant effect ( $P = 0.004$ ) on the HR rise from 0 to 3 min standing. At 3 min, HR increase (mean  $\pm$  SD) was CC:  $53 \pm 17$ , TC:  $34 \pm 13$ , TT:  $34 \pm 16$  bpm. The increase in NE with standing was also significantly greater in patients who were homozygous for -786C. No significant effects were found for either polymorphism on blood pressure. The association between the -786C homozygote (CC) and HR and NE in POTS is consistent with increased sympathetic activity due to a reduction in central eNOS activity. Our data suggest that eNOS polymorphisms contribute to the POTS phenotype.

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### The soluble epoxide hydrolase is a main effector of angiotensin II-induced hypertension

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The soluble epoxide hydrolase (sEH) metabolises vasodilatory epoxy eicosatrienoic acids (EETs) to inactive dihydro-EETs. We hypothesized that the metabolism of EETs by the

sEH contributes to angiotensin II-induced hypertension and tested the effects of the new sEH inhibitor 4-(3-Adamantan-1-ylureido)butyryloxy (AUDA) on blood pressure in vivo and on vasodilator function ex vivo in normotensive mice as well as in animals with angiotensin II- or phenylephrine-induced hypertension. AUDA (130  $\mu\text{g}/\text{ml}$ ), given with the drinking water, did not affect blood pressure in normotensive animals but markedly lowered blood pressure in mice with angiotensin II-induced hypertension (1.1 mg/kg/d), reaching a plateau within 3 days. Systolic blood pressure was reduced from  $142 \pm 3$  to  $117 \pm 4$  mmHg. The effect of AUDA was accompanied by an increase in urinary sodium ( $341 \pm 15$  vs.  $277 \pm 13$  nmol/min) and water excretion ( $0.133 \pm 0.007$  vs.  $0.096 \pm 0.004$  ml/h). Intravenous application of AUDA (8 mg/kg) acutely lowered blood pressure in animals with angiotensin II-induced hypertension, but failed to affect blood pressure in animals with phenylephrine-induced hypertension (29 mg/kg/d). AUDA (0.1  $\mu\text{M}$ ) lowered vascular resistance in an isolated perfused kidney preparation but not in the perfused hind limb or in isolated carotid arteries from angiotensin II-treated mice. In contrast, DDMS (5  $\mu\text{M}$ ), an inhibitor of cytochrome P450 4A which attenuates formation of the potent vasoconstrictor 20-hydroxyeicosatetraenoic acid, induced the relaxation of carotid arteries from angiotensin II-treated mice but not from control mice. These data advocate inhibition of sEH as new therapeutic principle in angiotensin II-induced hypertension. The sustained blood pressure lowering effect of the substance can be attributed to pressure diuresis, but additional mechanisms contribute to the acute antihypertensive effects of AUDA.

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### Megalyn mediates Angiotensin II uptake

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Experiments aiming to inhibit Angiotensin II (Ang II) internalization on immortalized yolk sac cells (BN-16 Cells) with specific peptide inhibitors and direct competitors of the Angiotensin Type 1 Receptor produced only partial prevention of Ang II-cell accumulation. Because megalin is a known scavenger receptor involved in the uptake of several ligands and is abundantly expressed at the surface of epithelial cells lining tissues such as the yolk sac and the proximal tubule, we searched for its possible role as a receptor for Ang II. Flow cytometry analysis of Ang II uptake by BN-16 cells demonstrated that anti-megalyn and anti-cubilin antisera interfered with Ang II uptake. By the same approach it was also shown that pure Ang II competed for uptake of metallothionein (a known megalyn ligand). Also, in flow cytometry analysis of binding experiments performed in brush border membrane vesicles freshly isolated from kidney cortexes of CD-1 mice, anti-megalyn antisera interfered with Ang II binding. Last, in surface plasmon resonance experiments an injection of Ang II produced a small change in mass at the surface of a megalyn receptor-coated chip. This change of mass is consistent with an interaction of a small peptide like Ang II (~1 kDa) with a large protein like megalyn (600 kDa). From these diverse results, we concluded that megalyn and perhaps to a lesser extent cubilin are receptors for Ang II. The role of megalyn as a receptor for Ang II suggests that megalyn mediates physiological and pathophysiological mechanisms linked to endogenous ligands *in vivo* and raises important questions regarding the interactions between megalyn and renin-angiotensin system in tissues where they are co-expressed such as the yolk sac and the proximal tubule.

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### Role of AT<sub>1</sub> Receptor in Increased Distal Nephron Renin in Angiotensin II-Infused Hypertensive Rats

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Renin in distal nephron segments may provide a possible pathway for AngII generation from proximally delivered angiotensinogen. We previously demonstrated positive renin immunoreactivity in principal cells of cortical and medullary collecting ducts and increased distal nephron renin in chronically AngII-infused hypertensive rats. However, the regulatory mechanism involved still remains unclear. This study was designed to determine the role of AT<sub>1</sub> receptors in mediating the enhanced renin protein expression in collecting duct cells that occurs in Ang II-infused hypertensive rats. Kidneys from adult, male Sprague-Dawley rats subjected to sham operation (n=8), AngII-infusion (80ng/min/day, 13days, n=8) and AngII-infusion plus AT<sub>1</sub> receptor blocker (ARB) treatment (Olmesartan, 5 mg/day, 13days, n=12) were processed to evaluate renin by immunohistochemistry and Western blot analysis. Ang II infusions progressively increased systolic blood pressure ( $181 \pm 4$  vs  $115 \pm 5$  mmHg at day 11) and markedly suppressed plasma renin activity (PRA,  $0.08 \pm 0.1$  vs.  $5.3 \pm 0.8$  ng of AngI mL<sup>-1</sup>•h<sup>-1</sup>) compared to sham. ARB treatment prevented hypertension ( $113 \pm 6$  mm Hg at day 11) and significantly increased PRA ( $15.8 \pm 1.5$  ng of AngI•mL<sup>-1</sup>•h<sup>-1</sup>). Renin immunoreactivity in juxtaglomerular cells (JG) was significantly suppressed in Ang II-infused compared to sham-operated rats ( $0.1 \pm 0.0$  vs  $1.0 \pm 0.1$  DU relative to Sham) and markedly increased after ARB treatment ( $2.1 \pm 0.3$  DU relative to Sham). In contrast, increased renin immunoreactivity in cortical and medullary collecting duct cells of Ang II-infused rats ( $6.4 \pm 1.4$  vs  $1.0 \pm 0.1$  cortex;  $2.5 \pm 0.3$  vs  $1.0 \pm 0.2$  medulla;  $P < 0.001$ ) was prevented by ARB treatment ( $0.1 \pm 0.0$  cortex;  $0.1 \pm 0.0$  medulla;  $P < 0.001$ ). Renin protein levels in kidney medulla were significantly higher in Ang II-infused compared to sham ( $1.3 \pm 0.1$  vs  $1.0 \pm 0.1$  DU relative to Sham;  $P < 0.05$ ); however ARB prevented this increase ( $0.83 \pm 0.1$  DU relative to Sham). These data demonstrate that augmentation of distal nephron renin in AngII-infused rats is AT<sub>1</sub>-dependent and provide further evidence that renin in collecting duct cells may contribute to increased intrarenal AngII levels in AngII-dependent hypertension.

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### Glucagon-Induced Hyperfiltration and Proliferation in Glomerular Mesangial Cells Involve Specific Glucagon Receptors and Activation of the MAP Kinase Signaling Cascade

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Glucagon, a peptide hormone synthesized and released by pancreatic  $\alpha$  cells, plays an important role in the development of glomerular hyperfiltration and diabetic glomerulosclerosis in humans and experimental animals. We tested the hypothesis that glucagon induces glomerular hyperfiltration by inhibiting angiotensin II type 1 receptor (AT1R) expression in cultured rat glomerular mesangial cells (MCs), which involves a mitogen-activated protein (MAP) kinase signaling cascade. Rat MCs were cultured to 80% confluence and starved for 24 h before experiments. Compared with control, glucagon (1 nM) decreased AT1R expression in a time-dependent manner, which began at 5 min (-35%,  $p < 0.05$ ) and peaked at 30 min (-56%,  $p < 0.05$ ). The effects of glucagon on AT1R expression were associated with 228% increase in MAP kinase ERK1/2 phosphorylation (p-ERK1/2) at 5 min after glucagon stimulation ( $p < 0.05$ ), and the response slowly returned to control 2 hours later. Glucagon-stimulated ERK1/2 phosphorylation was blocked by a specific glucagon antagonist, [Des-His<sup>1</sup>-Glu<sup>2</sup>] glucagon (1  $\mu\text{M}$ ) or captopril (10  $\mu\text{M}$ ), which inhibits Ang II formation. To study whether glucagon stimulates glomerular MC proliferation by glucose-mediated increase in Ang II formation, MCs were incubated with high glucose (30 nM) and treated with either captopril or losartan for 24 h. High glucose and Ang II (1 nM) increased [<sup>3</sup>H]thymidine incorporation by 53% and 41%, respectively ( $p < 0.05$ ). The proliferative effects of high glucose and Ang II were completely blocked by captopril and losartan (10  $\mu\text{M}$ ). These results suggest that glucagon activates specific receptors in rat glomerular MCs, which in turn increases glucose-induced Ang II formation. Increased Ang II formation by glucagon/glucose might explain increased MAP kinase ERK1/2 phosphorylation and subsequent proliferation in rat glomerular mesangial cells.

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### Role of Rho-kinase and p27 in Angiotensin II-Induced Vascular Monocyte/Macrophage Infiltration and Medial Thickness

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Angiotensin II (Ang II) has been shown to enhance the development of atherosclerotic lesion in which VSMC and monocyte/macrophage proliferation as well as migration is a critical step. Recently, both RhoA/Rho-kinase and p27 (a cell cycle dependent kinase inhibitor) have been implicated as factors promoting VSMC migration cooperatively. Nevertheless, the role of p27 and RhoA/Rho-kinase pathway in Ang II-mediated monocyte/macrophage migration and vascular injury *in vivo* has not been elucidated. We therefore investigated the contribution of p27 and Rho-kinase in Ang II-induced vascular injury in p27<sup>-/-</sup> mouse. Two-week Ang II (1500ng/kg/min) infusion elicited similar degrees of elevation in systolic blood pressure in p27<sup>+/+</sup> ( $159 \pm 5$  mmHg) and p27<sup>-/-</sup> mouse ( $157 \pm 5$  mmHg,  $p > 0.05$ , n=6). In p27<sup>-/-</sup> mouse, however, a 38.7% increase in the medial thickness of aorta and a prominent increase in MAC3 (a marker of macrophages) were observed, compared with p27<sup>+/+</sup> mice. Treatment of p27<sup>+/+</sup> mice with fasudil, a selective Rho-kinase inhibitor, did not alter blood pressure, but significantly upregulated the expression of p27 and decreased medial thickness of aorta (51±8% reduction from p27<sup>+/+</sup> mice with Ang II) and prevented macrophage infiltration. These protective effects of fasudil were attenuated in p27<sup>-/-</sup> mice. These observations suggest that Ang II-induced downregulation of p27 through RhoA/Rho-kinase pathway constitutes an important determinant of monocyte/macrophage infiltration and vascular hyperplasia, independent of changes in blood pressure. Furthermore, inhibition of Rho-kinase activity improves Ang II-induced vascular injury through both p27-dependent and -independent mechanisms.

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### Inhibition of arterial tone of mesenteric arteries by visceral periaortic adipose tissue and adiponectin in C57BL/6 mice

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Virtually all systemic blood vessels are surrounded by adipose tissue, which produces a number of biologically active substances, including adiponectin. We have previously reported that perivascular adipose tissue secretes adipocyte-derived relaxing factor (ADRF) to relax aorta and mesenteric arteries by opening of smooth muscle K<sup>+</sup> channels in rats. The nature of ADRF and its putative role in other species are unknown. We tested the hypothesis that periaortic adipose tissue modulates contraction of small mesenteric arteries in mice and examined the role of adiponectin as possible candidate of ADRF. We studied mesenteric artery rings surrounded by periaortic adipose tissue from 6–8 weeks C57BL/6 mice. The contractile response to serotonin was markedly reduced in intact vessels compared to vessels without periaortic fat. The contractile response to depolarizing high K<sup>+</sup> containing solutions (60 mM) was similar in vessels with and without periaortic fat. The anti-contractile effect of periaortic fat was abolished by inhibition of delayed-rectifier K<sup>+</sup> (K<sub>d</sub>) channels with 4-aminopyridine (2 mM). Blocking other K<sup>+</sup> channels had no effect. Adiponectin inhibited serotonin-dependent contractions in a dose-dependent manner, with half-maximal effects at 5  $\mu\text{g}/\text{ml}$ . Similar adiponectin plasma levels were measured in rats and mice. We suggest that visceral periaortic adipose tissue controls mesenteric arterial tone locally by inducing vasorelaxation via K<sub>d</sub> channel activation in vascular smooth muscle cells. Furthermore, Downloaded from <http://hyper.ahajournals.org/> by on December 2, 2009

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### Central Neuropeptide Y Signaling Ameliorates L-NAME Hypertension In The Rat Via A Y1 Receptor Mechanism

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Neuropeptide Y (NPY) is expressed within the central neurons involved in the control of blood pressure (BP) and may serve as a sympatholytic neurotransmitter. Nitric oxide (NO) also serves as a sympathoinhibitory neurotransmitter and L-NAME-induced NO deficiency causes sympathetic overactivity, which then contributes to L-NAME-induced hypertension. We have tested a hypothesis that central NPY acting as a sympathoinhibitory neurotransmitter compensates for NO deficiency and prevents L-NAME hypertension. This form of hypertension was induced in Sprague Dawley NPY transgenic male rats (NTG) overexpressing NPY under its natural promoter and in non-transgenic littermates (CTR). Each animal (n=9-11) was implanted with a telemetric arterial BP transmitter and a guide cannula in the lateral cerebral ventricle (LCV), 7 days post surgery, baseline BP was measured for 3 days. The animals were then implanted with osmotic pumps connected to the LCV cannulas and all animals were treated with L-NAME in drinking water (200 mg/L) for 14 days. During this treatment, synthetic rat NPY (7.0 nmol/kg/12  $\mu$ L/day in saline, Gr.1) or saline (Gr.2) was administered LCV in CTR rats. At the same time, NTG subjects were treated LCV either with a Y1 receptor antagonist, BIBP3226 (63.3 nmol/kg/12 mL/day, Gr.3) or with saline (Gr.4). Baseline BP was not different among the groups. Comparing to CTR, L-NAME hypertension was significantly (p<0.05) reduced in NTG subjects (122.7 $\pm$ 1.5 vs 132.4 $\pm$ 1.6 mmHg, Gr.4 vs Gr.2, respectively). Centrally given NPY reduced L-NAME hypertension in CTR rats (122.2 $\pm$ 1.7 vs 132.4 $\pm$ 1.6 mmHg in Gr.1 vs Gr.2, respectively, p<0.05), while the Y1 antagonist ameliorated the depressor effect of transgenic NPY upregulation (132.7 $\pm$ 1.7 vs 122.7 $\pm$ 1.5 in Gr.3 vs Gr.4, respectively, p<0.05). Thus, (1) central NPY ameliorated L-NAME hypertension; (2) upregulation of the NPY gene had a similar depressor effect; (3) blockade of the central NPY Y1 receptor abolished the hypotensive effect of NPY upregulation. In conclusion, central NPY Y1 receptor has potent antihypertensive effects and compensates for NO deficiency. The mechanism for the central NPY Y1 receptor-induced depressor effect may include inhibition of the central sympathetic outflow.

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### Eplerenone Enhances Adrenal and Excreted Aldosterone during Angiotensin-II Induced Hypertension

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Blockade of aldosterone receptors with eplerenone (epl) has been shown to alleviate cardiovascular and renal injury induced by aldosterone during hypertension. Despite its benefits, epl has been reported to increase plasma aldosterone levels; however, this mechanism has not been elucidated. Therefore, the present study addressed the hypothesis that aldosterone receptor blockade with epl induces an increase in adrenal aldosterone synthesis and release. Four groups of rats (n = 10/group) (1. normotensive (NT) controls, 2. NT + epl, 3. hypertensive (HT), and 4. HT + epl) were maintained in metabolic cages for 28 d and complete urine voids were collected daily. Angiotensin II (ang II; Phoenix Pharmaceuticals) was infused (60 ng/min) in the HT rats via an osmotic mini-pump implanted subQ at the beginning of the study and NT rats were sham operated. After 1 week, rats in the epl groups were fed a diet treated with the drug at a dose equivalent to 100 mg/kg/d. Blood pressure was monitored weekly by tail-cuff plethysmography. Epl increased both plasma aldosterone in NT (mean  $\pm$  SE; 159  $\pm$  44 vs. 751  $\pm$  139 pg/mL) and HT rats (901  $\pm$  293 vs. 2946  $\pm$  947 pg/mL) and adrenal aldosterone content (NT: 46  $\pm$  9 vs 141  $\pm$  13 pg/ mg tissue and HT: 214  $\pm$  43 vs 435  $\pm$  53 pg/ mg tissue). Within 24 hr of epl treatment, urinary aldosterone excretion increased in both NT and HT rats and remained elevated. Adrenal ang II was increased in the HT rats with epl augmenting the increase (4.0  $\pm$  0.5 vs. 6.2  $\pm$  0.9 fmol/mg tissue). Renal ang II was also increased in the HT rats; however epl did not alter the ang II content further. Epl abated urinary total protein excretion in HT rats after 1 week of treatment; however, treatment did not alleviate blood pressure. Despite the epl-induced increase in aldosterone, the attenuation of total protein excretion suggests that the dosage of epl was sufficiently effective to abate renal damage. The data suggests that aldosterone receptors play a role in the feedback mechanism regulating adrenal aldosterone content resulting in increased circulating aldosterone.

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### Vascular AT<sub>1</sub> Receptors But Not Arterial Pressure Regulate ACE2 Expression in Spontaneously Hypertensive Rats

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Angiotensin-converting enzyme (ACE) 2, a newly recognized homologue of ACE that converts angiotensin (Ang) II into Ang-(1-7), is found in endothelial and vascular smooth muscle cells. Expression of ACE2 may be a local determinant of vascular Ang-(1-7) production and, when increased, augment the increasingly recognized protective effects of this peptide within injured tissues. We showed previously in Lewis normotensive rats with coronary ligation-induced myocardial infarction that treatment with the AT<sub>1</sub> receptor blocker olmesartan increased cardiac ACE2 and Ang-(1-7) in conjunction with improved cardiac remodeling and function. In the present study, we investigated hypertension-related ACE2 in vasculature by determining the effect of sustained AT<sub>1</sub> blockade on ACE2 gene and protein expression in aortas and carotid arteries of twelve-week old male SHR treated with either vehicle (n=8) or 10 mg/kg olmesartan (n=8) in the drinking water for 14 days. Blood pressures measured before treatment by tail-cuff plethysmography were not different between groups. Compared to vehicle-treated SHR, olmesartan-treated SHR had lower systolic pressures (152 $\pm$ 3 mm Hg vs. 209 $\pm$ 3 mm Hg, p<0.05) but comparable heart rates. Real time RT-PCR analysis showed that ACE2 mRNA levels in aortas and carotid arteries were five-fold greater (p<0.05) compared to aortas of vehicle-treated SHR. In a separate set of SHR, treatment with olmesartan (n=5) compared to vehicle (n=5) was associated with increased (p<0.05) ACE2 immunostaining in the media of the proximal thoracic aorta (33 $\pm$ 10 vs. 9 $\pm$ 2 arbitrary units). In contrast, ACE2 mRNA and immunostaining intensities were not different in carotid arteries of olmesartan- and vehicle-treated SHR. Taken together, these data represent the first report of ACE2 in aorta and carotid arteries of SHR, as well as differential regulation of ACE2 by AT<sub>1</sub> receptor blockade in vascular smooth muscle of aorta and carotid arteries. Furthermore, the studies provide evidence that changes in ACE2 within the vascular system of SHR are regulated by a factor other than arterial pressure.

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### PHENOTYPIC CHARACTERIZATION OF NEW CONGENIC LINES FOR A STROKE-QTL ON RAT CHROMOSOME 1

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We previously identified a stroke-related quantitative trait locus on rat chromosome 1 (STR1) contributing about 20% to the overall genetic variance of blood pressure-independent stroke susceptibility in the stroke-prone, spontaneously hypertensive rat (SHRSP). To further investigate this finding we created a series of congenic SHRSP/SHRSR (stroke-resistant) strains, isolating portions of the STR1-locus from either strain on the other's background. After 10 consecutive back-crossings and a final round of interbreeding, 3 congenic rat strains were obtained carrying the entire SHRSP STR1 locus, its telomeric half, and its centromeric half, respectively within an SHRSR background, as well as the 3 reciprocal congenic strains. Phenotypic experiments were carried out in these congenic strains, as well as in the reference progenitor strains, administering the established, requisite stroke-permissive dietary regimen ("Japanese diet") starting at the 6th week of age. Whereas 100% of SHRSP suffered a stroke within 6.5 weeks, no strokes were observed in SHRSR during 12 weeks of observation. Congenic SHRSP carrying either the entire or the telomeric half of SHRSR-STR1 showed a reduction of stroke incidence to 50% at 6.5 weeks compared to the progenitor strain; in contrast, 20% of congenic SHRSR carrying the SHRSR STR1 suffered a stroke within 12 weeks. Tail-cuff blood pressure measurements demonstrated similar blood pressure levels among all congenic strains during the experiment. The results of this phenotypic characterization allow further studies aimed at the precise localization and identification of the STR1 stroke gene.

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### CHRONIC DEPLETION OF RENAL ANGIOTENSIN II DOES NOT INFLUENCE THE INTRACELLULAR DISTRIBUTION OF THE RENAL AT<sub>1</sub> RECEPTOR

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We and others have reported the predominant nuclear localization of the Ang II type 1 receptor (AT<sub>1</sub>) in the renal cortex of both normotensive and hypertensive rat strains. Although various studies *in vitro* demonstrate internalization of the AngII-AT<sub>1</sub> receptor complex following binding, the extent that nuclear accumulation of the AT<sub>1</sub> receptor within the kidney is dependent on Ang II is not known. In the present study, we examined the distribution of the AT<sub>1</sub> receptor in the plasma membrane and nuclear fractions isolated from the kidney of wildtype (Wt) and tissue ACE null mice (*tisACE*<sup>-/-</sup>) - a model of severely depleted intrarenal Ang II. In comparison to Wt, the *tisACE*<sup>-/-</sup> mice exhibit reduced blood pressure, polyuria and >80% reduction in the renal content of Ang II. Binding studies using a saturating concentration of [<sup>125</sup>I]-[Sar, Thr]-Ang II [2.5 nM] and losartan [10  $\mu$ M] revealed that the AT<sub>1</sub> subtype comprised essentially all the binding in the nuclear (>95%) and plasma membrane (>99%) fractions from the kidneys of both strains. Immunoblot analysis of the AT<sub>1</sub> receptor revealed a single molecular form of the protein [molecular weight of 52 kD] in both the plasma membrane and nuclear fractions from Wt and *tisACE*<sup>-/-</sup> kidneys. The apparent density of the renal AT<sub>1</sub> receptors was higher in the nuclear fraction for Wt [nuclear: 64.8  $\pm$  26 fmol/mg protein; plasma membrane: 10.4  $\pm$  1.6 fmol/mg protein, n=5] and *tisACE*<sup>-/-</sup> [nuclear 69.1  $\pm$  30 fmol/mg protein; plasma membrane: 21.7  $\pm$  8.7 fmol/mg protein n=4]. However, comparison of the binding densities for the nuclear AT<sub>1</sub> receptor revealed no difference between the Wt and *tisACE*<sup>-/-</sup> mice. In summary, the mouse kidney exhibits a high density of nuclear AT<sub>1</sub> binding sites in greater abundance than that of the plasma membrane receptor - a distribution similar to that of the rat kidney. Chronic depletion of intrarenal Ang II did not influence the expression of AT<sub>1</sub> binding sites in the nuclear compartment, although the density of plasma membrane AT<sub>1</sub> sites tended to be higher in the ACE null mice. We conclude that the localization and/or trafficking of the renal AT<sub>1</sub> receptor to the nucleus may be independent of tissue Ang II.

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### Angiotensin II-Dependent Hypertension in Rats with Reduced Kidney Mass at the Onset of Diabetes

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We have reported consistently that onset of diabetes in L-NAME treated rats causes significant hypertension that is associated with the failure of GFR to increase. Subsequently, we showed that reduced renal mass (RRM) also prevented GFR from increasing during the onset of diabetes, and onset of diabetes in those rats caused hypertension associated with an increase in plasma renin activity. This study tested the role of the renin-angiotensin system in mediating the diabetes-induced hypertension in rats with RRM. RRM was induced by surgical ablation of the poles of the left kidney. Two weeks later, the right kidney was removed during the surgery and 2000 Ang II osmotic minipumps were implanted. Total renal mass reduction was

approximately 70%. RRM rats were divided into Control (C, n=5) and Captopril (CP, n=4; 14  $\mu\text{g}/\text{kg}/\text{min}$ , iv.) groups. Baseline MAP (measured 18 hr/day) averaged  $97 \pm 4$  and  $79 \pm 4$  mmHg in the C and CP groups, respectively. By the third day after induction of diabetes with STZ (40mg/kg, iv.), MAP had increased to  $109 \pm 2$  mmHg in the C group and was  $79 \pm 5$  mmHg in the CP rats. MAP remained elevated in the C rats, similar to our previous study, and averaged  $112 \pm 4$  mmHg ( $\sim 15$  mmHg above baseline) on day 7 of diabetes. In the CP rats, on the other hand, MAP did not increase during diabetes, averaging  $79 \pm 2$  mmHg over the last 3 days of diabetes. Insulin replacement (iv.) was begun after 7 days of diabetes, and by day 4 MAP averaged  $103 \pm 2$  and  $71 \pm 10$  mmHg in the C and CP groups, respectively. GFR averaged  $1.3 \pm 0.2$  and  $1.0 \pm 0.1$  ml/min in the C and CP groups during baseline and did not change in either group during the diabetic or insulin replacement periods. There also were no significant differences in sodium excretion between the groups. These results indicate that the renin-angiotensin system is required for diabetes to cause hypertension in rats with reduced renal mass, and also support our hypothesis that an inability of GFR to increase early in diabetes may lead to an angiotensin-II dependent increase blood pressure.

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### Residual Intrarenal Effects Following Cessation of Angiotensin II Infusion in Angiotensin II-Dependent Hypertension

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Angiotensin II (AngII)-infused hypertensive rat is one of the established AngII-dependent hypertension models. However, there have been few investigations of the renal condition after the termination of AngII infusion. In this study, we investigated the recovery of the intrarenal and residual structural alternations following 3 days after the cessation of AngII infusion. Male Sprague-Dawley rats (175–200 g, N=30) were maintained on a normal diet and divided into 2 groups. One group (N=11) received a sham operation on day 0 and was sacrificed on day 12. The other group received continuous AngII infusion (120 ng/min, N=19) via osmotic minipumps implanted on day 0. This AngII-infused group was further subdivided into 2 subgroups. One subgroup (N=13) was sacrificed on day 12. In the other subgroup (N=6), the minipump was removed on day 12 and animals were terminated on day 15. Both kidneys were harvested and renal AngII contents, immunohistological analysis (IHC), Masson's trichrome staining (MT), and quantitative real-time RT-PCR (QRT-PCR) were evaluated. AngII infusion progressively increased all parameters. At day 12, systolic blood pressure (SBP) measured  $201 \pm 5$  vs.  $134 \pm 3$  mmHg, excretion of albumin in 24-hr urine (UAlb) measured  $19 \pm 2$  vs.  $6 \pm 1$  mg/day, renal AngII measured  $98 \pm 6$  vs.  $78 \pm 14$  pg/g, afferent arteriolar wall thickness (AA, IHC) measured  $4.5 \pm 0.1$  vs.  $3.3 \pm 0.1$   $\mu\text{m}$ , CD68-positive cell number in interstitium (IHC) was  $24 \pm 2$  vs.  $17 \pm 1$  /mm<sup>2</sup>, nuclear factor (NF) kappa B (IHC) was  $1.4 \pm 0.2$  vs.  $1.0 \pm 0.1$ , relative ratio, MT-positive area in interstitium was  $0.16 \pm 0.02$  vs.  $0.07 \pm 0.01\%$ , and angiotensinogen (AGT) mRNA (QRT-PCR) was  $1.4 \pm 0.2$  vs.  $1.0 \pm 0.1$ , relative ratio. Cessation of continuous AngII infusion rapidly normalized SBP, UAlb, renal AngII, NF kappa B, and AGT mRNA expression (at day 15, SBP:  $137 \pm 5$ ; UAlb:  $7 \pm 2$ ; renal AngII:  $62 \pm 11$ ; NF kappa B:  $1.1 \pm 0.1$ ; AGT mRNA:  $0.8 \pm 0.1$ ). However, the changes in some parameters were still remaining (AA:  $4.0 \pm 0.2$ ; CD68:  $28 \pm 3$ ; MT:  $0.17 \pm 0.02$ ). These data indicated that intrarenal AngII level returns to the basal levels within 3 days following the cessation of AngII infusion but some inflammatory changes are still remaining, suggesting that AngII-induced inflammation progresses, in part, independently from intrarenal AngII levels.

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### CGS 35601, a single molecule as a triple vasopeptidase (ACE, NEP, ECE) inhibitor, normalize blood pressure in chronically instrumented, conscious and unrestrained spontaneously hypertensive rats

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Objective: CGS 35601 is a potent inhibitor of angiotensin converting enzyme (ACE), neutral endopeptidase (NEP), and endothelin converting enzyme-1 (ECE-1) with respective IC50 values of 22, 2, and 55 nM. The aim of the present study was to determine the effects of this triple vasopeptidase inhibitor (VPI) on the hemodynamic and biochemical profiles of instrumented, conscious and unrestrained SHR. Methods: Male SHR (retired breeders) weighing  $385 \pm 10$  g (32 weeks; n=23–29) were instrumented with an arterial catheter in the aorta through the femoral artery and were placed individually in a metabolic cage for 28 days. The hemodynamic, hematological and biochemical profiles were assessed daily. After a 7-day stabilization period, the rats were divided into 2 groups: Gr. 1, SHR (n=13–18) received CGS 35601 at increasing doses (0.01, 0.1, 1 and 5 mg/kg/d x 5d each, i.a. constant infusion) followed by a 5d wash out period, while the Gr. 2 control SHR received the vehicle (250  $\mu\text{l}/\text{h}$ ; n=10). Results: CGS 35601 had no effect at 0.01 mg/kg/d, but it reduced the mean arterial blood pressure (MABP) by 10% ( $156 \pm 4$  to  $141 \pm 7$  mmHg) at 0.1 mg/kg/d. At 1 and 5 mg/kg/d, MABP was reduced to  $122 \pm 4$  and  $94 \pm 5$  mmHg (MABP of conscious Wistar rats are 110 mmHg), respectively. Heart rate was unaffected throughout the experiment. Plasma concentrations of angiotensin II and endothelin-1 decreased 50% at 5 mg/kg/day. Conversely, atrial natriuretic peptide and bradykinin concentrations steadily increased over CGS 35601 dose-range. The hemodynamic profile of SHR returned to normal during the 5d wash out period as was accompanied by the reduction in plasma concentrations of vasodilators and a rebound in vasoconstrictors. No pharmacotoxicology (>40 plasma and urinalysis parameters measured, mostly renal and hepatic) was observed in both groups, including diuresis. Conclusion: This novel single molecule triple VPI is potent, effective at lowering blood pressure and present a solid safety profile that may be of interest for treating hypertension and other cardiovascular diseases.

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### Hyperglycemia-induced Oxidative Stress Impairs Renal Dopamine D1 Receptor Function via Protein Kinase C Activation and G Protein Coupled Receptor Kinase 2 Translocation

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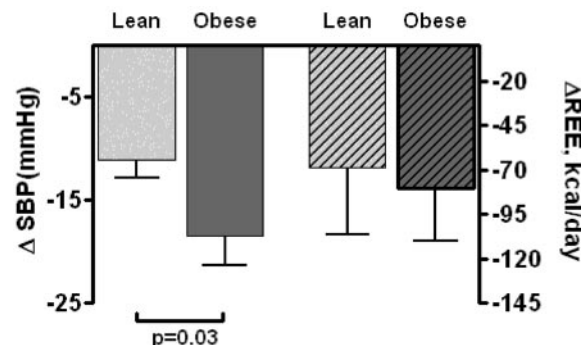
Hyperglycemia produces a bewildering array of changes in animal models and diabetic patients. The main challenge in this area is to identify a single unifying mechanism for hyperglycemia-induced biochemical changes responsible for diabetic complications. There is strong evidence that diabetes is associated with increased oxidative stress, however, the source of this oxidative stress is unclear. Recently, we reported that in rats, streptozotocin (STZ)-induced hyperglycemia caused renal dopamine D1 receptor (D1R)-G protein uncoupling resulting in loss of dopamine-induced Na,K-ATPase (NKA) inhibition and natriuretic response. In present study, we have investigated the mechanism of hyperglycemia-induced D1R-G protein uncoupling. We also examined direct effect of high glucose on D1R function under controlled conditions. In rats, STZ-induced hyperglycemia increased oxidative stress as determined by carboxymethyllysine (CML) and malondialdehyde (MDA), elevated basal protein kinase C (PKC) activity and increased expression of PKC isoforms  $\beta$  and  $\delta$ . Also, STZ-treated rats showed increased D1R serine-phosphorylation and translocation of G protein coupled receptor kinase 2 (GRK-2) from cytosol to cell membrane. SKF-38393, a D1 receptor agonist, failed to stimulate [<sup>35</sup>S]GTP- $\gamma$ S membrane binding and inhibit NKA activity in proximal tubules from STZ-treated rats. To ascertain the role of glucose, renal proximal tubular epithelial cell cultures were exposed to 5mM glucose (control) and 25mM glucose (hi-glc)-serum free media for 72 hours. Exposure of cells to hi-glc increased CML and MDA, elevated basal PKC activity and increased expression of PKC  $\beta$  and  $\delta$ . In addition, we observed increased D1R serine-phosphorylation and decreased D1R expression in cells exposed to hi-glc compared to control cells. In cells exposed to hi-glc, SKF-38393 failed to stimulate [<sup>35</sup>S]GTP- $\gamma$ S membrane binding and inhibit NKA activity. These results both from *in vivo* and *in vitro* studies suggest that glucose causes oxidative stress, increases PKC activity and expression of  $\beta$  and  $\delta$  isoforms leading to D1R serine-phosphorylation via translocation of GRK-2. These D1R do not couple to G proteins resulting in loss of dopamine-induced NKA inhibition and natriuretic response.

P225

### Metabolic and Cardiovascular Impact of the Autonomic Nervous System in Obesity

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An estimated 30% of U.S adults are obese and the incidence of this disorder is increasing. We tested the hypothesis that obesity increases sympathetic activity to induce a compensatory increase in energy expenditure, but this result in hypertension and cardiovascular complications. We studied 17 non-hypertensive subjects, 7 lean (BMI < 25 Kg/m<sup>2</sup>) and 10 obese (BMI  $\geq 30$ ). To assess the contribution of the sympathetic nervous system to blood pressure and energy expenditure we infused trimethaphan at doses (4–5 mg/min) that produced complete autonomic blockade, as determined by removal of baroreflex responses and elimination of low frequency variability of blood pressure, using finger plethysmography (Finapres). At baseline, supine systolic blood pressure (SBP) was  $107 \pm 2$  and  $121 \pm 4$  mmHg in lean and obese subjects, respectively, and resting energy expenditure was  $1410 \pm 77$  and  $1852 \pm 127$  Kcal/day, respectively. The fall in blood pressure after autonomic blockade was greater in obese compared to lean ( $-18 \pm 3$  vs.  $-11 \pm 2$ ,  $p < 0.05$ , figure, clear bars), and "intrinsic" blood pressure in the absence of autonomic influences was similar in both groups ( $p > 0.05$ ). In contrast, autonomic blockade produced similar reductions in resting energy expenditure in lean and obese subjects ( $-69 \pm 38$  and  $-80 \pm 30$ , respectively,  $p = \text{NS}$ , figure hatched bars). We conclude that sympathetic activation associated with obesity is not effective in inducing a compensatory increase in energy expenditure, but is an important determinant of the increase in blood pressure associated with obesity, even within the normotensive range.



P226

### Glibenclamide, an ATP-sensitive potassium channel blocker, suppress sodium-induced hypertension through increased secretion of urinary kallikrein

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Object: High sodium in diet is known to induce hypertension. Secretion of the renal kallikrein (G) is increased by sodium and glibenclamide (GbC) in the kidney cortex

slice in vitro. The object is to test if GbC suppresses sodium-induced hypertension. **Methods:** Hypertension was introduced in rats (8 weeks old) of normal Brown-Norway Kitasato (BN-Ki) and mutant kininogen-deficient BN-Katholik (BN-Ka) strains and of Sprague-Dawley (SD) strain under 8% NaCl in diet for one week. The systolic blood pressure (SBP) of unanesthetized rats was measured twice a week with tail-cuff plethysmography. GbC (100 mg/kg) or PNU-37883A (PNU, a kidney selective potassium channel ( $K_{ATP}$ ) blocker, Pharmacia & Upjohn, MI, 10 mg/kg) were injected S.C. twice a day. The urinary levels of kallikrein and sodium were determined. **Results:** The SBP in normal BN-Ki rats ( $n=6$ ) was increased under sodium diet from  $113.4 \pm 2.6$  (mean  $\pm$  SE) to  $121.2 \pm 2.0$  mmHg ( $P < 0.05$ ), whereas the SBP in GbC-treated rats was decreased from  $118.1 \pm 1.2$  to  $109.5 \pm 0.5$  ( $P < 0.05$ , GbC vs vehicle). The urinary kallikrein level in the GbC-treated group was largely increased from  $0.034 \pm 0.004$  to  $0.064 \pm 0.010$  micromol/mg Cr ( $P < 0.05$ ), whereas no change in the vehicle-treated rats ( $0.033 \pm 0.004$ ). The urinary sodium levels were increased in both the GbC-treated and the vehicle-treated groups, but that in the GbC-group ( $69.8 \pm 1.4$  mg/day) was higher than in the vehicle-group ( $43.2 \pm 4.1$ ) ( $P < 0.01$ ). The same results were obtained in the PNU-treated rats. In mutant kininogen deficient BN-Ka rats ( $n=6$ ), the GbC treatment excreted higher urinary kallikrein ( $0.057 \pm 0.004$  micromol/mg Cr), compared with that in the vehicle treatment ( $0.038 \pm 0.005$ ) ( $P < 0.01$ ). However, there was no difference in increase in the SBP and in urinary sodium between in the GbC- and the vehicle-treated rats. A bradykinin  $B_2$  receptor antagonist (FR173657, Fujisawa Co., Japan), together with GbC, to SD rats, canceled the hypotensive and natriuretic effects of GbC in sodium hypertension, although the secretion of urinary kallikrein was accelerated. **Conclusion:**  $K_{ATP}$  blockers accelerated the renal kallikrein excretion, which caused natriuresis and suppressed the high sodium-induced hyperension in rats.

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### Disparate Regulation of the Cardiac, Renal and Pulmonary Renin-Angiotensin Systems in the Tissue ACE Knockout Mouse

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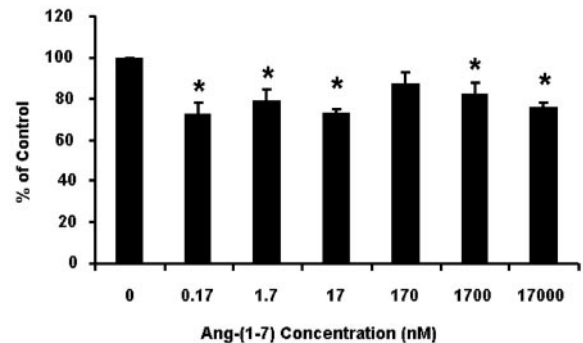
The renin-angiotensin system (RAS) comprises both circulating and distinct local or tissue systems. The interplay of the blood and tissue RAS is complex and may contribute to the development and progression of tissue injury in the kidney, heart and lung. We utilized the tissue ACE knockout [ $tACE^{-/-}$ ] mouse in which tissue-bound ACE is essentially abolished to elaborate the interrelationship of the tissue and circulating RAS by quantifying the precursor peptide Ang I and its two active products Ang II and Ang-(1-7) in adult wildtype (Wt) and  $tACE^{-/-}$  mice. In the  $tACE^{-/-}$  plasma, Ang II levels were suppressed [ $23 \pm 4$  vs.  $58 \pm 16$  pM for Wt], while both Ang-(1-7) [ $36 \pm 6$  vs.  $102 \pm 9$  pM for Wt] and Ang I [ $69 \pm 21$  vs.  $216 \pm 50$  pM for Wt] increased 3 fold. In the kidneys of  $tACE^{-/-}$  mice, both Ang II [ $5.2 \pm 0.6$  vs.  $26 \pm 2$  fmol/mg protein for Wt,  $p < 0.05$ ] and Ang I [ $8.2 \pm 1$  vs.  $2.2 \pm 0.5$  fmol/mg for Wt,  $p < 0.05$ ] were markedly suppressed, however, renal levels of Ang-(1-7) did not change. In the  $tACE^{-/-}$  lung, Ang II levels were reduced by 70% [ $1.7 \pm 0.2$  vs.  $5.8 \pm 0.7$  fmol/mg for Wt], however, Ang-(1-7) [ $7.4 \pm 0.7$  vs.  $4.7 \pm 0.5$  fmol/mg for Wt] and Ang I [ $0.99 \pm 0.07$  vs.  $0.53 \pm 0.06$  fmol/mg Wt,  $p < 0.05$ ] increased 1.5 and 1.9 fold, respectively. In contrast to the kidney and lung, the levels for all three peptides were not altered in the  $tACE^{-/-}$  heart. We then assessed the tissue peptides following Ang II infusion [40 ng/kg/day for 14 days] in  $tACE^{-/-}$  mice. In the lung, tissue levels of Ang-(1-7) increased 2.2 fold [ $16.3 \pm 3.0$  fmol/mg,  $p < 0.01$ ] with no changes in either Ang I or Ang II. Consistent with the accumulation of Ang-(1-7), pulmonary membranes expressed significant ACE2 activity in the  $tACE^{-/-}$  mice. In contrast to the lung, both renal Ang I and Ang II levels significantly increased without alterations in Ang-(1-7) following infusion of Ang II. We conclude that ACE is the predominant pathway for Ang II formation in both kidney and lung with no evidence of compensatory pathways for Ang II production. However, there are marked differences in the regulation of both Ang I and Ang-(1-7) in the lung and kidney of  $tACE^{-/-}$  mice. The absence of Ang II accumulation and increased pulmonary Ang-(1-7) following chronic infusion of Ang II suggests an efficient uptake and conversion pathway that may involve the Ang-(1-7)-forming enzyme ACE2.

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### Ang-(1-7) inhibits circulating levels of angiogenic growth factors in normal pregnant women

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Ang-(1-7) is increased in normal pregnancy, but its functional role is unknown. Recent studies have suggested that it opposes the action of Ang II on vascular growth. Because angiogenesis is critical to normal embryonic growth and development during human pregnancy, these studies were designed to assess the effects of Ang-(1-7) on angiogenesis in human umbilical vein endothelial cells (HUVECs) in culture. In addition, the effects of Ang-(1-7) infusion on two angiogenic growth factors, vascular endothelial growth factor (VEGF) and placenta growth factor (PLGF), were also studied in 3<sup>rd</sup> trimester normotensive pregnant human subjects ( $n=9$ ). HUVECs were cultured in EGM-2 medium, plated in wells precoated with ECMatrix, and then treated with Ang-(1-7) [0.17–17000 nM] for 18 hours. The growth of capillary-like tubules was quantified, and Ang-(1-7) inhibited angiogenesis by approximately 24% ( $p < 0.05$ ) at all doses tested (Figure). Infusion of Ang-(1-7) [1000–3000 pmol/min] was well tolerated without blood pressure reduction and resulted in a significant reduction in plasma VEGF ( $450 \pm 18$  vs  $413 \pm 8$  pg/ml,  $p < 0.05$ ) and PLGF ( $13 \pm 1$  vs  $10 \pm 1$  pg/ml,  $p < 0.05$ ) at 3000 pmol/min. Plasma Ang-(1-7) at that dose averaged  $127 \pm 12$  pg/ml. There was a significant inverse correlation of plasma Ang-(1-7) with PLGF ( $r = -0.9845$ ,  $p < 0.05$ ) and Ang-(1-7) with VEGF ( $r = -0.9718$ ,  $p < 0.05$ ). These studies show that Ang-(1-7) may exert a modulatory role by inhibiting angiogenesis. We have also shown for the first time that in pregnancy Ang-(1-7)'s effects may be mediated through attenuation of the release of angiogenic factors.



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### Chronic Central Hyperinsulinemia Transiently Decreases Food Intake without Changing Arterial Pressure and Heart Rate in Rats

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Acute studies suggest that insulin has anorexic actions and may increase arterial pressure through direct effects on the central nervous system. However, the importance of these actions in long-term regulation of food intake and cardiovascular function is still unclear. The goal of the present study was to determine whether chronic intracerebroventricular (ICV) infusion of insulin decreases food intake and raises arterial pressure in rats. An ICV cannula was placed in the lateral ventricle of male Sprague-Dawley rats for chronic insulin or vehicle (artificial cerebrospinal fluid, 0.5  $\mu$ l/hr) infusion, via osmotic minipump, and arterial and venous catheters were implanted for measurement of mean arterial pressure (MAP) and heart rate (HR) 24 hr/d and IV infusions. After 4 days of control measurements, insulin (6  $\mu$ U/hr,  $n=7$  or 12  $\mu$ U/hr,  $n=6$ ) or vehicle ( $n=4$ ) was infused ICV for 7 days. Insulin infusion at these doses caused only a transient decrease in food intake ( $-6$ g on day 2 and 3) and did not significantly alter MAP or HR ( $106 \pm 2$  vs.  $104 \pm 2$  mmHg and  $411 \pm 8$  vs.  $410 \pm 8$  bpm, respectively for 6  $\mu$ U/hr, and  $105 \pm 5$  vs.  $103 \pm 4$  mmHg and  $407 \pm 9$  vs.  $411 \pm 7$  bpm, respectively for 12  $\mu$ U/hr). Food intake, MAP and HR remained unchanged in the vehicle-treated group. These findings provide no evidence that direct central actions of insulin play a major role in long-term regulation of appetite, arterial pressure or heart rate. However, insulin may exert modest acute effects on appetite that could play a role in limiting meal size (Supported by NHLBI grant P01 HL51971)

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### Activation of NADPH Oxidase Contributes to RhoA/Rho-kinase Dependent Contraction Induced by Angiotensin II

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Evidence suggests that enhanced production of reactive oxygen species (ROS) contributes to elevated blood pressure in several hypertensive rat models; and administration of antioxidants has been reported to decrease the blood pressure. Previously, we reported that ROS are able to activate RhoA/Rho-kinase, a calcium sensitization pathway. Because angiotensin II (ANGII) has been shown to increase ROS production through activation of NADPH oxidase, we hypothesize that activation of NADPH oxidase contributes to ANGII-induced RhoA/Rho-kinase activation. Endothelium-denuded rat aortic rings were mounted in muscle baths and isometric force was recorded. RhoA translocation and phosphorylation of myosin light chain phosphatase targeting subunit (MYPT1) were analyzed by western blot. Our results showed that ANGII (100 nM) induced 78% of phenylephrine-induced maximum contraction (10  $\mu$ M). Pre-incubation with Y-27632 (0.5  $\mu$ M), a Rho-kinase inhibitor, or diphenyleneiodonium chloride (DPI, 10  $\mu$ M), a NADPH oxidase inhibitor, reduced vessel contractions in response to ANGII by 26% and 90%, respectively. However, we did not observe the additive effects when the rings were pre-incubated with Y-27632 and DPI together, suggesting that ROS and Rho-kinase may partly act in the same pathway. Furthermore, ANGII significantly increased RhoA translocation to the plasma membrane by  $1.63 \pm 0.21$  fold of control; and DPI decreased this translocation to  $1.04 \pm 0.18$  fold ( $n=3$ ,  $p < 0.05$ ). In addition, DPI decreased ANGII-induced MYPT1 phosphorylation level from  $1.61 \pm 0.11$  to  $1.16 \pm 0.04$  fold of control ( $n=3$ ,  $p < 0.01$ ). In conclusion, these results suggest that activation of NADPH oxidase contributes to ANGII-induced calcium sensitization.

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### Angiotensinogen gene polymorphism and hypertension

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Hypertension is a serious risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure. It is estimated that hypertension affects 50 million Americans with a prevalence rate of 25–30% in the adult Caucasian population. The incidence of hypertension and complications due to this disease are even greater in the African American population. The renin-angiotensin system plays an important role in the regulation of blood pressure. Previous studies have suggested that angiotensinogen (AGT) gene locus is associated with human essential hypertension. The human AGT gene has an A/G polymorphism at -217 and we report here that frequency of allele A at -217 is significantly increased in the genomic DNA of African-American as well as Caucasian hypertensive patients. We also show that reporter constructs containing AGT gene promoter with nucleoside A at -217 have about 90% increased promoter activity on transient transfection in SV-40 transformed human hepatocytes. Our gel shift assays suggest that C/EBP family of transcription factors bind preferentially to this region of the promoter. We also show that this region of the promoter is present at -217. We also show that this region of the

promoter has partial homology with glucocorticoid receptor binding site (GRE). Our gel shift assays suggest that recombinant GR binds more strongly to an oligonucleotide containing nucleoside A at -217. In addition, transient transfection of reporter constructs containing nucleoside A at -217 have increased glucocorticoid induced promoter activity in human liver cells. These data suggest that A/G polymorphism at -217 of the human AGT gene affects its expression in liver cells and plays an important role in human hypertension. Supported by grants from NHLBI and Philip-Morris Inc.

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### Sexual Dimorphism in Oxidative Stress and Renal Production of Endothelin-1 and Prostanoids in Spontaneously Hypertensive Rats

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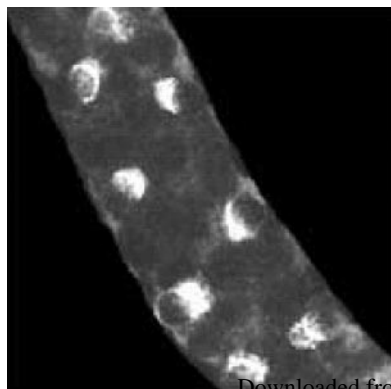
Male spontaneously hypertensive rats (SHR) have higher blood pressure, a blunted pressure-natriuresis relationship, and accelerated progression of renal injury compared to female SHR. Oxidative stress, ET-1 and prostanoids contribute to the regulation of kidney function and blood pressure. The aim of this study was to determine if there is a gender difference in oxidative stress or renal production of ET-1 or prostanoids in SHR and examine the role of sex steroids in regulating these factors. Urine from 13 week old male, orchidectomized (ORX) male, female, and ovariectomized (OVX) female SHR was analyzed for protein, sodium,  $H_2O_2$ , ET-1, and prostanoids (n=6). Proteinuria was greater in male SHR ( $55 \pm 8$  mg/day) compared to female ( $25 \pm 6$  mg/day), ORX ( $30 \pm 6$  mg/day), and OVX ( $25 \pm 6$  mg/day) ( $p < 0.05$ ). Sodium excretion was not different among groups. Urinary  $H_2O_2$  was greater in male SHR ( $21 \pm 3$  nmol/day) compared to female ( $5 \pm 1$  nmol/day), ORX ( $5 \pm 1$  nmol/day), and OVX ( $3 \pm 1$  nmol/day) ( $p < 0.05$ ). Male SHR had greater ET-1 excretion ( $926 \pm 48$  fmol/day) compared to female ( $651 \pm 55$  fmol/day), ORX ( $708 \pm 34$  fmol/day) and OVX ( $603 \pm 29$  fmol/day) ( $p < 0.01$ ). TXB2 excretion, an index of renal TX production, was lower in male ( $26 \pm 1$  ng/day) than female SHR ( $37 \pm 4$  ng/day,  $p < 0.005$ ), and was not affected by gonadectomy (ORX:  $23 \pm 2$  ng/day, OVX:  $42 \pm 1$  ng/day). PGE metabolite excretion was lower in male SHR ( $54 \pm 4$  ng/day) compared to female SHR ( $174 \pm 12$  ng/day) and gonadectomy increased PGE metabolite excretion (ORX:  $91 \pm 3$  ng/day, OVX:  $240 \pm 8$  ng/day). There were no differences in 2,3 dinorTXB2, an index of systemic TX, or 6-keto PGF1 $\alpha$  between the groups. In male SHR, testosterone-dependent increases in  $H_2O_2$  and renal production of ET-1 may contribute to accelerated renal injury and increased blood pressure. In hypertensive females, prostanoids such as TX and PGE may play a greater role in the determination of blood pressure than in hypertensive males.

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### Translocation of the H-ATPase pump in the collecting duct in diabetic kidney

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The vacuolar H-ATPase is responsible for acidifying endomembrane compartments in eukaryotic cells, and is required for many intracellular processes. Our previous studies have shown that glucose activates H-ATPase activity in the kidney epithelial cells LLC-PK<sub>1</sub> through the glycolysis pathway by signals that require PI3-kinase activity (Am J Physiol Cell Physiol 287: C97-C105, 2004). The present study examines the effect of glucose on H-ATPase activity in the distribution of the H-ATPase in the isolated OMCD tubules in normal and diabetic rats. Following immunostaining with E11 anti-H-ATPase antibody, tubular images were analyzed by the scanning laser confocal microscopy. In normal rat OMCD, the intercalated cells demonstrate H-ATPase staining which is polarized to either apical or basolateral pole or distributed diffusely throughout the cell. In diabetic OMCD, there is prominent degree of H-ATPase staining which is sharply delineated along the apical cytoplasmic vesicular staining. In order to confirm the effect of glucose on H-ATPase activity in the distribution of the H-ATPase, we added high glucose (50 mM glucose) to the staining media in the normal OMCD tubules and observed bright H-ATPase staining with well polarized from the basal to the apical poles. These results are similar with those from the diabetic OMCDs. Translocation of the H-ATPase pump between cytoplasmic vesicle and apical plasma membrane of the intercalated cells is an important mechanism of the regulation in acidification. These findings suggest that the glucose is an important physiologic regulator of vacuolar H-ATPase activities in the cultured renal tubular epithelial cells and in the OMCD tubules.



### H-ATPase Activity in Selective Disruption of H-K-ATPase alpha 1 Gene of Mice Under Normal and K-depleted Conditions

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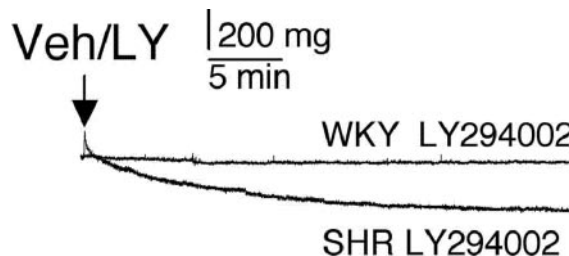
The renal outer medullary collecting duct (OMCD) plays an important role in acid-base homeostasis by two luminal proton ATPases, H-ATPase and H-K-ATPase (HKA), both of which are in the intercalated cells (ICs) of the OMCD. We showed previously that the HKA $\alpha$ 1 activity is the essential H-K-ATPase activity under normal conditions, and that HKA $\alpha$ 2 is induced and mediates increased H-secretion under K-depleted conditions. To better understand the role of H-ATPase in acid secretion and the relationship between H-ATPase (K-independent) and a specific HKA isoform, we examined H-ATPase activity in the HKA $\alpha$ 1 knockout (KO) mice under normal and K-depleted conditions. Mice were fed a K-free diet and studied after 7 days. The OMCD were perfused in vitro and intracellular pH (pHi) was measured by using the pH-sensitive indicator BCECF-AM. The isolated OMCD tubules obtained from mice fed a K-free diet were examined by fluorescent immunocytochemistry with an antibody to the 31-kDa subunit of H-ATPase and were compared with those obtained from a normal diet. In the absence of Na and K, the H-ATPase-mediate pHi recovery rates were  $6.7 \pm 1.1 \times 10^{-4}$  units/sec (n=7 ICs) in wild type (WT) mice and increased to  $8.7 \pm 1.8 \times 10^{-4}$  ( $p < 0.05$ ; n=6) in KO mice. K-independent proton transport activity was significantly inhibited by the H-ATPase inhibitor bafilomycin A1 (BAF, 10 nM) with luminal applied in both WT and KO mice. Comparison of the results indicated upregulation of BAF-sensitive H-ATPase activity in KO mice. To determine the intracellular localization of H-ATPase in the ICs of the OMCD, we dissected the OMCD and performed the immunocytochemistry with the H-ATPase antibody in the WT and KO mice. In the WT mice on normal diet, H-ATPase staining distributed diffusely throughout the ICs and was slightly polarized to the apical plasma membrane in the KO mice, consistent with increase in the H-ATPase-mediate pHi recovery in the KO mice. One week of a K-free diet resulted in a significant increase in the degree of H-ATPase polarization at the apical plasma membrane in both WT and KO mice. In conclusion, Hypokalemia stimulates H-ATPase in the ICs of OMCD of both WT and KO mice. The enhanced activity of H-ATPase plays an important role in compensatory proton secretion in the KO mice under normal conditions.

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### PHOSPHOINOSITIDE 3-KINASE STRIKES AGAIN; ENHANCED ARTERIAL CONTRACTION AND TONE IN SPONTANEOUSLY HYPERTENSIVE RATS

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Phosphoinositide-3-kinase (PI3-K) has been implicated in mediation of arterial spontaneous tone and enhanced contraction, findings observed in arteries from experimentally-based hypertensive but not normotensive subjects and which contribute to changes in total peripheral resistance. As hypertension is largely genetically-based, we tested the hypothesis that a similar change occurs in genetically hypertensive animals. Aorta from Spontaneously Hypertensive rats (SHR) (systolic blood pressure =  $175 \pm 9$  mm Hg) and Wistar Kyoto rats (WKY) ( $114 \pm 3$  mm Hg) were isolated for measurement of isometric contractile force and biochemical expression of the p110 catalytic and p85 regulatory subunits of PI3-K. Aorta from SHR displayed variable increases in spontaneous tone; WKY aorta displayed no tone. The PI3-K inhibitor LY294002 (20  $\mu$ mol/L) reduced basal tone in SHR aorta (figure;  $20 \pm 7.3$  % initial phenylephrine contraction) greater than in WKY ( $2 \pm 0.2$  %) and normalized the increased potency of NE (-log EC50 [M] Vehicle: WKY =  $7.5 \pm 0.1$  vs. SHR =  $7.8 \pm 0.1$ ; LY294002: WKY =  $7.0 \pm 0.1$  vs. SHR  $7.0 \pm 0.1$ ). Western analyses revealed no differences in expression of the regulatory p85 $\alpha$  or catalytic p110 $\alpha$  subunits between WKY and SHR; p110 $\gamma$  was not detected. By contrast, p110 $\delta$  expression was increased by over 30% in aorta from SHR compared to WKY ( $827.6 \pm 88.5$  vs.  $576.8 \pm 53.4$  arbitrary densitometry units). These data underscore the relevance of an enzyme historically classified as one committed to growth to modifying contractility. Moreover, they support a clear involvement of this enzyme in genetically-based hypertension.



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### Regulation of membrane-bound matrix metalloproteinases and integrins in monocytes/macrophages by proprotein convertases

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Inflammatory mechanisms contribute to vascular remodeling. Monocyte/macrophage functions are partly controlled by the ECM-degrading MT1-MMP and the ECM-linking  $\alpha$ v integrin. Both are synthesized as larger precursor proproteins, which are activated by endoproteolytic cleavage. This type of activation requires the presence of proprotein convertases (PCs), namely furin and PC5. This study investigated the cellular expression pattern of furin and PC5 in human arteriosclerotic lesions as well as their function in monocytes/macrophages. Immunohistochemistry of different arteriosclerosis stages (human femoral arteries; n = 15) revealed the expression of furin and PC5 in VSMCs, endothelial cells and macrophages/foam cells. Whereas little furin and PC5 were found in early stages (Stary I-III), their expression was increased in advanced lesions (Stary > IV) and prominent staining of furin and PC5 was localized to foam cells/macrophages. Furin and PC5 colocalized with their putative substrates MT1-MMP and  $\alpha$ v in macrophages. To investigate the function of furin/PC5 in macrophages, monocytic THP-1 cells were differentiated to THP-1/macrophages by PMA stimulation (100 nM; 48h). FACS analysis and immunoblotting demonstrated, that differentiation to macrophages was accompanied by increased expression of  $\alpha$ v and MT1-MMP as well as their activating enzymes furin and PC5. Inhibition of furin/PC5 with the specific pharmacological inhibitor dec-CMK inhibited  $\alpha$ v integrin endoproteolytic activation, as well as MT1-MMP activation, but had no significant effect on their cell surface expression, indicating that sorting and routing occurs independently. No significant MMP-2 levels were detected in THP-1 monocytes/macrophages, but inhibition of PCs and thus consequently inhibiting MT1-MMP activation, inhibited MT1-MMP-dependent activation of exogenous MMP-2, as found by gelatin zymography. In conclusion, the present study demonstrates, that furin and PC5 are expressed in monocytes/macrophages in the advanced arteriosclerotic plaque, where they colocalize with MT1-MMP and  $\alpha$ v integrin. These PCs are required for  $\alpha$ v and MT1-MMP activation in macrophages and are thus potential targets in the vascular remodeling process.

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### Reduced Aortic but not Venous Contraction to Big Endothelin-1 (ET-1) in Deoxycorticosterone Acetate (DOCA) -salt Hypertension: the Role of Endothelin Converting Enzyme (ECE)

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In DOCA-salt hypertension, aortic but not venous tissue ET-1 levels are elevated. Aortae from DOCA-salt hypertensive rats display reduced ET-1-induced contraction, while thoracic vena cae do not. One historical report suggests that ECE, a metalloprotease that cleaves big ET-1 to ET-1, is functional in rabbit saphenous artery but not saphenous vein, indicating an alternate route for ET-1 synthesis in veins. We challenge this hypothesis by proposing that ECE is present and functional in both thoracic aorta and vena cava from normotensive sham and DOCA-salt hypertensive rats. Immunohistochemically, we detected ECE on adventitial and endothelial cells of thoracic aorta from sham and DOCA-salt rats, and throughout the vessel wall of thoracic vena cava from sham and DOCA-salt rats. ET-1 staining was present throughout sham aorta and vena cava, suggesting ET-1 is synthesized via ECE in these tissues. We examined the effects of two ECE/neutral endopeptidase inhibitors, phosphoramidon and CGS-26393 (CGS), on big ET-1-induced contraction in sham aorta and vena cava. Phosphoramidon (10  $\mu$ M) significantly reduced maximal aortic big ET-1-induced contraction [control: 48.6 $\pm$ 16.0% of initial phenylephrine (PE, 10  $\mu$ M) contraction; phosphoramidon: 20.1 $\pm$ 9.6%], and venous contraction [control: 478.1 $\pm$ 61.5% of initial norepinephrine (NE, 10  $\mu$ M) contraction; phosphoramidon: 176.5 $\pm$ 35.2%]. CGS (10  $\mu$ M) significantly reduced maximal aortic big ET-1-induced contraction (control: 83.9 $\pm$ 2.9%; CGS: 21.5 $\pm$ 1.6%), but not maximal venous contraction (control: 349.6 $\pm$ 43.9%; CGS: 281.5 $\pm$ 53.4%). We compared big ET-1-induced contraction of aorta and vena cava from sham and DOCA-salt rats. Maximal big ET-1 (100 nM)-induced contraction was significantly reduced in aorta from DOCA-salt rats [DOCA: 17.7 $\pm$ 5.3% of initial PE contraction; sham: 60.7 $\pm$ 17.8%], but not vena cava from DOCA-salt rats [DOCA: 479.1 $\pm$ 51.3% of initial NE contraction; sham: 478.1 $\pm$ 61.5%], suggesting that aorta from DOCA-salt rats are desensitized to big ET-1, while vena cava are not. Our findings suggest that ECE is present in aorta and vena cava, and that differences in ET-1 synthesis between aorta and vena cava of hypertensive rats are not caused by different ECE activity between the two vessel types.

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### Endothelium-dependent responses mediated by cyclic GMP in rat aorta

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The intracellular mechanism most commonly described as mediating the actions of nitric oxide (NO) in vascular smooth muscle cells involves the production of the second messenger 3', 5'-cyclic guanosine monophosphate (cGMP), following NO activation of soluble guanylyl cyclase (sGC). It has been shown that endothelium-intact vascular segments have a higher level of cGMP than denuded preparations, although little attention has been given to endothelium-dependent relaxation induced by this second messenger. This work tested the hypothesis that the endothelium contributes to cGMP-induced relaxation in a positive feedback way. Concentration-effect curves (CEC) stimulated with endothelium-intact rat aorta (100  $\mu$ m) were

2'furyl)-1-benzylindazole (YC-1) (1 nmol/L to 30  $\mu$ mol/L) or with exogenous cGMP (8-Br-cGMP; 10 nmol/L to 0.3 mol/L) were constructed in rat aortic rings precontracted with phenylephrine (PE: 0.1 or 0.3  $\mu$ mol/L) in the absence and in the presence of endothelium. The magnitude of contraction induced by PE was not different between intact or denuded segments. YC-1 was more effective in relaxing intact ( $pD_2=6.5 \pm 0.10$ ) than denuded ( $pD_2=5.8 \pm 0.05$ ) aortic rings. 8-Br-cGMP-induced relaxation was impaired in endothelium-denuded aortic rings or in intact arterial segments preincubated with the inhibitors of NO synthase or sGC. The relaxation induced by 8-Br-cGMP in the presence of the sGC inhibitor was not different between intact and denuded aortic rings. These data support the hypothesis that endothelium facilitates cGMP-induced relaxation in rat aorta. This may be due to cGMP inducing NO release, coupled with an increase in cGMP production followed by subsequent increase in smooth muscle relaxation. Interestingly, these data provide evidence of a positive feedback mechanism for the vasodilation induced by the NO/cGMP pathway mediated by the endothelium.

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### Paracrine Role of Periadventitial Fat on Mesenteric Vascular Function in Spontaneously Hypertensive Rats

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Perivascular adipose tissue induces vasorelaxation by opening of 4-aminopyridine (4-AP)-sensitive  $K^+$  channels in normotensive rats. We characterized the mesenteric perivascular adipose tissue from 3-month-old Wistar Kyoto (WKY) and aged-matched spontaneously hypertensive rats (SHR) and tested the hypothesis that this tissue contributes to differences in vascular resistance in hypertension. Mesenteric bed (MB) weight and MB total lipid content were lower in SHR than in WKY rats. Freshly isolated MB adipocytes were smaller in size but not in number between strains. Plasma triglycerides, glycerol, non-esterified free-fatty acids, cholesterol, and leptin were also lower in SHR than in WKY rats. To study vascular function, the MB was cannulated and perfused at a constant flow of 2 ml/min. 4-AP (2 mM) induced a smaller increase in perfusion pressure in MB of SHR rats, compared to WKY rats. In isolated mesenteric artery rings, 4-AP (2 mM) induced a contractile effect that was significantly reduced in SHR, compared to WKY rats. The anti-contractile effects of perivascular fat were smaller in mesenteric artery rings from SHR rats than from WKY rats. These results suggest that the lower content of perivascular adipose tissue in SHR might contribute to the increased vascular resistance in this strain and support a paracrine role for periadventitial fat in vascular tone regulation. Supported by MCyT (BFI 2001-0638) and DFG Go766/8-1.

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### The role of the vanilloid receptor in hypertension

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We have recently demonstrated that endocannabinoids acting at cannabinoid-1 receptors (CB1) decrease blood pressure and myocardial contractility in hypertension. In addition to its CB1 agonist properties, the endocannabinoid anandamide also activates vanilloid TRPV1 receptors on sensory nerve terminals, triggering the release of calcitonin-gene related peptide that elicits vasorelaxation in isolated blood vessels in vitro. However, the contribution of TRPV1 receptors to the in vivo hypotensive effect of endocannabinoids is equivocal. In this study, we analyzed in detail the hemodynamic effects of anandamide using the Millar pressure-volume conductance catheter system in anesthetized TRPV1 knockout (TRPV1 $^{-/-}$ ) mice and their wild-type (TRPV1 $^{+/+}$ ) littermates. Baseline cardiovascular parameters as well as systolic and diastolic function at different preloads were similar in TRPV1 $^{-/-}$  and TRPV1 $^{+/+}$  mice. The predominant hypotensive response to bolus intravenous injections of anandamide and the associated decrease in cardiac contractility and total peripheral resistance (TPR) were similar in TRPV1 $^{+/+}$  and TRPV1 $^{-/-}$  mice, as was the ability of the CB1 antagonist, SR141716 to completely block these effects. In TRPV1 $^{+/+}$  mice, this hypotensive response was preceded by a transient, profound drop in cardiac contractility and heart rate and increase in TPR, followed by a brief pressor response, which were unaffected by SR141716 and were absent in TRPV1 $^{-/-}$  mice. In spontaneously hypertensive rats (SHR), the hypotensive effect of anandamide is completely blocked by SR141716, which does not affect TRPV1-mediated vasodilation by capsaicin. Furthermore, the TRPV1 antagonist capsazepine failed to influence the hypotensive response to anandamide in both hypertensive and normotensive rats. These results indicate that mice lacking TRPV1 receptors have a normal cardiovascular profile and their predominant cardiovascular depressor response to anandamide is mediated through CB1 receptors. The role of TRPV1 receptors may be limited to the transient activation of cardiogenic sympathetic reflex by very high plasma concentrations of anandamide and these receptors are not likely involved in the antihypertensive effects of endocannabinoids.

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### Protective effect of Angiotensin II receptor blocker (ARB) in the progression of peritoneal damage in hypertensive rats on peritoneal dialysis

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Although the effect of ARBs has been studied, less extensively in hypertensive patients undergoing dialysis. In the present study, to evaluate the effect of angiotensin type 1 receptor blocker (ARB) on the progression of peritoneal fibrosis, we examined the effect of ARB,

CS866, in a model of peritoneal fibrosis in hypertensive rat. Thirty two male wistar rats with 2 kidney 1 clip renovascular hypertension (2K1C-RVH) were divided into four groups and dialyzed with various solutions for 40 days as follows: (1) pH3.5 DS 10ml (pH3.5 containing 1.35% glucose, n=8), (2) pH3.5 DS 10ml+ARB (olmesartan 5mg/kg/day, n=8), (3) pH3.5 DS 10ml (n=6)+Ca blocker (amlodipine 3mg/kg/day, n=8) and (4) pH7.0 DS 10ml (n=8). 40 days later, all rats were decapitated. Expression of mRNA of ACE, AT1, AQP and glucose transporters (GLTs) were studied by RT-PCR. ARB and amlodipine treatment induced the significant reduction of blood pressure in RVH. 2K1C-RVH induced significant elevation of the expression of ACE- and AT1-mRNA in the peritoneum. In pH3.5 DS treated rats, necropsy findings show evidence of peritoneal sclerosis. The typical appearance was multiple surfaces covered with granulation tissue or fibrosis tissue or both. Multiple adhesions were present. Microscopic findings revealed that low pH DS induced peritoneal fibrosis and loss of mesothelium. AQP1, AQP4, GLT1, and GLT4 mRNA were expressed in peritoneum. The expression of AQPs was significantly suppressed and GLTs were significantly enhanced in pH3.5 DS treated rats. ARB treatment prevented the progression of peritoneal fibrosis and adhesion of peritoneum. In pH7.0 DS treated rats, there was no signs of EPS. Peritoneal function test by using PET was maintained in ARB treated rats. From these data, we concluded that acidic DS induced the development of peritoneal fibrosis. Activation of R-A system aggravates the peritoneal fibrosis. On the contrary, ARB treatment protects the peritoneum from the progression of peritoneal damage undergoing peritoneal dialysis.

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WITHDRAWN

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### Design And Implementation Of A Multi-Species Hypertension Gene Array

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We are currently performing studies on salt-sensitive hypertension in both a rodent model and primate model. In order to perform parallel gene expression studies, we have designed and constructed a hypertension-related gene array composed of oligonucleotide (oligo) targets for each gene that are compatible with both rat and baboon. These targets are also compatible with mouse and human genes. The multi-species hypertension gene array contains 310 genes relevant to hypertension and/or sodium management and 122 genes that show two fold or more change in baboon renal RNA in response to dietary salt. The array also contains 18 positive control genes that show consistent expression in baboons with high and low RBC sodium-lithium countertransport and high and low dietary salt. Ten Arabidopsis genes were included as negative controls. Each gene on the array is represented by duplicate 65-mer oligos. In addition, each array was designed to align to rat and human with a minimum sequence identity of 92%. To do so, oligos were designed from each human gene's coding region. The human oligo was then aligned with the homologous rat gene coding sequence to ensure 92% or greater sequence identity. To ensure specificity of each oligo for the target gene, oligos were then subjected to BLAT analysis. If sequence homology greater than 85% existed between the target gene's oligo and other genes, then the oligo was rejected. This cutoff was based on data that show baboon gene sequences contain 92 to 100% sequence identity with human gene coding regions and that previous work in our laboratory demonstrate the ability to discriminate between 89% and 90% probe to target sequence identity by hybridization wash temperatures. Using this multi-species array, we have identified genes expressed in baboon cortex and medulla. In addition, we have compared renal cortex and medulla gene expression in sham and DOCA treated rats. In comparing renal cortical gene expression in three sham and three DOCA rats, differentially expressed genes include a glutathione transferase and prostasin. Prostasin, a serine peptidase previously shown to regulate sodium channel activity and to be upregulated by aldosterone, is upregulated in DOCA versus sham (70.99 +/- 8.58 versus 24.98 +/- 13.15, p = 0.043).

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### Apelin and Myocardial Stretch

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Apelin (A), in a molar basis, is the most potent inotropic agent known and exhibits high levels of mRNA expression in the heart. Although A and angiotensin II (Ang II) as well their corresponding receptors, APJ and AT<sub>1</sub>, share identity the possible physiological role of A following myocardial stretch has not been established. This is potentially important since A-like immunoreactivity is present in endocardial endothelial cells suggesting that the peptide may play a role as a local mediator following the shear stress supported by endothelial cells after stretch. We studied the effect of myocardial stretch on A expression in cat papillary muscles (pm) paced at 0.2 Hz. The pm were randomly assigned to: a) 15 minutes stretch (S); b) 15 minutes stretch under AT<sub>1</sub> receptor blockade with 1 μmol/L losartan (SL) or c) no stretch control (C). At the end of each protocol, total RNA was isolated and A mRNA was quantified by real time RT-PCR. Myocardial stretch significantly upregulated the expression of A as indicated by the abundance of its specific mRNA (167.34 ± 17.63% vs. 100 ± 20.1% in C; P < 0.05, n = 4 each). AT<sub>1</sub> receptors blockade with losartan abolished the increase in A mRNA induced by stretch (96.15 ± 28.30%; n = 4). In summary myocardial stretch induced within 15 minutes a significant increase in the expression of A, probably as a result of stretch-induced Ang II secretion. This upregulation of A was not observed in unstimulated pm. <http://www.physiology.org/journal/ajp-hypertension>

preliminary results suggest for the first time a novel role, not yet clarified, for A in the chain of events triggered by myocardial stretch.

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### RhoA/Rho-kinase dependent Ca<sup>2+</sup> sensitization is modulated by age: RGS domain containing RhoGEFs is the culprit?

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Signaling via G protein coupled receptors (GPCR) plays a critical role in the regulation of vascular tone. It is established that both calcium/calmodulin pathway and RhoA/Rho-kinase pathway are able to mediate vasoconstriction by ligand binding to GPCR. In addition to vasoconstriction, both signaling pathways mediate other vascular responses to vasoactive agents. For example, the migration of smooth muscle cells induced by vasoactive agents is mediated by calcium/calmodulin pathway, but inhibited by RhoA/Rho-kinase pathway. The calcium/calmodulin pathway may lead to apoptosis of smooth muscle cells, whereas RhoA/Rho-kinase pathway inhibit pharmacologically induced apoptosis of smooth muscle cells. Rho specific guanine exchange factors (RhoGEFs) are the critical mediators of RhoA activation. Three RhoGEFs, known as regulators of G-protein signalling (RGS) domain containing RhoGEFs, were demonstrated to directly link RhoA to GPCR, which include p115RhoGEF, LARG and PDZ-RhoGEF. Here, we examined the effect of age on the expression of these RGS domain containing RhoGEFs in aorta from 4-week-old and 18-week-old wistar-kyoto (WKY) rats. Results showed that the mRNA expression level of all three RGS domain containing RhoGEFs in aorta from 4-week-old WKY rats was higher than that from 18-week-old WKY rats. Then we investigated the relaxation dose-response curve to Y-27632, a Rho-kinase inhibitor, from phenylephrine (PE)-induced maximum contraction. The maximum contraction by 10 μM PE was different in those animals (13.3 ± 0.58 mN and 21.4 ± 1.33mN, 4-week-old and 18-week-old respectively; n=4). As expected, Y-27632 caused more relaxation in 4-week-old group (95.63%, 79.22% and 23.38% of PE<sub>MAX</sub> at the presence of 1 μM, 4 μM and 16 μM Y-27632, respectively), when compared to 18-week-old (100%, 94.18% and 68.07% of PE<sub>MAX</sub> at the present of 1 μM, 4 μM and 16 μM Y-27632, respectively), suggesting that RhoA/Rho-kinase pathway play a more significant role in response to vasoconstrictors in young animals. In conclusion, the present study supports the hypothesis that signaling pathway downstream from GPCR is modulated by age, and provides an insight into the relationship between age and vascular diseases.

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### Swimming Training Induces Increase of Angiotensin (1-7) and Mas Receptor Expression in the Left Ventricle of SHR

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It has been reported that swimming training improves heart function. However, it is not known if this effect is related to changes in RAS in plasma and/or the heart. We aimed to investigate whether the effects of physical training are associated with changes in the cardioprotective peptide Ang-(1-7) and its receptor Mas. Normotensive Wistar rats and SHR were divided in four groups: normotensive sedentary (NOR S), normotensive trained (NOR T), SHR sedentary (SHR S), and SHR trained (SHR T). The period of 5% overload swimming training was 8 weeks. Blood pressure was measured once a week by tail plethysmography. Angiotensins were measured by RIA. The expression of Ang-(1-7) receptor Mas was evaluated by RT-PCR and immunofluorescence using a polyclonal anti-body. The physical training did not reduce tail blood pressure at rest. In NOR rats the physical training induced an increase in the cardiomyocytes transversal axis (NOR S: 14.83 ± 0.38 μm vs NOR T: 16.72 ± 0.54 μm, p < 0.05) and a significant increase in the left ventricle (LV) body weight ratio (NOR S: 0.20 ± 0.03 vs NOR T: 0.21 ± 0.03, p < 0.05). In the SHR group training also induced an increase in the LV body weight ratio (SHR S 0.30 ± 0.06 vs SHR T: 0.32 ± 0.04, p < 0.05). A significant decrease of plasma Ang II levels in both groups was observed (NOR S: 77.52 ± 7.78 pg/ml vs NOR T: 50.75 ± 5.93 pg/ml, p < 0.05 and SHR S: 59.90 ± 6.38 pg/ml vs SHR T: 44.13 ± 4.82 pg/ml, p < 0.05). Plasma Ang-(1-7) did not change. Strikingly, swimming training in SHR induced a 2-fold increase in Ang-(1-7) concentration in the LV (SHR S: 1.16 ± 0.09 pg/mg protein vs SHR T: 2.76 ± 0.37 pg/mg protein, p < 0.05). In addition the mRNA expression and immunofluorescence staining for the Ang-(1-7) receptor Mas in the LV were substantially increased in trained SHR. Only slight, non significant, changes in Ang-(1-7) concentration or AT<sub>1-7</sub> receptor expression were observed in trained normotensive rats. LV Ang II concentration in LV did not change in both groups. These results show that swimming training produces significant changes in the tissue and plasma profile of angiotensin peptides. These results suggest that changes in Ang-(1-7) and its receptor may participate in the cardiac effects of swimming training in SHR.

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### Silencing of p21-activated kinase (PAK) attenuates phosphorylation and reorientation of the vimentin network during serotonin (5-HT) stimulation of smooth muscle cells

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The intermediate filament protein vimentin has been implicated in affecting tension development in non-muscle cells and smooth muscle. However, how vimentin filaments affect tension development is currently unknown. We hypothesize that agonist stimulation may induce phosphorylation and spatial reorganization of vimentin intermediate filaments, which may facilitate force transmission in smooth muscle. Cultured canine tracheal smooth muscle cells were stimulated with 5-HT and stained for vimentin. The vimentin network exhibited a curved filamentous structure in unstimulated smooth muscle cells. Vimentin filaments became straight, and arranged along the long axis of cells upon stimulation with 5-HT. Using

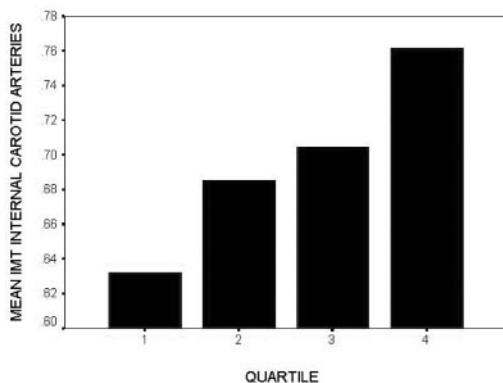
triggered vimentin phosphorylation on ser-56. We also hypothesize that PAK may regulate vimentin filaments in smooth muscle. Treatment of cells with siRNA selectively downregulated the expression of PAK1 without affecting the content of smooth muscle  $\alpha$ -actin. The silencing of PAK1 inhibited the site-specific phosphorylation and spatial rearrangement of the vimentin network in response to 5-HT stimulation. Neither the disruption of stress fibers by cytochalasin D nor the inhibition of protein tyrosine phosphorylation affects the spatial reorganization of vimentin intermediate filaments in response to 5-HT stimulation. In addition, stimulation of smooth muscle cells with 5-HT increased the ratio of soluble vimentin to insoluble vimentin. PAK1 silencing attenuated increases in the ratio of soluble vimentin to insoluble vimentin upon 5-HT stimulation. These results suggest that the PAK-mediated vimentin site-specific phosphorylation may play a role in regulating the reorganization of vimentin intermediate filaments during 5-HT stimulation of smooth muscle cells. The reorganization of vimentin filaments may be important for mechanical force transmission during contractile activation of smooth muscle. Supported by HL75388, AHASDG, and Indiana Showalter Foundation.

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#### IMPACT OF OBESITY IN INTIMA MEDIA THICKNESS OF CAROTID ARTERIES

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The purpose of the present study was to explore differences in intima media thickness (IMT) of carotid arteries among lean, normal BMI subjects, moderate and severe obese subjects. Our population consisted of five hundred twelve subjects, who visited our hospital for a routine check up and had never been treated with antihypertensive medication. All patients underwent 24h blood pressure monitoring and ultrasonography of the carotid arteries. Body weight was measured with the subject in light clothing and without shoes. Body mass index (BMI) was calculated as weight (Kg)/height (m<sup>2</sup>). Our population was divided into quartiles according to their BMI: 1) LEAN, from the lowest value of BMI to 25% percentile, 2) NORMAL, BMI from 25% to 50%, 3) MODERATE obese, from BMI 50% to 75%, and 4) SEVERE obese group from BMI 75% to the highest value of BMI. IMT of carotid arteries was  $0.63 \pm 0.20$  mm in LEAN group,  $0.68 \pm 0.22$  in NORMAL group, and  $0.70 \pm 0.26$  in MODERATE obese group and  $0.76 \pm 0.50$  in SEVERE obese group. There was a significant difference between the lowest and the highest quartile  $P < 0.005$  (One-Way Anova Tukey post hoc tests). Mean 24h systolic/diastolic blood pressures, mean 24h heart rate, 24h pulse pressure and daytime or nighttime parameters of ambulatory blood pressure did not statistically differ among the groups. The groups also did not differ in age or sex. Mean IMT of internal carotid arteries was increased from the lowest to the higher quartile of BMI. Mean IMT was significantly higher in severe obese subjects compared to lean ones. Thus, obesity is an important factor for carotid atherosclerosis and at least some of the effects of obesity are independent of blood pressure.



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#### Elevation of Detached and Circulating Endothelial Cells is a Marker for Vascular Injury in Patients with Type 2 Diabetes Mellitus

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Patients with diabetes mellitus are well known to be at high risk for vascular disease. Circulating endothelial cells have been reported to be an *ex vivo* indicator of vascular injury. We investigated the presence of circulating endothelial cells in the peripheral blood of 25 patients with diabetes mellitus and in 9 non-diabetic control donors. Endothelial cells were isolated from peripheral blood with anti-CD-146-coated immunomagnetic Dynabeads™, and were stained with acridine orange dye and counted by fluorescence microscopy. Anti-CD-146 recognizes mature endothelial cells, but not progenitor endothelial cells. The cells were also stained for von Willibrand factor and *Ulex Europaeus* Lectin 1. Patients with diabetes mellitus had an elevated number of circulating endothelial cells (mean 54, range 35–126 cells/ml) compared to healthy controls (mean 10, range 3–18 cells/ml) ( $p < 0.01$ ). The increase in circulating endothelial cells did not correlate with the levels of HbA1C. Circulating endothelial cell numbers were elevated regardless of glucose levels, suggesting that, even with control of glucose levels, there is increased endothelial cell sloughing. There was, however, a trend toward higher numbers of circulating endothelial cells in patients with a higher body mass index (BMI). There was no correlation between the number of circulating endothelial cells and age. Similarly, plotting the number of circulating endothelial cells against gender also demonstrated no significant association. Our study suggests that the higher number of circulating endothelial cells in diabetics may reflect ongoing vascular injury, which is not directly dependant on glucose control.

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#### ANGIOTENSIN DEPENDENT AND 2K1C HYPERTENSION DO NOT CAUSE SIGNIFICANT IMPAIRED ERECTILE RESPONSE IN RATS

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It has been shown that hypertension is a risk factor contributing to erectile dysfunction in adult men. In previous studies from our group we have shown that in different models of hypertension such as SHRSP and DOCA-salt, hypertensive rats present a significant impaired erectile response when compared to their normotensive controls. The erectile response is attributed to vasodilation due to NO release. Therefore, since in some models of hypertension such as those dependent on the rennin-angiotensin system, there are evidences of increased NO synthase activity in the vasculature, we hypothesized that the erectile response in rennin/angiotensin dependent hypertension could be normal due to a compensated increased activity of NO signaling. Using continuous monitoring of corpus cavernosum pressure (ICP) and mean arterial pressure (MAP), erectile function was assessed by electrical stimulation of the major pelvic ganglion (MPG) of 14 days Angiotensin II (Ang II)-treated and 30 days two-kidney, one-clip (2K-1C) hypertensive and respective sham operated rats. A voltage curve (1–5 V) was elicited in those rats and the erectile response was measured and expressed as the ICP/MAP ratio. ICP/MAP curves obtained by increasing voltage stimulation of the MPG were not different between those hypertensive and normotensive control rats. In Ang II-treated and sham rats, the maximal ICP/MAP response was  $0.82 \pm 0.07$  and  $0.85 \pm 0.02$ , respectively. In 2K-1C and sham rats, no differences were observed in the ICP/MAP induced by 5 V stimulation of the ganglion ( $0.52 \pm 0.06$  vs  $0.54 \pm 0.05$ ). Our data strongly suggest that the lack of erectile dysfunction in Ang II-treated and 2K-1C hypertensive rats, unlike that observed in SHRSP and DOCA-salt, may be due to an increased NO synthase activity.