Role of Reactive Oxygen Species in Hyperadrenergic Hypertension

Biochemical, Physiological, and Pharmacological Evidence From Targeted Ablation of the Chromogranin A (Chga) Gene

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Background—Oxidative stress, an excessive production of reactive oxygen species (ROS) outstripping antioxidant defense mechanisms, occurs in cardiovascular pathologies, including hypertension. In the present study, we used biochemical, physiological, and pharmacological approaches to explore the role of derangements of catecholamines, ROS, and the endothelium-derived relaxing factor nitric oxide (NO) in the development of hyperadrenergic hypertension: targeted ablation (knockout [KO]) of chromogranin A (Chga) in the mouse.

Methods and Results—Homozygous (−/−) Chga gene knockout (KO) mice were compared with wild-type (WT, +/+) control mice. In the KO mouse, elevations of systolic and diastolic blood pressure were accompanied by not only elevated catecholamine (norepinephrine and epinephrine) concentrations but also increased ROS (H2O2) and isoprostane (an index of lipid peroxidation), as well as depletion of NO. Renal transcript analyses implicated changes in Nox1/2, Xo/Xdh, and Sod1,2 mRNAs in ROS elevation by the KO state. KO alterations in blood pressure, catecholamines, H2O2, isoprostane, and NO could be abrogated or even normalized (rescued) by either sympathetic outflow inhibition (with clonidine) or NADPH oxidase inhibition (with apocynin). In cultured renal podocytes, H2O2 production was substantially augmented by epinephrine (probably through β2-adrenergic receptors) and modestly diminished by norepinephrine (probably through α1-adrenergic receptors).

Conclusions—ROS appear to play a necessary role in the development of hyperadrenergic hypertension in this model, in a process mechanistically linking elevated blood pressure with catecholamine excess, renal transcriptional responses, ROS elevation, lipid peroxidation, and NO depletion. Some of the changes appear to be dependent on transcription, whereas others are immediate. The cycle could be disrupted by inhibition of either sympathetic outflow or NADPH oxidase. Because common genetic variation at the human CHGA locus alters BP, the results have implications for antihypertensive treatment as well as prevention of target-organ consequences of the disease. The results document novel pathophysiological links between the adrenergic system and oxidative stress and suggest new strategies to probe the role and actions of ROS within this setting. (Circ Cardiovasc Genet. 2010;3:414-425.)

Key Words: chromogranin A ■ hypertension ■ reactive oxygen species ■ nitric oxide

Increased sympathoadrenal activity plays a role in the development or maintenance of elevated blood pressure (BP) in both hypertensive patients and animal models of hypertension.1–4 Chromogranin A (CHGA human, Chga rodent), the index member of the chromogranin/secretogranin protein family, is costored and coreleased with catecholamines from secretory vesicles in adrenal medulla5–7 and postganglionic sympathetic axons.8 Although CHGA is over-expressed in human essential (hereditary) hypertension, the plasma concentration of its catestatin (catecholamine release-inhibitory) fragment9 is decreased in not only established cases of hypertension but also still-normotensive subjects with a family history of hypertension.10,11 Genetic variation at the human CHGA locus predicts substantial alterations in BP.12–14 Expanding on the human findings, we found that Chga knockout (KO, −/−) mice are hyperten-
sive and hyperadrenergic.\textsuperscript{15} To get better insight into the development of hypertension in KO mice, we looked at the status of reactive oxygen species (ROS) and nitric oxide (NO) in these mice.

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Oxidative stress, characterized by excessive production of ROS outstripping antioxidant defense mechanisms, has been associated with several cardiovascular pathologies, including hypertension,\textsuperscript{16,17} hypercholesterolemia, and diabetes. ROS are a family of short-lived, highly reactive byproducts of oxygen ($O_2$) metabolism. They include oxygen ions, free radicals, and peroxides, both inorganic and organic. ROS are generated by $O_2$ metabolism through NADH dehydrogenase in mitochondria as well as by specific oxidases, including NADPH oxidase (Nox), xanthine oxidase (Xdh/Xo), and arachidonic acid–metabolizing enzymes.\textsuperscript{18} ROS may promote vascular smooth muscle cell contraction and proliferation, enhancing cell death in part by depleting the endothelium-derived relaxing factor nitric oxide (NO).\textsuperscript{19}

In animal models, oxidative stress has been observed in the spontaneous (genetically) hypertensive rat,\textsuperscript{20} renovascular hypertension,\textsuperscript{21} salt-sensitive hypertension,\textsuperscript{22} and obesity-induced hypertension.\textsuperscript{23} Although its pathogenesis is complex, human hypertension also displays signs of increased oxidative stress\textsuperscript{16,17,24,25} associated with a decreased antioxidative activity and a reduced ability to scavenge oxygen-derived free radicals.\textsuperscript{26,27} Indeed, enhanced ROS accumulation may be a heritable trait in hypertensive pedigrees,\textsuperscript{17} with penetrance even before the onset of overt hypertension.\textsuperscript{16}

In the present study, we explore the involvement of ROS and NO\textsuperscript{1} in the development of a hyperadrenergic model of hereditary hypertension: the Chga-KO mouse. Our results suggest a role for catecholamine excess in generating a hypertensive state partially dependent on ROS activation and NO\textsuperscript{1} depletion, a state amenable to pharmacological correction by inhibition of either adrenergic outflow or ROS generation.

Methods

Targeted Ablation of the Chga Locus in the Mouse

Mice were studied according to a protocol approved by the Animal Subjects Committee of the University of California at San Diego, and research was conducted in accordance with institutional guidelines. Ablation of Chga gene was done as described previously.\textsuperscript{15} The homozygous ($\sim\sim$) Chga gene knockout (KO) mouse line was maintained and used for the experiments and compared with wild-type (WT, $+/+$) strain controls; 5- to 6-month-old animals were studied from each strain. Based on preliminary calculations of statistical power for BP, a threshold number of animals was included in each study, but we used as many animals as were available at each step from our local breeding colony (as many as 10 to 12) to optimize our ability to find true-positive and avoid false-negative results.

Renal Podocyte Cultures

Because we found ROS changes in urine and kidney during hyperadrenergic hypertension, we studied ROS generation by kidney podocytes as a renal cell type in contact with urine as it is generated in the Bowman space. Conditionally immortalized mouse podocytes were a gift of Peter Mundel and were cultured at 33°C in the presence of IFN-γ, as previously described\textsuperscript{28} for multipurpose purposes, in the presence of mouse γ-interferon for 7 days until the cells reached 90% confluence. Podocytes were trypsinized at this point and split at 1:5 ratio for differentiation at 37°C for 8 to 10 days without IFN-γ in DMEM containing 5.5 mmol/L glucose and 5% FCS. Differentiated podocytes were serum-starved overnight when confluent and then used for the experiments.

Biochemical Measurements: Catecholamines, $H_2O_2$, Isoprostane, Creatinine, and NO

Catecholamines

Catecholamines were measured in mouse plasma from anesthetized animals by high-performance liquid chromatography (HPLC) (Waters 600E Multisolvnet Delivery System; Waters, Milford, Mass) using an electrochemical detector (Waters 2465 Electrochemical Detector). Sample purification was done using activated alumina. The data were analyzed using Empower software from Waters, and catecholamine levels were normalized according to the recovery of internal standard 3,4-dihydroxybenzylamine, as previously described.\textsuperscript{15}

$H_2O_2$

As a quantitative index of $H_2O_2$ generation, fluorescence generated by the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; Amplex Red Hydrogen Peroxide/ Peroxidase Assay Kit; Molecular Probes [A-22188]; Invitrogen, Carlsbad, Calif) was monitored. Amplex Red reacts with hydrogen peroxide in the presence of horseradish peroxidase with 1:1 stoichiometry to form the fluorescent resorufin. Amplex Red and peroxidase in Krebs Ringer phosphate buffer (pH 7.4) were introduced into each well (podocyte or urine) in the dark. Catecholamines (epinephrine or norepinephrine) or synthetic adrenergic agonists ($\alpha_1$: phenylephrine; $\alpha_2$: clonidine; $\beta$: isoproterenol) were tested for effects on $H_2O_2$ production in podocytes. The contents of the plate were incubated at 37°C for 60 minutes before the commencement of the first cycle of measurement. Fluorescence intensity of resorufin was kinetically recorded with excitation at 544 nm and emission of 590 nm at 37°C, via fluorescent plate reader (POLARStar OPTIMA; BMG Labtech, Offenburg, Germany). Measurements were made over 10 to 20 cycles, at 19 to 67 seconds per cycle. The data are reported as the mean value from each well over the recording period, with n=6 wells per experimental condition. $H_2O_2$ levels were also measured in mouse urine and then normalized by urine creatinine values or in kidney cortex homogenate, with normalization to protein concentration in the same sample.

Nitric Oxide

Urine or plasma NO\textsuperscript{1} was measured according to manufacturer’s protocol using a colorimetric assay kit for conversion of nitrate---nitrite by nitrate reductase, followed by quantification of nitrite with the Griess reaction and absorbance at 540 nm (Cayman Chemical, Ann Arbor, Mich). Urinary NO\textsuperscript{1} levels were normalized by urine creatinine values or in kidney cortex homogenate, with normalization to protein concentration in the same sample.

Isoprostane

As an index of oxidative stress, we used the urinary content of 8-isop-PGF2α (also known as 8-epi-PGF2α, or 15-isoprostane F2α), a stable product of arachidonic acid formed on nonenzymatic oxidation. Urinary isoprostane was measured using an ELISA kit (Northwest Life Science Specialties LLC, Vancouver, Wash). The assay sensitivity was 50 pg, with a dynamic range of 0.1 to 10 ng/mL, and the average intrassay coefficient of variation for 24 replicate samples was 1.72%. Cross-reactivities were undetectable (<0.01%) for prostaglandins F2α, E2, D2, or arachidonic acid. The correlation between this ELISA method and GC/MS was $r=0.89$. Urinary isoprostane levels were normalized by urine creatinine values.

Creatinine

Diluted urine samples (0.5 mL) were mixed with 6 mL water and 1 mL saturated alkaline picrate solution. The mixture was kept for 45 minutes at 20°C, absorbance measured at 505 nm, and calculated using known standard concentrations.
Blood Pressure
BP of mice was measured by the noninvasive tail-cuff method using the BP-2000 Blood Pressure Analysis System (Visitek Systems, Apex, NC <http://www.visiteksystems.com/>), as previously described. Mice were placed into individual rodent restraint holders on a preheated specimen platform at 38°C as measured by a LCD strip thermometer. For each treatment state, BP was measured at 3 sessions daily, between 1 PM and 3 PM, for 5 consecutive days (Mon→Fri). The first 2 days (Mon/Tues) were for acclimation of mice with the BP system, whereas data from the last 3 days (Wed/Thurs/Fri) were analyzed. At each of the 3 daily sessions, BP was measured 10 times in succession (with Visitek hardware/software), and the results were averaged and saved as one measurement (with standard deviation) in a notebook computer running the BP analysis software package (Visitek Systems) via a PCMCIA data acquisition card. As per the manufacturer’s protocol, BP readings having a standard deviation of <10 mm Hg were accepted for further analysis. We thus collected 9 measurements (3 days; 3 measurements per day) at each state for each mouse. Elevations of BP were monitored over 3 weeks.

Drug Treatments

Inhibition of Sympathetic Outflow
Clonidine HCl (C7897 at >98% purity by TLC; Sigma-Aldrich, St Louis, Mo) was embedded in a clonidine-chow diet for rodents by Harlan-Teklad (Madison, Wis), beginning with 1% (wt/wt) clonidine and increasing to 3% (wt/wt) clonidine by the end of the 3-week treatment. For each treatment state, BP was measured at 8 AM, 11 AM, 2 PM, and 4 PM over 3 days (Mon/Tues/Thurs). The first 2 days were for acclimation of mice with the BP system, whereas data from the last 3 days (Wed/Thurs/Fri) were analyzed. At each of the 3 daily sessions, BP was measured 10 times in succession (with Visitek hardware/software), and the results were averaged and saved as one measurement (with standard deviation) in a notebook computer running the BP analysis software package (Visitek Systems) via a PCMCIA data acquisition card. As per the manufacturer’s protocol, BP readings having a standard deviation of <10 mm Hg were accepted for further analysis. We thus collected 9 measurements (3 days; 3 measurements per day) at each state for each mouse. Elevations of BP were monitored over 3 weeks.

Inhibition of NADPH Oxidase
Apocynin (acetoxyanilone, A10809 at 98% purity; Sigma-Aldrich) was used at 2 mmol/L in drinking water for 3 weeks.

mRNA Abundance by Real-Time RT-PCR
Total RNA was extracted from 1 kidney of each mouse, and real-time PCR was done with fluorescent reporter-tagged oligonucleotide primers on an ABI-7700 TaqMan platform (Life Technologies, Carlsbad, Calif), with normalization of data to β-actin expression. Ct (threshold cycle) is determined for both the specific target mRNA/cDNA as well as β-actin, and the difference in Ct (from target mRNA versus β-actin mRNA) is normalized to the average for that state (eg, control versus experimental) and expressed on a percent change (difference) scale.

Renal Cortex Biochemistry
Renal cortical homogenates were prepared from freshly harvested adult male kidneys (WT and KO) and assayed for H2O2 and NO, as described.29 Overall mitochondrial mass (per milligram of tissue protein) was indexed by citrate synthase activity, and electron transport chain enzymatic activity of mitochondrial complexes I, II, II/III, and IV was determined and normalized to citrate synthase activity as described.

Statistical Analyses
Results are expressed as mean±1 SEM (standard error of the mean). The reported “n” refers to the number of mice rather than to the number of BP measurements taken (because, under each condition, BP was measured multiple times in each mouse and then averaged for that mouse). Data were evaluated by ANOVA, followed by post hoc tests, in Excel (Microsoft, Bellevue, Wash), Kaleidagraph (Synergy Software, Reading, Pa), or SPSS-17 (Chicago, Ill). MANOVA (multivariate ANOVA) was used to test the effect of strain on more than 1 dependent variable. Repeated measures (eg, multiple BP values for each animal at each treatment state) were approached by linear mixed effect models (MIXED) in SPSS-17 to account for correlated data, factoring also for treatment. Probability values <0.05 were considered statistically significant.

Results

Chga Ablation on H2O2 and Lipid Peroxidation (Isoprostane) in Urine and Kidney
Because increased ROS activity generates H2O2, with resulting lipid peroxidation (isoprostane),31 we measured H2O2 and isoprostane renal excretion as well as H2O2 in kidney. Chga ablation caused significant increases in both urinary H2O2 and isoprostane excretions (Figure 1A). There was a 20% increase in urinary isoprostane excretion in KO mice (10.33±0.51 versus 12.86±0.61 ng/mg creatinine, P<0.02) (Figure 1A), whereas urinary H2O2 excretion was 2.5 times higher in the KO mice (1851.6±256.6 versus 4574.7±306.6 fluorescence units/mg creatinine, P<0.002) (Figure 1A). MANOVA confirmed the simultaneous effect of mouse strain on the 2 oxidative traits (P<8.55E-06). In renal cortex, H2O2 was elevated by ~45% in the KO (online-only Data Supplement Figure 1).

Catecholamines on H2O2 in Cultured Glomerular Podocytes
Epinephrine caused a substantial increase (P<0.001) in H2O2 production, whereas norepinephrine caused a modest decrease (also P<0.001) (Figure 1B). When adrenergic receptor type-specific agonists were tested (Figure 1C), both the α1 agonist phenylephrine (P<0.001) and the α2 agonist clonidine reduced H2O2, whereas the β agonist isoproterenol substantially increased H2O2 (P<0.001). Because epinephrine displays preferential activity at β2 receptors, epinephrine probably increases H2O2 production via β2 activation; because norepinephrine displays preferential activity at α1 over α2 receptors, norepinephrine probably inhibits H2O2 production via α1 activation.

Sympathetic Outflow Inhibition (Clonidine) on BP
Chga ablation caused substantial elevations of both systolic BP (SBP) (P<0.0001) and diastolic BP (DBP) (P<0.003) in KO mice (Figure 2). Three weeks of sympathoinhibitory treatment with the α2-adrenergic agonist clonidine caused significant reductions of both SBP (by ~9.1 mm Hg; from 139.3±1.8 to 130.2±2.7 mm Hg, P<0.02) and DBP (by ~9.9 mm Hg; from 101.1±2.6 to 91.2±3.3 mm Hg, P<0.03) in the KO mice (Figure 2), and the effects on DBP virtually normalized the trait.

Nox Inhibition (Apocynin) on BP
After treatment of the KO with the NADPH oxidase inhibitor apocynin for 3 weeks, SBP was reduced by ~10.9 mm Hg (from 139.3±1.8 to 128.4±2.0 mm Hg, P<0.001), whereas DBP fell by ~11.2 mm Hg (from 101.1±2.6 to 89.9±2.5 mm Hg, P<0.007) (Figure 2), and the effects on DBP virtually normalized the trait.
BP Repeated Measures

At each state (strain, treatment), BP was analyzed from 9 sets of measurements in each animal (3 days, 3 times per day). To account statistically for correlated values during repeated BP determinations, we also used a linear mixed-effect model, specifying random effect per mouse. Treatment effects on SBP (F_H11005/40.3, P_H11005/2.8E-11) and DBP (F_H11005/11.9, P_H11005/1.7E-7) remained significant. Repeated-measures ANOVA also yielded significant effects for treatment regimens.

Renal mRNA Expression

Nox mRNA Abundance in Kidney

Relative abundance of all isoforms of Nox mRNA were examined by real-time PCR and normalized to beta-actin. In KO mice, Nox1 and Nox2 were significantly overexpressed by ~4.7-fold and ~0.8-fold, respectively, compared with WT mice (Figure 3A). There were no significant differences in Nox3 or Nox4 mRNA abundance, whereas p22Phox (Cyba) was actually reduced in the KO by ~40% (P=0.001) (Figure 3A).

Other RedOx Enzymes

Xdh/Xo expression was also augmented by ~0.7-fold (P=0.03) in the KO (Figure 3B). Sod1 was reduced in the KO by ~30% (P=0.009), as was Sod2 (by ~40%, P<0.0001), though not Sod3 (P=0.207) (Figure 3B).

NO Synthases (Nos Isoforms)

Nos3 (eNos) was increased by ~0.6-fold (P=0.0154) in the KO; Nos1 (nNos) was increased marginally (P=0.073), whereas Nos2 (bNos) was unchanged (P=0.929) (Figure 3C).
Response of ROS to Drug Treatment: Clonidine or Apocynin

ROS were reduced significantly in KO mice either by sympathetic inhibition (α₂-agonist: clonidine) or NADPH oxidase blockade (Nox inhibitor: apocynin).

**Clonidine**

Treatment with clonidine for 3 weeks reduced urinary H₂O₂ excretion significantly in KO mice (from 4574.7±306.6 to 3023.4±400.4 fluorescence units/mg creatinine, P<0.02), a value comparable to the WT level (Figure 4A). 

**Apocynin**

Three weeks of apocynin also corrected the elevated urinary H₂O₂ excretion in KO mice (from 4574.4±306.6 to 2389.2±376.7 fluorescence units/mg creatinine, P<0.005), once again comparable to the WT level (Figure 4A). In KO kidney cortex, apocynin decreased H₂O₂ by ∼31% (online-only Data Supplement Figure 1).

Response of Lipid Peroxidation (Isoprostane) to Drug Treatment: Clonidine or Apocynin

Elevated ROS may activate lipid peroxidation, indexed by isoprostane from arachidonic acid. Formation of isoprostane was reduced significantly in KO mice either by sympathetic inhibition (α₂ agonist: clonidine) or NADPH oxidase blockade (Nox inhibitor: apocynin).

**Clonidine**

Three weeks of oral clonidine reduced urine isoprostane excretion significantly in KO mice (from 12.86±0.61 to 9.78±0.99 ng/mg creatinine, P<0.04), which is comparable to WT (Figure 4B).

**Apocynin**

Treatment with apocynin for 3 weeks also reduced urinary isoprostane excretion in KO mice (from 12.86±0.61 to 7.81±0.81 ng/mg creatinine, P<0.01), a final value even lower than that seen in WT mice (10.33±0.51 versus 7.81±0.81 ng/mg creatinine, P<0.04) (Figure 4B).

Adrenergic Overactivity: Response to Drug Treatment by Clonidine or Apocynin

In KO mice, circulating catecholamines were significantly higher than in WT (norepinephrine: 4.22±0.50 versus 2.21±0.33 ng/mL, P<0.006; epinephrine: 1.19±0.07 versus 0.81±0.08 ng/mL, P<0.005) (Figure 4C).

**Clonidine**

Treatment with clonidine corrected the elevated catecholamine levels in KO mice (norepinephrine: from 4.22±0.50 to 2.62±0.45 ng/mL, P<0.004; epinephrine: from 1.19±0.07 to 0.90±0.10 ng/mL, P<0.04), thus returning to levels that are comparable to WT (Figure 4C).

**Apocynin**

Three weeks of apocynin also reduced catecholamine levels in KO mice (norepinephrine: from 4.22±0.50 to 2.01±0.42 ng/mL, P<0.01; epinephrine: from 1.19±0.07 to 0.86±0.12 ng/mL, P<0.04), once again achieving levels that are comparable to the WT (Figure 4C).

NO Depletion: Response to Drug Treatment by Clonidine or Apocynin

In KO mice, urine NO excretion was reduced by ∼50% as compared with WT (3273±193 versus 1546±146 μmol/mg creatinine, P<0.001) (Figure 4D). In kidney cortex, NO was reduced by ∼33% in the KO (online-only Data Supplement Figure 1). Likewise, KO mice also displayed a reduction of circulating (plasma) NO by ∼23% (from 24.8±2.0 to 19.1±1.5 nmol/mL, P<0.036) (Figure 4E).

**Clonidine**

Clonidine “rescued” the NO depletion in KO mice (renal excretion: from 1546±146 to 3231±416 μmol/mg creat-
nine, \( P < 0.002 \), which is comparable with WT (Figure 4D). Likewise, clonidine increased circulating NO (from 19.1±1.5 to 28.3±2.7 nmol/mL, \( P < 0.01 \)), and the resulting level of NO in plasma after clonidine treatment of KO mice was comparable to WT (Figure 4E).

**Apocynin**

After apocynin treatment in KO mice, NO renal excretion increased (from 1546±146 to 2199±260 \( \mu \text{mol/mg creatinine}, P < 0.039 \)) to WT level (Figure 4D). In KO kidney cortex, apocynin increased NO by \( \approx 46\% \) (online-only Data Supplement Figure 1). Likewise, circulating NO concentration increased (from 19.1±1.5 to 26.2±2.6 nmol/mL, \( P < 0.03 \)) back to the WT level (Figure 4E).

**Mitochondria**

There was a \( \approx 28\% \) decline in mitochondrial complex I activity in the KO animals (normalized to citrate synthase activity, from 318±24 to 230±10 \( \text{U/ citrate synthase unit}, P < 0.03 \)), but no changes in the activities of complex II, complex II–III, or complex IV. Overall mitochondrial mass,
Figure 4. A, Oxygen radicals (H$_2$O$_2$): Response to treatment by sympathetic inhibition or NADPH oxidase blockade. Urine H$_2$O$_2$ level (amplex red fluorescence/mg creatinine) is presented in WT (n=8), KO (n=8), KO+clonidine (n=8), or KO+apocynin (n=8) mice. Results are shown as mean±SEM.

B, Lipid peroxidation (isoprostane): Response to treatment by sympathetic inhibition or Nox blockade. Urine isoprostane level (ng/mg creatinine) is presented in WT (n=8), KO (n=8), KO+clonidine (n=8), or KO+apocynin (n=8) mice. Results are shown as mean±SEM.

C, Catecholamine secretion: Response to treatment by sympathetic inhibition or Nox blockade. Plasma catecholamine levels (ng/mL) of WT, KO, KO+clonidine, and KO+apocynin mice [n=8 per condition] were measured by HPLC in plasma obtained from anesthetized mice. Results are shown as mean±SEM.

D, NO• depletion: Response to treatment by sympathetic inhibition or Nox blockade. Urinary excretion of nitrate+nitrite was taken as an index of NO• production. Urine levels of NO• (mol/mg creatinine) were measured in WT (n=5), KO (n=8), KO+clonidine (n=7), and KO+apocynin (n=7) mice. Results are shown as mean±SEM.

E, NO• depletion in the circulation: Response to treatment by sympathetic inhibition or Nox blockade. Plasma levels of NO• (nmol/mL) were measured in WT (n=10), KO (n=10), KO+clonidine (n=8), and KO+apocynin (n=8) mice. Results are shown as mean±SEM.
indexed by citrate synthase activity itself, was unchanged from WT to KO (from 0.269±0.009 to 0.261±0.026 U/mg protein, P=0.79).

Discussion

Overview

ROS (such as H$_2$O$_2$ or O$_2^-$ [superoxide anion]) are implicated in a variety of cellular inflammatory responses, including pathological changes in cardiovascular disease, aging, hypercholesterolemia, and diabetes. ROS synthesized by cytoplasmic/membrane NADPH oxidases (Nox), mitochondrial NADH oxidases, or xanthine oxidase (Xo), are to some extent unavoidable byproducts of cellular respiration. Oxygen radicals resulting from increased ROS activity may cause cellular damage, for example, in the form of peroxidation of arachidonic acid yielding isoprostanes, which may themselves be vasoactive. Elevated ROS may deplete the endogenous vasodilator NO, thereby leading to elevation of BP. In the present study, we probed the role of ROS in the hyperadrenergic hypertension created by targeted ablation of the Chga gene.

The Sympathetic Nervous System and CHGA in Hypertension

Studies in animal models of genetic hypertension as well as investigations in hypertensive patients suggest that elevated sympathoadrenal activity may play an important role in the pathogenesis of genetically determined blood pressure elevations. CHGA (a 48-kDa acidic polypeptide) is targeted into the regulated secretory pathway and may act as an “off/on switch” in neuroendocrine cells to trigger secretory granule biogenesis as well as the precursor of the catecholamine release-inhibitory peptide catestatin (human CHGA, mouse Chga). We developed a mouse model of targeted Chga ablation to delineate the in vivo role of Chga. Our findings in the Chga−/− mouse confirmed the putative functions of Chga: Chga KO mice displayed extreme phenotypic changes including elevated BP, loss of diurnal BP variation, and evidence of disturbed storage/release of sympathetic transmitters, with depleted adrenal but augmented plasma concentrations. In the present study, we uncover additional pathogenic consequences of catecholamine excess in the model.

ROS: Role in Hypertension and Renal Disease

Although NADPH oxidase–generated ROS were originally characterized biochemically as “attack” molecules in the phagocyte antimicrobial response or later as mediators of target organ damage in cardiorenal or metabolic disease, increasingly, roles for ROS in autonomic signaling have been described. For example, recent evidence suggests that NADPH oxides may mediate central nervous system control of sympathetic outflow, and roles for NAPDH and ROS in signal transduction for several adrenergic receptors have been explored, including α$_1$, α$_2$, β$_2$, and dopamine.

Because we found ROS changes in urine and kidneys during hyperadrenergic hypertension, we studied ROS generation by podocytes, a renal cell type in contact with urine as it is generated from the glomerulus in the Bowman space. In the renal podocyte, cellular functions (such ion currents, signal transduction) are also influenced by adrenergic agonists, especially α$_1$- and β$_2$-adrenergic agonists, yielding the potential for NADPH/ROS signaling in the podocyte (Figure 1B) as well. The selective effect of epinephrine to elevate H$_2$O$_2$ suggests involvement of a β$_2$-adrenergic receptor, whereas the decline by norepinephrine suggests an action on the α$_1$ receptor.

Source of Elevated ROS in Hyperadrenergic Hypertension

We indexed ROS production principally by H$_2$O$_2$ excretion in urine (Figure 1A). The net effect of Chga−/− to elevate ROS production would seem to involve the counterbalancing effects of several processes to both increase and decrease ROS (online-only Data Supplement Table 1). Two very proximate sources of elevated ROS became likely on studies of renal transcripts: among Nox isoforms, Nox1 and Nox2 (phagocyte form) were elevated, despite unchanged Nox3 and Nox4 (Renox) and an actual decline in the p22Phox (Cyba) subunit (which is shared across Nox1/2/3/4). The Nox system is well described to catalyze the formation of superoxide (O$_2^-$) by the pathway: 2H$_2$O$_2$ → H$_2$O → O$_2$ → O$_2^-$ + H$_2$O + OH$^-$; superoxide can then dismutate (dissproportionate) to H$_2$O$_2$ in a Sod-catalyzed pathway: 2O$_2^-$ + 2H$_2$O → O$_2$ + H$_2$O + 2OH$^-$. When the enzyme xanthine dehydrogenase (Xdh) is posttranslationally modified to xanthine oxidase (Xo) by reversible dithiol oxidation, it then catalyzes the formation of H$_2$O$_2$ by oxidation of hypoxanthine, bypassing O$_2^-$ as follows: hypoxanthine + H$_2$O + O$_2$ → xanthine + H$_2$O$_2$. Finally, even Nos itself (especially Nos3/eNos) can generate O$_2^-$, especially when its activity is “uncoupled” by shortage of its cofactor BH$_4$ (tetrahydrobiopterin).

Thus, at least 3 potential sources of ROS excess in Chga−/− emerge from transcriptional studies: elevations in mRNAs for Nox1/Nox2 (Figure 3A), Xdh/XO and Nos3/eNos (because Nos3/eNos may give rise to O$_2^-$ when “uncoupled”) (Figure 3B). Furthermore, depletion of Sod1/Sod2 (Figure 3B) would tend to sustain the duration of action of any ROS generated.

Apocynin inhibits Nox activity by interfering with the assembly of the Nox monomers into the final heteromultimeric active complex; normalization of elevated ROS by apocynin (Figure 4A and 4B) further implicates the Nox pathway in the excess ROS state of Chga−/−; although apocynin is a relatively low potency inhibitor of Nox4 (Renox), the transcript abundance studies (Figure 3A) suggest that within Nox pathways, apocynin-sensitive isoforms Nox1/Nox2 are principally at work in the Chga−/− state.

At least 2 transcriptional changes would tend to decrease ROS in the Chga−/− state: decreased p22Phox/Cyba mRNA (because p22Phox is a shared subunit among Nox1–4 isoforms) and increased catalase/Cat mRNA (because catalase catalyzes the reaction: 2 H$_2$O$_2$ → 2 H$_2$O + O$_2$).

Although mitochondria can also generate ROS, particularly O$_2^-$ arising through incomplete NADH-mediated reduction of O$_2$ in complex I (by NADH dehydrogenase) or complex III (by coenzyme Q/cytochrome c reductase), we found that...
overall mitochondrial mass was unchanged, and the activity of complex I was actually reduced ($P < 0.03$), whereas the activities of complex II, complex II-III, or complex IV were unchanged. This reduction in complex I activity would have the effect of decreasing the electron flow and superoxide production in mitochondria and may be seen as compensatory to increased superoxide production by Nox1/2.

**Origin of NO’ Depletion in Hyperadrenergic Hypertension**

A likely scenario is that $O_2^-$ generated by Nox activation ($2O_2 + 2H_2O \rightarrow O_2^+ + H_2O_2 + OH^-$) then depletes NO’ by forming peroxynitrite ($O_2^- + NO' \rightarrow ONO_2^-$). $H_2O_2$ can also react with NO’ to produce singlet oxygen. Although our measurements of NO’ were indirect (NO’→NO→NO3 quantified by the colorimetric Griess reaction), the results were directionally coordinate in urine and plasma (Figure 4D and 4E) and consistent with known responses of NO’ to oxygen radicals.

By contrast, the enzymatic sources of NO’, Nos1/2/3, did not appear to be depleted in the KO, at least at a transcriptional level: the renal transcript for Nos3 (eNos) was actually increased in the Chga+/− mouse ($P = 0.0154$), whereas Nos1 and Nos2 transcripts were unchanged (Figure 3C). Nos enzymes are subject to posttranslational modifications, and we have no direct evidence to exclude posttranslational inactivation of Nos enzymes. However, in the face of diminished NO’ in the KO (Figure 4D and 4E), a predicted decline in Nos nitrosylation would be expected to increase Nos enzymatic activity and, likewise, elevated catecholamines in the KO (Figure 4C) would be predicted to signal through protein kinases A and B to Nos phosphorylation and hence enzymatic activation. Thus, it would be difficult to invoke known signaling pathways to postulate posttranslational inactivation of Nos enzymes.

**Treatment Implications**

Both sympathetic outflow inhibition with clonidine and NADPH oxidase inhibition with apocynin appeared to normalize the diverse phenotypes deranged in the Chga+/− state, including increased DBP, ROS excess, lipid peroxidation, catecholamine elevation, and NO’ depletion (Figure 4A through 4E). Because the Chga+/− model displays many features in common with human hypertension, and common genetic variation at the human CHGA locus predicts changes in BP, these treatment results may prompt novel therapeutic strategies for human hypertension. Of note for pathophysiology, both agents (clonidine and apocynin) normalized not only DBP but also ROS and catecholamine excess. These findings suggest that there may be bidirectional or reciprocal influences of catecholamines and ROS on each other’s production. Why did clonidine and apocynin normalize DBP though not SBP in Chga+/− mice (Figure 2)? We evaluated only 1 dose for each drug and cannot exclude the possibility that greater doses would normalize SBP as well. Alternatively, SBP elevations in the face of chronic (5 to 6 months) hypertension may involve structural adaptations of large arteries that cannot be completely reversed by only 3 weeks of treatment. Finally, the SBP elevation in Chga+/− mice may involve additional mechanisms that cannot be completely reversed by sympathetic outflow or NADPH inhibition.

**Advantages and Limitations to the Present Study**

Our experiments took advantage of a definitive biological reagent for evaluation of the consequences of Chga: that is, the Chga−/− mouse. In this system, we were able to probe a spectrum of properties of the strain: molecular/transcriptional (mRNA profiling), biochemical (ROS, catecholamines, NO’, isoprostane), physiological (BP), and pharmacological (sympathetic blockade with clonidine, Nox blockade with apocynin). Although our experiments point to a unifying explanation (Figure 5) for the diverse adrenergic, oxidative, and nitroxidergic manifestations of the hypertension consequent on Chga ablation, the work does raise new questions, both methodological and conceptual. We identified changes...
in several transcripts that might account for the oxidative consequences of Chga: Nox1/2, Xdh/Xo, Sod1/2, Nos3, and Cat. However, we do not yet understand the relative quantitative contributions of these gene products to the altered oxidative state in Chga−/− hypertension.

Clonidine acts centrally to effectively reduce sympathetic outflow (Figure 4A through 4E), as evidenced by substantial reductions of elevated epinephrine and norepinephrine; however, we have not characterized the particular target-organ adrenergic receptors by which the elevated catecholamines exert their oxidative and nitroxidergic consequences, though the selective action of epinephrine (but not norepinephrine) to elevate podocyte H2O2 production (Figure 1B and 1C) may implicate β2 (ADRB2) receptor subtypes.52 Nor do we yet understand precisely how catecholamine’s postreceptor signal transduction apparatus (heterotrimeric GTP-binding [“G”] proteins) might contact and influence expression of Nox, Nos, or Sod isoforms or Xdh/Xo.

Catecholamines themselves can undergo base-catalyzed, nonenzymatic autoxidation, thereby generating ROS; indeed, antioxidant drugs may ameliorate catecholamine-mediated organ toxicity in some settings.62 However, catecholamine oxidation is quite slow at physiological pH,63 and plasma catecholamine concentrations are typically only in the high fM to pM range. In addition, catecholamine metabolism via monoamine oxidase proceeds by oxidative deamination, yielding H2O2.64 However, others have shown that ROS-mediated responses to catecholamines are mediated by adrenergic receptors.55,56

Finally, we used the inhibitor apocynin to probe the role of Nox in these processes because apocynin is known to interfere with assembly of Nox monomers into active heteromultimeric enzymatic complexes; whereas apocynin has the advantages of previous characterization of its particular Nox enzymatic isoform targets57 and tolerability with administration in vivo (in the present study, 3 weeks at 2 mmol/L in drinking water), a recent report suggests that apocynin may have other effects on oxidative pathways, such as a direct antioxidant (electron donor) property.58 Nonetheless, hypertension is perhaps the quantitatively most potent risk factor for cardiovascular disease,69 and because common genetic variation at the CHGA locus has substantial effects on BP in humans,12–14 our results are likely to have implications for not only the pathogenesis but also the target-organ consequences of human hypertension.

Conclusions and Perspectives

Dysregulated ROS production has the potential to “hitjack” physiological excitatory pathways (especially in brain) to elevate BP.41 Such changes in ROS may result from catecholaminergic signaling42–50 (Figure 1B and 1C); thus, the catecholamine excess resulting from decline in Chga expression15 (Figure 4C) may initiate the pathogenic ROS cascade (Figure 5) in processes that are both transcriptional (Figure 3) and at a level not requiring new mRNA or protein synthesis (Figure 1B). Pharmacological disruption of this cascade, at the level of either sympathetic outflow or ROS synthesis, not only corrects disturbances in ROS (Figure 4A and 4B) and NO (Figure 4D and 4E) production but also normalizes elevated BP. Our results thus point to a role for novel pathways in the genesis and consequences of hypertension and suggest new strategies for approaching the pathogenesis and treatment of hypertension as well as the amelioration of its target organ consequences.

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Disclosures

None.

References


Oxidative stress, in which reactive oxygen species (ROS) outstrip antioxidant defenses, contributes to cardiovascular disease. In the present investigation, we studied derangements of ROS in the development of a hyperadrenergic model of hereditary hypertension: targeted ablation (knockout [KO]) of chromogranin A (*Chga*) in the mouse. In the KO mouse, BP elevation was accompanied by not only catecholamine excess but also by increased ROS (H$_2$O$_2$) and isoprostane levels (index of lipid peroxidation). Renal transcript analyses implicated changes in several redox enzymes. KO alterations in BP as well as biochemical traits could be abrogated by inhibition of either sympathetic outflow or of NADPH oxidase. In cultured renal podocytes, H$_2$O$_2$ production was augmented by epinephrine (probably through $\beta_2$ receptors). Thus, ROS may play an important role in the development of hyperadrenergic hypertension in this experimental model, in a process mechanistically linking elevated BP with catecholamine excess, renal transcriptional responses, ROS elevation, lipid peroxidation, and nitric oxide depletion. Overall, our results demonstrate the existence of novel pathophysiological links between the adrenergic system and oxidative stress and suggest new strategies to probe the role and actions of ROS in this setting.
Role of Reactive Oxygen Species in Hyperadrenergic Hypertension: Biochemical, Physiological, and Pharmacological Evidence From Targeted Ablation of the Chromogranin A (Chga) Gene

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Supplemental material
Adrenergic genetic hypertension in targeted ablation of Chga: Alterations in processes likely to influence reactive oxygen species (ROS)

**Tend to increase ROS:**
- ↑ NADPH oxidase (Nox1, Nox2) mRNAs
- ↑ Xanthine oxidase (Xdh/Xo) mRNA
- ↑ Nitric oxide synthase (Nos3/eNos) mRNA
- ↓ Superoxide dismutase (Sod1, Sod2) mRNAs
- ↑ Epinephrine secretion

**Tend to decrease ROS:**
- ↓ p22Phox/Cyba mRNA
- ↑ Catalase (Cat) mRNA
- ↓ Mitochondrial complex I (NADH oxidase) activity
- ↑ Norepinephrine secretion

Gayen et al, on-line Table 1
Kidney cortex $H_2O_2$ and $NO^*$

$NO^*$ (mmol/mg protein)

$H_2O_2$ (Amplex red fluorescence/mg protein)

WT (10)

KO + Apocynin (10)

KO (9)

ANOVA:
$H_2O_2$ p<0.017
$NO$ p<0.015

Gayen et al, on-line Fig. 1