Role of Reactive Oxygen Species in Hyper-Adrenergic Hypertension: Biochemical, Physiological, and Pharmacological Evidence from Targeted Ablation of the Chromogranin A (*Chga*) Gene

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Abstract

Background—Oxidative stress, an excessive production of reactive oxygen species (ROS) outstripping antioxidant defense mechanisms, occurs in cardiovascular pathologies including hypertension. Here, we used biochemical, physiological, and pharmacological approaches to explore the role of derangements of catecholamines, ROS, and NO• in the development of a hyper-adrenergic model of hereditary hypertension: targeted ablation (knockout, KO) of chromogranin A (*Chga*) in the mouse.

Methods and Results—Homozygous (−/−) Chga gene knockout (KO) mice were compared to wild-type (WT, +/+) controls. In the KO mouse, elevations of systolic and diastolic BP were accompanied by not only elevated catecholamine (norepinephrine and epinephrine) concentrations, but also increased ROS (H_2O_2) 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 BP, catecholamines, H_2O_2, 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, H_2O_2 production was substantially augmented by epinephrine (likely through b_2-adrenergic receptors) and modestly diminished by norepinephrine (likely through a_1-adrenergic receptors).

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Conclusions—ROS seem to play a necessary role in the development of hyper-adrenergic hypertension in this model, in a process mechanistically linking elevated BP with catecholamine excess, renal transcriptional responses, ROS elevation, lipid peroxidation, and NO• depletion. Some of the changes seem to be dependent on transcription, while others are immediate. The cycle could be disrupted by inhibition of either sympathetic outflow or NADPH oxidase. Since 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 andc oxidative stress, and suggest new strategies to probe the role and actions of ROS within this setting.

Keywords

Chromogranin A; hypertension; reactive oxygen species; nitric oxide

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Introduction

Increased sympathoadrenal activity plays a role in the development or maintenance of elevated blood pressure 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 co-stored and co-released with catecholamines from secretory vesicles in adrenal medulla[5–7] and post-ganglionic sympathetic axons[8]. Although CHGA is over-expressed in human essential (hereditary) hypertension, the plasma concentration of its catestatin (catecholamine release-inhibitory) fragment[9] 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 upon the human findings, we found that *Chga* knockout (KO, −/−) mice are hypertensive and hyper-adrenergic[15]. To get a 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.

Oxidative stress, characterized by excessive production of reactive oxygen species (ROS) outstripping antioxidant defense mechanisms, has been associated with several cardiovascular pathologies including hypertension[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 (AA)-metabolizing enzymes[18]. ROS may promote vascular smooth muscle cell contraction and proliferation, enhancing contraction in part by depleting the endothelium derived relaxing factor nitric oxide (NO•)[19].

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

In the present study, we explore the involvement of ROS and NO• in the development of a hyper-adrenergic model of hereditary hypertension: the *Chga*-KO mouse. Our results suggest a role for catecholamine excess in generating a hypertensive state partially

consequent upon ROS activation and NO• 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[15]. The homozygous (−/−) *Chga* gene knockout (KO) mouse line was maintained and used for the experiments, and compared with wild-type (WT, +/+) strain controls; 5–6 month-old animals were studied from each strain. Based upon 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 10–12), to optimize our ability to find true positive and avoid false negative results.

Renal podocyte cultures

Since we found ROS changes in urine and kidney during hyper-adrenergic hypertension, we studied ROS generation by kidney podocytes, as a renal cell type in contact with urine as it is generated in Bowman's 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[28] for multiplication purposes, in the presence of mouse gamma 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 8–10 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, nitric oxide (NO•)

Catecholamines—Catecholamines were measured in mouse plasma from anesthetized animals by high performance liquid chromatography (HPLC) (Waters 600E Multisolvent Delivery System; Waters, Milford, MA) using an electrochemical detector (ECD) (Waters 2465 Electrochemical Detector). Sample purification was done using activated alumina. The data were analyzed using Empower software from Waters, and catecholamine levels normalized according to the recovery of internal standard 3,4-dihydroxybenzylamine (DHBA) as previously described[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, CA] was monitored. Amplex Red reacts with hydrogen peroxide in the presence of horseradish peroxidase (HRP) with 1:1 stoichiometry to form the fluorophore resorufin. Amplex Red and peroxidase in Kreb's 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 min 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 10–20 cycles, at 19–67 sec/ 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 nitric oxide (NO•) 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, MI). Urinary NO• 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 systemic oxidative stress, we used the urinary content of 8-iso-PGF2α (also known as 8-epi-PGF2α, or 15-isoprostane F2t) a stable product of arachidonic acid formed on non-enzymatic oxidation. Urinary isoprostane was measured using an ELISA kit (Northwest Life Science Specialties LLC, Vancouver, WA). The assay sensitivity was 50 pg, with a dynamic range of 0.1–10 ng/ml, and the average intra-assay 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 min at 20°C, absorbance measured at 505 nm and calculated using known standard concentrations.

Blood pressure

BP of mice was measured by the non-invasive tail-cuff method using the BP-2000 Blood Pressure Analysis System (Visitech Systems, Apex, NC <http://www.visitechsystems.com/>), as previously described[15]. 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 three sessions daily, between 1 PM to 3 PM, for 5 consecutive days (Mon→Fri). The first 2 days (Mon/Tues) were for acclimation of mice with the BP system, while 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 Visitech 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 (Visitech Systems) via a PCMCIA data acquisition card. As per the manufacturer's protocol, BP readings having a standard deviation of less than 10 mmHg were accepted for further analysis. We thus collected 9 measurements (3 days; 3 measurements/day) at each state for each mouse. Elevations of BP in the *Chga*(−/−) mouse can be detected by either this non-invasive method, or by implanted carotid transducers with telemetric monitoring[15].

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, WI), beginning with 1% (w/w) clonidine HCl in sucrose, and embedding in Harlan formula 5001 standard laboratory rodent diet, at 1 mg/kg chow. The dose was calculated to achieve delivery of 125 μg clonidine/kg body weight/day, and used for 3 weeks.

Inhibition of NADPH oxidase—Apocynin (acetovanillone, A10809 at 98% purity; Sigma-Aldrich, St. Louis, MO) was used at 2 mmol/L in drinking water for 3 weeks.

mRNA abundance by real-time RT-PCR

Total RNA was extracted form one 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, CA), with normalization of data to β-actin expression. C_t (threshold cycle) is determined for both the specific target mRNA/cDNA as well as β-actin, and the difference in C_t (from target mRNA versus β-actin mRNA) is normalized to the average for that state (e.g., control versus experimental), and expressed on a % change (difference) scale.

Renal cortex biochemistry

Renal cortical homogenates were prepared from freshly harvested adult male kidneys (WT and KO), and assayed for H_2O_2 and NO•, as described above. Results were normalized to homogenate protein concentration (Bradford Coomassie Brilliant Blue R-250 assay method; BioRad, Hercules, CA).

Mitochondrial electron transport chain

Mitochondria were prepared from freshly harvested adult male livers (n=3 WT, n=3 KO), as described[29]. Overall mitochondrial mass (per mg 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[29].

Statistical analyses

Results are expressed as the mean value ± one SEM (standard error of the mean). The reported "n" refers to the number of mice, rather than to the number of BP measurements taken (since, 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, WA), Kaleidagraph (Synergy Software, Reading, PA) or SPSS-17 (Chicago, IL). MANOVA (multivariate ANOVA) was used to test the effect of strain on more than one dependent variable. Repeated measures (e.g., 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. *P* values less than 0.05 were considered statistically significant.

Results

Chga ablation on H_2O_2 and lipid peroxidation (isoprostane) in urine and kidney

Since increased ROS activity generates H_2O_2[19, 30] with resulting lipid peroxidation (isoprostane)[31], we measured H_2O_2 and isoprostane renal excretion, as well as H_2O_2 in kidney. *Chga* ablation caused significant increases in both urinary H_2O_2 and isoprostane excretions (Fig. 1A). There was a ~20% increase in urinary isoprostane excretion in KO mice (10.33±0.51 vs 12.86±0.61 ng/mg creatinine, p<0.02) (Fig. 1A), while urinary H_2O_2 excretion was ~2.5 times higher in the KO mice (1851.6±256.6 vs 4574.7±306.6 fluorescence units/mg creatinine, p<0.002) (Fig. 1A). MANOVA confirmed the simultaneous effect of mouse strain on the two oxidative traits (p<8.55E-06). In renal cortex, H_2O_2 was elevated by ~45% in the KO (on-line Fig. 1).

Catecholamines on H_2O_2 in cultured glomerular podocytes

Epinephrine caused a substantial increase (p<0.001) in H_2O_2 production, while norepinephrine caused a modest decrease (also p<0.001) (Fig. 1B). When adrenergic receptor type-specific agonists were tested (Figure 1C), both the alpha-1 agonist

phenylephrine (p<0.001) and the alpha-2 agonist clonidine reduced H_2O_2, while the beta agonist isoproterenol substantially increased H_2O_2 (p<0.001). Since epinephrine displays preferential activity at beta-2 receptors, epinephrine likely increases H_2O_2 production via beta-2 activation[32]; since norepinephrine displays preferential activity at alpha-1 over alpha-2 receptors, norepinephrine likely inhibits H_2O_2 production via alpha-1 activation[32].

Sympathetic outflow inhibition (clonidine) on BP

Chga ablation caused substantial elevations of both systolic (p<0.0001) and diastolic (p<0.003) BP[15] (Fig. 2). 3 weeks of sympatho-inhibitory treatment with the α_2-adrenergic agonist clonidine caused significant reductions of both SBP (by ~9.1 mmHg; from 139.3±1.8 to 130.2±2.7 mmHg, p<0.02) and DBP (by ~9.9 mmHg; from 101.1±2.6 to 91.2±3.3 mmHg, p<0.03) in the KO mice (Fig. 2), and the effects on DBP virtually normalized the trait.

NADPH oxidase (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 mmHg (from 139.3±1.8 to 128.4±2.0 mmHg, p<0.001) while DBP fell by ~11.2 mmHg (from 101.1±2.6 to 89.9±2.5 mmHg, p<0.007) (Fig. 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/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=40.3, p=2.8E-11) and DBP (F=11.9, p=1.7E-7) remained significant. Repeated measures ANOVA also yielded significant effects for treatment regimens.

Renal mRNA expression

NADPH oxidase (Nox) mRNA abundance in kidney—Relative abundance of all isoforms of Nox mRNA were examined by real-time PCR and normalized to β-actin. In KO mice, *Nox1* and *Nox2* were significantly over-expressed by ~4.7- and ~0.8-fold respectively compared to WT mice (Fig. 3A). There were no significant differences in *Nox3* or *Nox4* mRNA abundance, while *p22Phox* (*Cyba*) was actually *reduced* in the KO by ~40% (p=0.001)(Fig. 3A).

Other RedOx enzymes—*Xdh/Xo* expression was also augmented by ~0.7-fold (p=0.03) in the KO (Fig. 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) (Fig. 3B).

Nitric oxide 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), while *Nos2* (bNos) was unchanged (p=0.929) (Fig. 3C).

Response of ROS to drug treatment: Clonidine or apocynin

ROS were reduced significantly in KO mice either by sympathetic inhibition (α_2-agonist: clonidine) or NADPH oxidase blockade (Nox inhibitor: apocynin).

Clonidine—Treatment with clonidine for 3 weeks reduced urinary H_2O_2 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 (Fig. 4A).

Apocynin—3 weeks of apocynin also corrected the elevated urinary H_2O_2 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 (Fig. 4A). In KO kidney cortex, apocynin decreased H_2O_2 by ~31% (on-line Fig. 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 (α_2-agonist: clonidine) or NADPH oxidase blockade (Nox inhibitor: apocynin).

Clonidine—3 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 (Fig. 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 vs 7.81 ± 0.81 ng/mg creatinine, $p<0.04$) (Fig. 4B).

Adrenergic over-activity: Response to drug treatment by clonidine or apocynin

In KO mice, circulating catecholamines were significantly higher than in WT (norepinephrine: 4.22 ± 0.50 vs 2.21 ± 0.33 ng/ml, $p<0.006$; epinephrine: 1.19 ± 0.07 vs 0.81 ± 0.08 ng/ml, $p<0.005$)(Fig. 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, $p<0.04$; 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 (Fig. 4C).

Apocynin—3 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 a again achieving levels that are comparable to the WT (Fig. 4C).

Nitric oxide (NO•) depletion: Response to drug treatment by clonidine or apocynin

In KO mice urine NO• excretion was reduced by ~50% as compared to WT (3273 ± 193 vs 1546 ± 146 μ-mol/mg creatinine, $p<0.001$) (Fig. 4D). In kidney cortex, NO• was reduced by ~33% in the KO (on-line Fig. 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, $p<0.036$) (Fig. 4E).

Clonidine—Clonidine "rescued" the NO• depletion in KO mice (renal excretion: from 1546 ± 146 to 3231 ± 416 μmol/mg creatinine, $p<0.002$), which is comparable with WT (Fig. 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 (Fig. 4E).

Apocynin—After apocynin treatment in KO mice, NO• renal excretion increased (from 1546 ± 146 to 2199 ± 260 μ-mol/mg creatinine, $p<0.039$) to WT level (Fig. 4D).). In KO kidney cortex, apocynin increased NO• by ~46% (on-line Fig. 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 (Fig. 4E

Mitochondria

There was a ~28% *decline* in mitochondrial complex I activity in the KO animals (normalized to citrate synthase [CS] activity, from 318 ± 24 to 230 ± 10 units/CS unit, $p<0.03$), but no changes in the activities of complex II, complex II-III, or complex IV. Overall mitochondrial mass, indexed by citrate synthase activity itself, was unchanged from WT to KO (from 0.269 ± 0.009 to 0.261 ± 0.026 units/mg protein, $p=0.79$).

Discussion

Overview

Reactive oxygen species (ROS, such as H_2O_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.[33] 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[34], which may themselves be vasoactive[35]. Elevated ROS may deplete the endogenous vasodilator NO•, thereby leading to elevation of BP. Here we probed the role of ROS in the hyper-adrenergic hypertension created by targeted ablation of the *Chga* gene[15].

The sympathetic nervous system and CHGA in hypertension

Studies in animal models of genetic hypertension[36] as well as investigations in hypertensive patients[13] 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[37] and may act as an "on/off switch" in neuroendocrine cells to trigger secretory granule biogenesis[38][39], as well as the precursor of the catecholamine release-inhibitory peptide catestatin (human CHGA 9$_{352-372}$; mouse Chga$_{364-384}$)[9]. 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[15]: *Chga* KO mice displayed extreme phenotypic changes including elevated BP, loss of diurnal BP variation, and evidence of disturbed storage/release of sympathochromaffin transmitters, with depleted adrenal but augmented plasma concentrations. Here we uncover additional pathogenic consequences of catecholamine excess in the model.

ROS: Role in hypertension and renal disease

While NADPH oxide-generated ROS were originally characterized biologically as "attack" molecules in the phagocyte antimicrobial response[40] or later as mediators of target organ damage in cardio-renal or metabolic disease[41], increasingly roles for ROS in autonomic signaling have been described. For example, recent evidence suggests that NADPH oxidases may mediate central nervous system control of sympathetic outflow[42–44], and roles for NAPDH and ROS in signal transduction for several adrenergic receptors have been explored, including alpha-1[45–47], alpha-2[48], beta-2[49], and dopamine[50].

Since we found ROS changes in urine and kidneys during hyper-adrenergic hypertension, we studied ROS generation by podocytes, a renal cell type in contact with urine as it is generated from the glomerulus in Bowman's space. In the renal podocyte[51], cellular functions (such ion currents, signal transduction) are also influenced by adrenergic agonists, especially alpha-1 and beta-2 adrenergic agonists[52,53], yielding the potential for NADPH/ ROS signaling in the podocyte (Fig. 1B), as well. The selective effect of epinephrine to

elevate H_2O_2 suggests involvement of a beta-2 adrenergic receptor, while the decline by norepinephrine suggests an action on the alpha-1 receptor[32].

Source of elevated ROS in hyper-adrenergic hypertension (on-line Table 1)

We indexed ROS production principally by H_2O_2 excretion in urine (Fig. 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 (on-line Table 1). Two very proximate sources of elevated ROS became likely upon 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: $2O_2^- + 2H_2O \rightarrow 2O_2^- + H_2O_2 + OH^-$; superoxide can then dismute (disproportionate) to H_2O_2 in a Sod-catalyzed pathway: $2O_2^- + 2H_2O \rightarrow O_2 + H_2O_2 + 2OH^-$. When the enzyme xanthine dehydrogenase (Xdh) is post-translationally modified to xanthine oxidase (Xo) by reversible disulfide oxidation[54,55], it then catalyzes the formation of H_2O_2 by oxidation of hypoxanthine, bypassing O_2^- as follows: hypoxanthine $+ H_2O + O_2 \rightarrow$ xanthine $+ H_2O_2$. 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)[56].

Thus, at least three potential sources of ROS excess in *Chga−/−* emerge from transcriptional studies: elevations in mRNAs for Nox1/Nox2 (Fig. 3A), Xdh/XO and Nos3/eNos (since Nos3/eNos may give rise to O_2^- when "uncoupled") (Fig. 3B). Furthermore, depletion of Sod1/Sod2 (Fig. 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 (Fig. 4A&B) further implicates the Nox pathway in the excess ROS state of *Chga−/−*; although apocynin is a relatively low potency inhibitor of Nox4 (Renox)[57], the transcript abundance studies (Fig. 3A) suggest that, within Nox pathways, apocynin-sensitive isoforms Nox1/Nox2 are principally at work in the *Chga−/−* state.

At least two transcriptional changes would tend to *decrease* ROS in the *Chga−/−* state: decreased *p22Phox/Cyba* mRNA (since p22Phox is a shared subunit among Nox1–4 isoforms), and increased catalase/*Cat* mRNA (since catalase catalyzes the reaction: $2 H_2O_2 \rightarrow 2 H_2O + O_2$)

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), here we found that overall mitochondrial mass was unchanged, and the activity of complex I was actually *reduced* (p<0.03), while 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 hyper-adrenergic hypertension

A likely scenario is that O_2^- generated by Nox activation ($2O_2 + 2H_2O \rightarrow 2O_2^- + H_2O_2 + OH^-$) then depletes NO• by forming peroxynitrite ($O_2^- + NO• \rightarrow ONO_2^-$). H_2O_2 can also react with NO• to produce singlet oxygen.[58, 59] While our measurements of NO• were indirect (NO• $\rightarrow NO_2 \rightarrow NO_3$ quantified by the colorimetric Griess reaction), the results were directionally coordinate in urine and plasma (Fig. 4D&E), 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), while Nos1 and Nos2 transcripts were unchanged (Fig. 3C). Nos enzymes are subject to post-translational modifications[60], and we have no direct evidence to exclude post-translational inactivation of Nos enzymes. However, in the face of diminished NO• in the KO (Fig. 4D&E), a predicted decline in Nos nitrosylation would be expected to *increase* Nos enzymatic activity[60]; likewise, elevated catecholamines in the KO (Fig. 4C) would be predicted to signal through protein kinases A and B to Nos phosphorylation and hence enzymatic activation[61]. Thus, it would be difficult to invoke known signaling pathways to postulate post-translational *inactivation* of Nos enzymes.

Treatment implications

Both sympathetic outflow inhibition with clonidine and NADPH oxidase inhibition with apocynin seemed to normalize the diverse phenotypes deranged in the *Chga−/−* state, including increased DBP, ROS excess, lipid peroxidation, catecholamine elevation, and NO• depletion (Fig. 4A–E). Since the *Chga−/−* model displays many features in common with human hypertension, and common genetic variation at the human *CHGA* locus predicts changes in BP[12, 13], 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 (Fig 2)? We evaluated only one 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 (5–6 month) 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 this study

Our experiments took advantage of a definitive biological reagent for evaluation of the consequences of Chga: i.e., 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). While our experiments point to a unifying explanation (Fig. 5) for the diverse adrenergic, oxidative, and nitroxidergic manifestations of the hypertension consequent upon *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 (Fig. 4A–E), 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 H_2O_2 production (Fig. 1B, 1C) may implicate beta-2 (ADRB2) receptor subtypes[32]. Nor do we yet understand precisely how catecholamines' post-receptor 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, non-enzymatic auto-oxidation, 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 (MAO) proceeds by oxidative deamination, yielding H_2O_2[64]. However, others have shown that ROS-mediated responses to catecholamines are mediated by adrenergic receptors[65, 66].

Finally, we used the inhibitor apocynin to probe the role of Nox in these processes, since aopocynin is known to interfere with assembly of Nox monomers into active heteromultimeric enzymatic complexes; while apocynin has the advantages of previous characterization of its particular Nox enzymatic isoform targets[57], and tolerability with administration *in vivo* (here, 3 weeks at 2 mM in drinking water), a recent report suggests that apocynin may have other effects on oxidative pathways, such as a direct antioxidant (electron donor) property[67].

Nonetheless, hypertension is perhaps the quantitatively most potent risk factor for cardiovascular disease[68], and since 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 "hijack" physiological excitatory pathways (especially in brain) to elevate BP[43]. Such changes in ROS may result from catecholaminergic signaling[42–50] (Fig. 1B&C); thus, the catecholamine excess resulting from decline in Chga expression[15] (Fig. 4C) may initiate the pathogenic ROS cascade (Fig. 5), in processes that are both transcriptional (Fig. 3) and at a level not requiring new mRNA or protein synthesis (Fig. 1B). Pharmacological disruption of this cascade, at the level of either sympathetic outflow or ROS synthesis, not only corrects disturbances in ROS (Fig. 4A&B) and NO• (Fig. 4D&E) 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.

Oxidative stress, in which reactive oxygen species (ROS) outstrip antioxidant defenses, contributed to cardiovascular disease. In the present investigation, we studied derangements of ROS in the development of a hyper-adrenergic 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_2O_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_2O_2 production was augmented by epinephrine (likely through beta-2-receptors). Thus, ROS may play an important role in the development of hyper-adrenergic hypertension in this experimental model, in a process mechanistically linking elevated BP with catecholamine excess, renal transcriptional responses, ROS elevation, lipid peroxidation, and NO• 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

BP	Blood pressure
CA	Catecholamines (epinephrine or norepinephrine)
Cat	Catalase
Chga	Chromogranin A (human CHGA, rodent Chga)
CS	Citrate synthase (index of mitochondrial mass)
KO	Knock-out (homozygous Chga−/−)
NO•	Nitric oxide
Nox	NADPH oxidase
O_2^-	Superoxide anion
Red/Ox	Reduction/Oxidation
ROS	Reactive oxygen species (H_2O_2 or O_2^-)
WT	Wild-type (homozygous Chga+/+)
Xdh/Xo	Xanthine dehydrogenase/Xanthine oxidase

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Targeted ablation of the *Chga* locus in the mouse: Coordinate effects on H_2O_2 and isoprostane formation
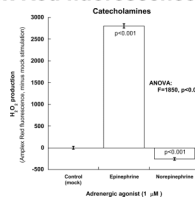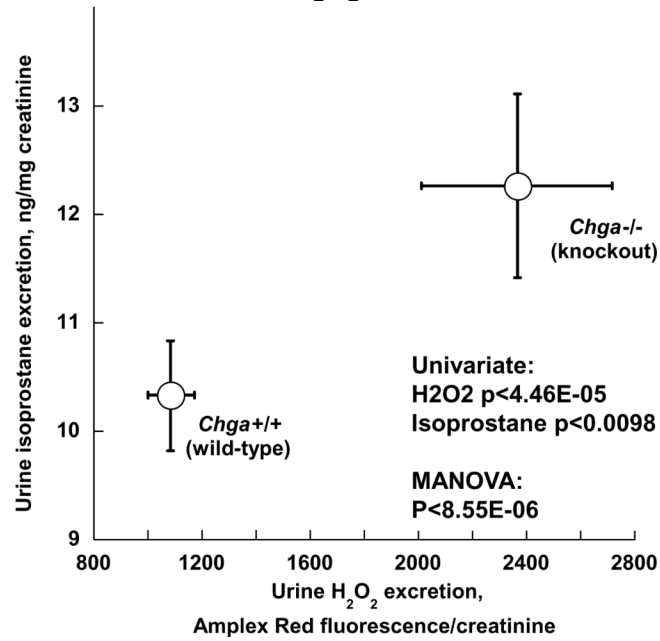


Synthetic adrenergic agonists
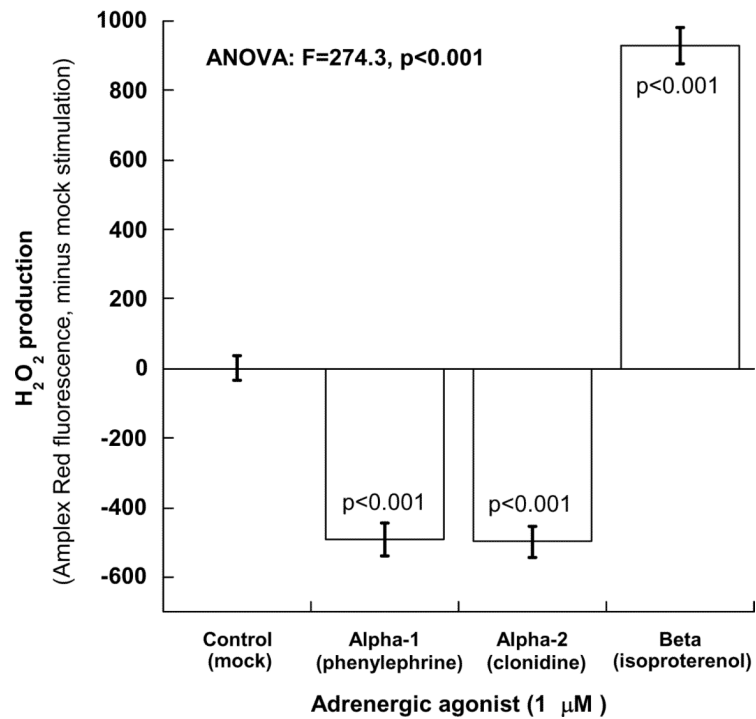
Figure 1.

A. *Chga* targeted ablation on H_2O_2 and lipid peroxidation (isoprostane). Urine isoprostane and H_2O_2 measured and normalized by creatinine. WT vs KO: H_2O_2 ($p<0.02$) and isoprostane ($p<0.002$), [n=6 animals/condition]. Results are shown as mean ± one SEM. **B. Renal podocytes, catecholamines and H_2O_2**. Catecholamines (both epinephrine and norepinephrine; each at 1 μM) were used to influence H_2O_2 production in mouse podocytes (n=6 replicate wells per condition). Results are shown as mean ± one SEM. C. Selective adrenergic agonists and renal podocyte H_2O_2. Selective adrenergic agonists (alpha-1: phenylephrine; alpha-2: clonidine; beta: isoproterenol), each at 1 μM, were used to influence H_2O_2 production in mouse podocytes (n=6 replicate wells per condition). Results are shown as mean ± one SEM.
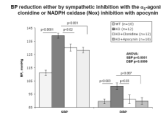
Figure 2. Adrenergic outflow inhibition (clonidine) or NADPH oxidase (Nox) inhibition (apocynin) on BP

Both SBP and DBP were reduced significantly in KO mice by sympathetic inhibition with the α$_2$-agonist clonidine (125 μg/kg body weight/day for 3 weeks). SBP and DBP were reduced significantly in KO mice by Nox inhibitor apocynin (2 mmol/L) in drinking water for 3 weeks. N for each group (number of mice) is given in the figure inset. Results are shown as mean ± one SEM.
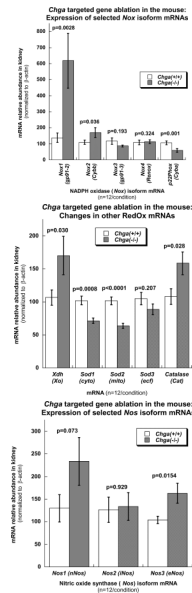
Figure 3. mRNA abundances in kidney
Relative abundances of mRNAs were normalized to β-actin in kidney by real-time PCR
(n=12 kidneys were studied from each strain, WT or KO). Differences in C_t (target mRNA
versus β-actin mRNA) are expressed on a % scale (see Methods). Results are shown as
mean ± one SEM. A. NADPH oxidase (*Nox*) isoforms. B. RedOx targets. C. *Nos* (nitric
oxide synthase) isoforms.
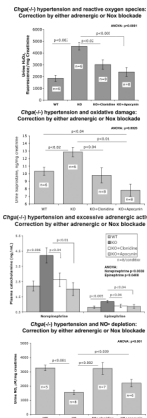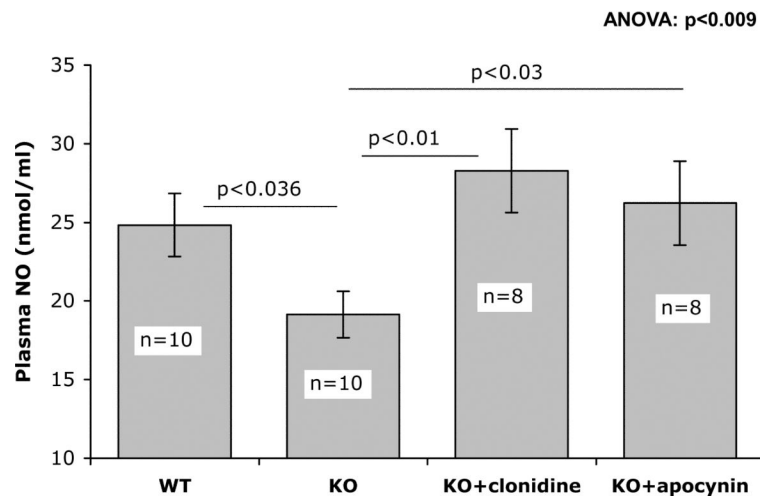
Figure 4A. Oxygen radicals (H_2O_2): Response to treatment by sympathetic inhibition or NADPH oxidase blockade

Urine H_2O_2 level (amplex red fluorescence/mg creatinine) is presented in WT (n=6), KO (n=6), KO+clonidine (n=8), or KO+apocynin (n=8) mice. Results are shown as mean ± one SEM. B. Lipid peroxidation (isoprostane): Response to treatment by sympathetic inhibition or NADPH oxidase (Nox) blockade. Urine isoprostane level (ng/mg creatinine) is presented in WT (n=6), KO (n=6), KO+clonidine (n=8), or KO+apocynin (n=8) mice. Results are shown as mean ± one SEM. C. Catecholamine secretion: Response to treatment by sympathetic inhibition or NADPH oxidase (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 ± one SEM. D. Nitric oxide (NO•) depletion: Response to treatment by sympathetic inhibition or NADPH oxidase (Nox) blockade. Urinary excretion of nitrate+nitrite was taken as an index of NO• production. Urine levels of NO• (μmol/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 ± one SEM. E. Nitric oxide (NO•) depletion in the circulation: Response to treatment by sympathetic inhibition or NADPH oxidase (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 ± one SEM.
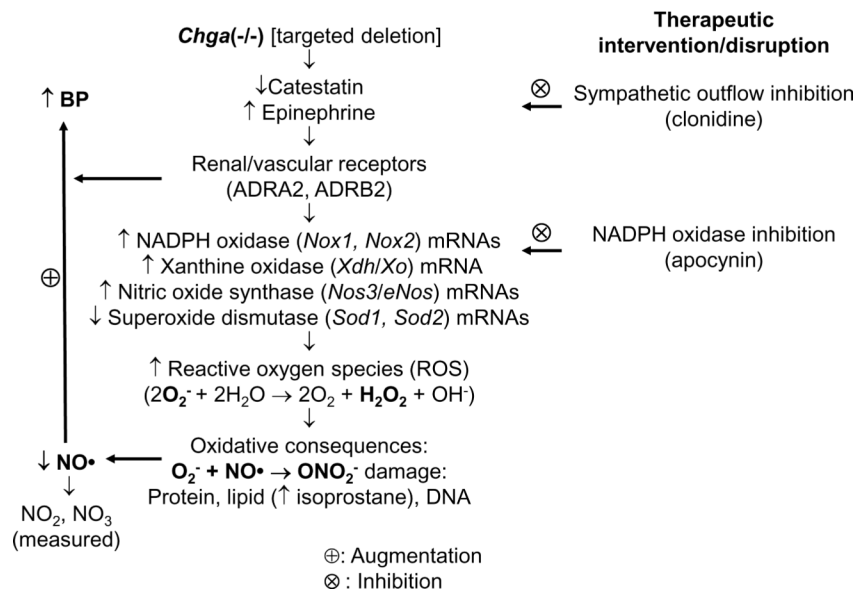
Figure 5. Oxygen radicals and hyper-adrenergic hypertension: Hypothesis integrating experimental results from this study in targeted ablation of the mouse *Chga* locus
Directional arrows indicate proposed cause-and-effect relationships.