Dual Oxidase 2 Mediates Cardiovascular, Autonomic and Baroreflex Function: An in vivo Study in Conscious Mice

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Abstract

Changes in circadian rhythm profiles including phase changes and classical dipper/non-dipper profiles have recently been shown to be affected by reactive oxygen species (ROS) in several hypertensive models. Dual oxidase 2 (DUOX2), a new member of the ROS-producing NADPH oxidase family of enzymes, is primarily responsible for the production of thyroid hormone through its generation of hydrogen peroxide. In addition to hypothyroidism, here we report that global knockout of the DUOX2 gene in mice causes both hypotension and bradycardia basally, which are independent of locomotor activity and that which cannot be rescued by thyroid hormone supplementation. Furthermore, DUOX2 knockout mice exhibit a classical "dipper" profile, which is, at least in part, the result of increased sympathetic control of basal heart rate. Moreover, we report that DUOX2 null mice exhibit an increased baroreflex sensitivity, which was tested by both spontaneous baroreflex analysis and classical vasoactive drug administration. Supplemental real-time PCR and in situ hybridization studies confirm the genotype of DUOX2 null mice and demonstrate that DUOX2 is not expressed in the thyroid of null mice, providing rationale for the low plasma T4 levels observed in DUOX2null mice at baseline. Current studies are underway to localize precisely where DUOX2 is expressed including neurocardiovascular control sites such as the subfornical organ (SFO), a forebrain circumventricular organ lacking a blood brain barrier that interfaces with factors (e.g., angiotensin-II, norepinephrine, etc.) circulating in the systemic circulation; the paraventricular nucleus of the hypothalamus (PVN), which is involved in vasopressin release and in regulating autonomic outflow; the rostral ventrolateral medulla (RVLM), a primary regulator of sympathetic outflow located in the brainstem; the nucleus tractus solitarius (NTS), a site responsible for baroreflex control;
and the suprachiasmatic nucleus (SCN), which controls circadian rhythm. In conclusion, we have determined that DUOX2-mediated pathways participate in the regulation of both blood pressure and heart rate and that this occurs via mechanisms independent of thyroid hormone synthesis.
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Introduction

The observation of a pattern in movement in leaves by the French geophysicist Jean-Jacques d'Ortous de Mairan in the 1700s provided some of the first intriguing evidence that natural, endogenous processes control the function of living things. Since then, the circadian rhythm, the endogenous biochemical and physiological process by which the body adjusts to environmental cues called Zeitgebers within a 24-hour period, has moved to the forefront of molecular and physiological research. While there are many different types of cues, one of the main Zeitgebers for the circadian rhythm is daylight, which influences the level of melatonin released from the pineal gland via interaction of the photoreceptive retinal ganglion cells with the suprachiasmatic nucleus (SCN). Molecular Zeitgebers include negative feedback loops formed by specific “clock genes” including the period genes and the transcriptional factors BMAL1 and CLOCK.

Considerable evidence suggests that there is a strong circadian component to blood pressure (BP) regulation. A typical change that occurs during circadian phases in healthy individuals is a decrease, or “dipping”, of the blood pressure during the nighttime; this phenomena is also observed in other organisms during the time of day corresponding to their “rest” period. Interestingly, hypertensive patients often present with a “non-dipping” BP profile in which the nocturnal blood pressure falls less than 10% of the daytime baseline value. This abnormal dipping pattern can occur as a result of a diminished cardiac output and/or a dramatic increase in nighttime systemic vascular resistance. This “non-dipping” BP profile has also been associated with autonomic dysfunction, whereby there is an increase in the sympathetic nervous system component of blood pressure regulation. Clinically, sympathetically controlled “non-dipping” BP can
be seen in patients with obstructive sleep apnea syndrome (OSAS)\(^4\) and essential hypertension.\(^5\)

Over the past few decades, reactive oxygen species (ROS) have risen to the forefront of cardiovascular and molecular research as key signaling mediators in the control of blood pressure and circadian rhythm. ROS are produced as natural byproducts of cellular metabolism. Specifically, one, two and three electron reductions of molecular oxygen yield superoxide (O\(_2^−\)), hydrogen peroxide (H\(_2\)O\(_2\)) and hydroxyl radicals (HO\(^\cdot\)), respectively. Generation of ROS typically begins with production of O\(_2^−\), which rapidly dismutates, either spontaneously at a low pH or catalyzed via superoxide dismutase (SOD), into H\(_2\)O\(_2\). Alternatively, O\(_2^−\) can react with nitric oxide (NO) to form peroxynitrite (ONOO\(^−\)) or enter the iron-catalyzed Fenton reaction to produce a hydroxyl radical.\(^6\) ROS have been shown to lead to protein nitrosylation, mutation of nucleic acids, lipid peroxidation and even cellular apoptosis via activation of the caspase-9, an aspartic acid-specific protease linked to mitochondrial death.\(^7-9\) As a result, the cell has developed sensitive mechanisms to protect itself against internal changes of the redox environment. One such protective measure includes multiple antioxidant enzymes targeted to specific locations, such as the presence of variations of the antioxidant enzyme superoxide dismutase (SOD in the mitochondria (MnSOD, SOD2), intracellular cytoplasm (CuZnSOD, SOD1) and the extracellular spaces (ecSOD, SOD3).\(^10\) In cases of oxidative stress, superoxide production exceeds well beyond the ability of the cell to reduce it to water and hydrogen peroxide. Oxidative stress can occur due to multiple conditions including mitochondrial disruption, over-activation of ROS-generating enzymes and/or dysfunction of ROS scavenging systems.\(^11\)
Studies exploring the molecular mechanisms involved in circadian rhythm have identified ROS such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) as key signaling molecules. The first of two major studies reported a correlation between circadian rhythm, H$_2$O$_2$ and the expression of two zebrafish-specific clock genes called zCry1a and zPer2. In this study, it was demonstrated that light facilitates an increase in intracellular H$_2$O$_2$ via flavin oxidases, which impacts the cyclical transcription of zCry1a and zPer2 via the MAPK signaling pathway. It was also observed that light increases the concentration of intracellular catalase, an enzymatic scavenger of H$_2$O$_2$, via zCat upregulation after the peak increases in zCry1a and zPer2. This creates a negative-feedback loop that correlates with the circadian cycle of the zebrafish. In a separate study by Krishnan et al., circadian rhythm was correlated with mortality due to oxidative stress in Drosophila melanogaster. This study demonstrated that daily oxidative stress-induced mortality rhythm is cyclical, with a maximum susceptibility occurring during the late light phase and a minimum susceptibility occurring during late dark phase. This rhythm disappeared when Drosophila were continuously exposed to light. The rhythm was also abolished in the period clock gene mutant, a strain in which one of the two “clock genes” known as period are deleted, demonstrating that a fully functional circadian rhythm is necessary for this phenotypic effect to arise. Furthermore, period mutants displayed increased susceptibility to H$_2$O$_2$ compared with wild-type flies, as indicated by an increase in H$_2$O$_2$ production and a decrease in catalase activity. This novel study concluded that period and other genes controlling circadian rhythm are involved in ROS homeostasis by balancing ROS production and removal during the shift between light and dark periods.
Both a change in rhythmic clock gene expression and a shift in BP profile have recently been identified in the TGR(mREN-2)27 hypertensive rat model. These animals possess an additional copy of the mouse renin gene in their genome, leading to overexpression of renin and, therefore, an overactive renin-angiotensin (RAS) system that is characterized by high circulating Ang-II levels. Because of the higher levels of circulating Ang-II, these animals exhibit an inverted blood pressure profile that resembles the “non-dipping” pattern observed in humans and have internal desynchronization of normal circadian rhythm. It was discovered that there was an upregulation of BMAL1 and CLOCK in the SCN and of period2 in the rostroventral lateral medulla (RVLM) of these animals. A phase delay of 3.5 hours of period2 in the nucleus tractus solitarius (NTS), a cardiovascular regulatory control site responsible for regulating baroreflex control of blood pressure, was also observed. The baroreflex response receives afferent signals from the arterial baroreceptors and sends an inhibitory response to the RVLM, which controls sympathetic drive of neurons responsible for blood pressure control (Figure 1), and there is an interaction between the NTS and RVLM. Considering this, a link was made between angiotensin-II (Ang-II)-related hypertension with the circadian inversion characteristic of this rat model. This postulate was supported by Li et al. in 2009 who demonstrated that the vasopressin receptor (V1a) in wild type mice, including the inversion of the circadian rhythm, is controlled by the SCN.

One of the major sources of ROS is the NADPH oxidase (NOX) family of enzymes, whose molecular structure was not discovered until 1986 with the cloning of the gene encoding for one of the major subunits gp91phox (also known as NOX2). This discovery, in addition to the development of cell-free systems for purifying cytosolic and
membrane fractions, sparked much research aimed at identifying additional components of NOX. Since then, the p22phox, p47phox, p67phox, p40phox and the small GTP-binding proteins Rac1 and Rac2 subunits have all been discovered. These subunits, localized either to the membrane or cytosol, are involved in proper enzyme assembly and activation. The family of NADPH oxidases based on the structural identity of the NOX subunits is now comprised of NOX1, NOX2, NOX3, NOX4 and NOX5 as well as two related members dual oxidase 1 (DUOX1) and dual oxidase 2 (DUOX2).

In addition to identifying the key components and structures of the various homologues of Nox-containing enzymes, additional studies have examined their tissue expression. While the NOXs exhibit close structural and functional similarities to each other, each oxidase is expressed and regulated across distinct cell types and tissues other than phagocytic cells, where they are involved in the respiratory burst. These tissues include, but are not limited to, fibroblasts, carcinomas and vascular smooth muscle. Of particular interest, expression of the NADPH oxidases have been observed in the brain. Infanger et al. identified high expression of NOX2 and NOX4 in the mouse forebrain and midbrain, respectively, suggesting that an increase in NOX-derived ROS could play a role in the neurocardiovascular dysfunction associated with heart failure, hypertension and cognitive diseases such as Alzheimer’s Disease and Parkinson’s Disease.

The newest members of the NADPH oxidase family are the dual oxidases (DUOXs), which were originally described as thyroid oxidases (Thox) due to their primary role in thyroid hormone synthesis. However, they were re-named “DUOX” due to the presence of a peroxidase-like domain at the most amino-terminal transmembrane
domain in addition to a NADPH oxidase domain. Currently, two isoforms of DUOX have been discovered – DUOX1 and DUOX2 – and they share 99% sequence homology. However, in thyroid tissue, DUOX2 has a 1.5 to 5 times higher expression than DUOX1, and it is believed to be the main oxidase responsible for the production of thyroid hormone. Briefly, inorganic iodine enters the thyroid follicular cell upon consumption via a basolateral Na+/I- symporter. It is then shuttled across the apical membrane into the colloid cells’ cytoplasm of the thyroid via the anion transporter pendrin. Upon entering the colloid, thyroid peroxidase (TPO) oxidizes iodide into atomic iodine (I) or iodinium (I''), which is subsequently incorporated into thyroglobulin. TPO is catalyzed via ROS, particularly H2O2, on the apical surface of colloid cells. It is through this process by which DUOX2 plays a significant role, as it is the main source of the H2O2 needed for the function of TPO. In addition to its role in thyroid hormone synthesis, DUOX2 also functions in the lactoperoxidase (LPO)-mediated anti-infection system present in the salivary glands and in controlling ROS production in the respiratory and intestinal tracts. Elevated DUOX2 expression, localized to the apical poles of the colon, has been linked to the pathogenesis of inflammatory bowel syndrome. Moreover, In situ hybridization of rat salivary glands has demonstrated expression in the terminal portions of the salivary ducts, suggesting that ROS produced by DUOX2 are secreted into the saliva during the final steps of saliva formation.

Because of the molecular action of DUOX in the generation of H2O2 used in iodide organification, DUOX has become one of the key candidates to explain the genetic basis of hypothyroid diseases including congenital hypothyroidism. Figueiredo et al. demonstrated that low thyroid NADPH oxidases, particularly DUOX2, are present in
thyroids of patients with hypothyroidism. DUOX2 deficiency has also been observed in a severe hypothyroidism patient. Genotypic analysis revealed this patient was homozygous for a nonsense mutation in the portion of DUOX2 responsible for its NADPH oxidase function, further supporting the notion that DUOX2 is primarily responsible for the $H_2O_2$ generation required for thyroid hormone synthesis. Moreno also noted, however, that three patients heterozygous for a nonsense mutation in DUOX2 were able to produce sufficient amounts of thyroid hormone to meet the body’s requirements. These patients, particularly the patient with the homozygous nonsense mutation, are the only current in vivo evidence in humans linking DUOX2 with thyroid hormone synthesis.

Hypothyroidism has many effects on the cardiovascular system including impaired cardiac contractility, diminished cardiac output, higher systemic vascular resistance and cardiac atrophy. As a result, patients with hypothyroidism typically present with slightly elevated blood pressure and a diminished heart rate. Foley et al. examined the vascular control of this change in blood pressure and heart rate via the baroreflex and changes in autonomic tone and concluded that hypothyroid rats exhibit depressed arterial baroreflex function and increased dependence of resting sympathetic tone in both the heart and the vasculature. To better understand the local mechanism by which these changes in baroreflex and autonomic tone are controlled, a 2007 study by Carneiro-Ramos et al. examined the behavior of cardiac Ang-II receptors in surgically-induced hypothyroid rats. The authors concluded that there was upregulation of Ang-II receptors in the right ventricle, providing one rationale for the physiological effects observed in these rats.
While substantial evidence indicates that NADPH oxidase-derived ROS in both the periphery and central nervous system are involved in circadian rhythm and in cardiovascular diseases such as Ang-II hypertension and hypothyroidism, considerably less is known about the role of DUOX2 in areas other than the thyroid. In these studies, we sought to assess the role of DUOX2 in mediating circadian cardiovascular function by examining blood pressure, heart rate and locomotor activity via telemetry. Locomotor activity was measured in order to determine whether or not potential differences in the cardiovascular profile of DUOX2 knockout mice were due to inactivity since lethargy is typically observed in human hypothyroid patients.\textsuperscript{38} For this, we utilized a transgenic mouse model functionally deficient in the DUOX2 gene (i.e., DUOX2 null mice; DUOX2\textsuperscript{−/−}), which was confirmed by real-time PCR and \textit{in situ} hybridization. Considering DUOX2's known role in thyroid hormone synthesis, additional experiments were performed to determine the extent to which thyroid hormone contributes to these processes.

Here, we report that DUOX2\textsuperscript{−/−} mice exhibit a unique cardiovascular profile characterized by lower baseline mean arterial pressure (MAP) and heart rate (HR) and a "dipping" circadian rhythm that is significantly different from DUOX2 heterozygous (DUOX2\textsuperscript{+/−}) littermates. Interestingly, dietary supplementation with thyroid hormone did not restore the cardiovascular profile of DUOX2\textsuperscript{+/−} mice in DUOX2\textsuperscript{−/−} mice. Furthermore, DUOX2\textsuperscript{−/−} mice displayed increased baroreflex sensitivity compared to DUOX2\textsuperscript{+/−} controls, which have previously been shown to be no different from DUOX2 wild-type (DUOX2\textsuperscript{+/+}) mice in regards to cardiovascular function and locomotor activity. Real-time PCR and \textit{in situ} hybridization analyses confirmed the genotype of DUOX2\textsuperscript{−/−} mice by
demonstrating the absence of DUOX2 gene expression in the thyroid. Taken together, these results demonstrate that the unique cardiovascular profile exhibited by DUOX2<sup>−/−</sup> mice is unrelated to thyroid hormone deficiency.
**Figure 1:** Schematic summarizing central cardiovascular regulatory pathways of the brain. A potential role of ROS as signaling intermediates has previously been established. Circulating factors such as Ang-II interact with the CNS via areas lacking a blood-brain barrier including the SFO, OVLT and AP (yellow), which, in turn, send projections to hypothalamic and hindbrain structures such as the PVN, RVLM and NTS. AP, area postrema, NTS, nucleus tractus solitarius OVLT, organum vasculosum of the lamina terminalis, PVN, paraventricular nucleus, RVLM, rostral ventrolateral medulla, SFO, subfornical organ. Adapted from Zimmerman and Davisson and Infanger et al.
Materials and Methods

Animals

All experiments were performed on 10-12 week old DUOX2-Cre heterozygous (DUOX2+/−) and knockout (DUOX2−/−) mice (bred on a C57BL/6 background) obtained from the breeding colonies maintained at Cornell University. Original breeding pairs were acquired from our colleague, Dr. Botond Banfi, at the University of Iowa. Mice were housed in a temperature-controlled room (72°F) set to a 12:12-hour light-dark cycle (6 a.m. – 6 p.m. light: dark cycle) and had access to standard mouse chow and water ad libitum. In telemetry studies, telemetered mice were individually housed in a separate room set to the same light-dark cycle. These animals also had access to standard mouse chow and water ad libitum except for a period during which the chow was supplemented with thyroxine (chow was soaked in 25 mg/ml; Sigma; Saint Louis, MO) for 14 days. All procedures and protocols were approved by the Cornell University Institutional Animal Care and Use Committee (IACUC).

Genotyping

Mice were genotyped by polymerase chain reaction (PCR) using primer pairs based on the region of the DUOX2 deletion, which is located at exons 26-28. Primers were designed using the National Center for Biotechnology Information’s Basic Local Alignment Search Tool (BLAST) program and Integrated DNA Technology’s PrimerQuest program. As shown in Figure 1, three primers were designed: (1) one forward primer before the deleted sequence; (2) one reverse primer in the deleted sequence and (3) one reverse primer located outside the deleted sequence. Additional
primer details are presented in Table 1. All primers were obtained from Integrated DNA Technologies (Coralville, IA).

Tail snips were collected when the mice were weaned (~2.5 weeks old) and DNA extracted using the HotSHOT technique (BioTechniques 29:52-54,2000). The PCR protocol was optimized using the TaKaRa Ex Taq PCR kit, using a total reaction volume of 25 μl and an additional 1.5 μl of 2.5mM magnesium chloride. The amplification product was then subjected to gel electrophoresis in a 1% agarose gel run at 125 volts for approximately one hour. Gels were scored using a BioRad Gel Doc XR system and associated Quantity One 1D-Analysis software. Genotypes were determined according to band size: the DUOX2+/+ band was 666 base pairs, whereas the DUOX2−/− band was 467 base pairs.

**Telemetry**

**Radiotelemetry Surgery**

Mice were anesthetized (ketamine, 117 mg/kg in combination with xylazine, 17 mg/kg intraperitoneally, i.p.) and implanted with TA11PA-C10 radiotelemetry probes (Data Science International, Arden Hills, MN). An incision was created in the cardiothoracic area of the mouse and the left common carotid artery bluntly dissected from the surrounding fascia. The telemeter catheter was inserted into the thoracic aorta via the carotid artery, and the telemeter body was placed in a subcutaneous pocket created in the right flank of the mouse. The cardiothoracic opening was then sutured, after which the mouse was allowed to recover on a heating pad in order to maintain constant body temperature at 37°C. Mice were then allowed to recover for one week in individual telemetry cages and checked twice daily for a period of five days for general
condition, food and water intake, and the state of healing around the surgery site. Further, mice received a subcutaneous dose of buprenorphine (0.05 mg/kg; Sigma) twice daily for a period of up to three days post-surgery.

*Telemetry Monitoring*

Following surgery, telemetered mice were transferred to individual cages and housed on an animal rack specially equipped with hardware for assessing cardiovascular function via telemetry. Mice were allowed to recover for 7 days before the beginning of experiments in order for normal circadian rhythm to be restored. Following this recovery period, telemetry probes were activated for continuous measurement of baseline mean arterial pressure (MAP) for a total of 7 days (sampling frequency=500 Hz), from which heart rate (HR) was derived. 2,000 Hz frequency recordings were also performed for a period of 1-2 hours between the hours of 9:00 a.m. and 12:00 p.m. for subsequent analysis of autonomic function via spectral analysis and spontaneous baroreflex function via the sequence method. Following baseline sampling, the diet of the mice was supplemented with thyroid hormone for a period of 9 days. After this period, continuous measurement was again continued for a period of 5 days. Circadian rhythm was analyzed using 4-hour averages over both periods of testing.

*Spectral and Baroreflex Analysis*

The high frequency 2,000 Hz recordings obtained during the baseline and thyroxine treatment periods were used to evaluate spontaneous baroreflex sensitivity (BRS) and autonomic tone. Baroreflex gain was derived from spontaneous, reciprocal fluctuations in MAP and HR by the sequence method and analyzed using the HemoLab
software provided by Dr. Harald Stauss (University of Iowa). First, recordings were loaded into the Analysis portion of the HemoLab software and all artifacts removed. The regions of interest, separated by periods of activity and inactivity, were then selected and a baroreflex test performed. Periods of activity and inactivity were determined using a built-in activity meter in the telemetry receivers. The parameters used for the baroreflex test were: a correlation coefficient of 0.85 between MAP and HR, a pressure threshold of 15 mmHg, and a lag of four beats. In this analysis, a baroreflex sequence (BRR) was defined as four consecutive heart beats in which both MAP and pulse interval either increased (“up” sequence) or decreased (“down” sequence). A non-baroreflex sequence (nBRR) was defined as four consecutive heartbeats in which MAP and pulse interval were inversely correlated (i.e., one increased while the other decreased).

Spectral analysis of autonomic tone was also performed using the HemoLab software. Four channels corresponding to HR, systolic blood pressure (SBP), MAP, and diastolic blood pressure (DBP) were derived from the cleaned tracings used in the baroreflex analysis. A linear transformation of the HR data was then performed to convert HR from beats per second to beats per minute. The 2,000 Hz data were then converted to 25 Hz using a spline interpolation. Next, using the Batch Processor program in HemoLab, frequency histograms were created for each 60-minute interval using a 4,096-point Fast Fourier Transformation (FFT) with percent overlap equal to 50%. Next, the area under the curve of each Overlapping Segment file was calculated after manual corrections for the respiration frequency, which was determined by manually locating a natural peak in the Overlapping Segment file typically found between 2.0 and 5.0 Hz. Spectra were then divided into three distinct frequency ranges: very low frequency (0.08-
0.4 Hz), low frequency (0.4-1.0 Hz), and high frequency (1.0-3 Hz) as described previously by Janssen et al.\textsuperscript{41} From these data, LF/HF ratios were calculated to determine the relative impact of the parasympathetic and sympathetic nervous systems on heart rate and blood pressure variability.

**Pharmacological Testing**

In addition to analyzing baroreflex and autonomic function via the sequence method and spectral analysis, respectively, supplemental studies were performed using a classical pharmacological approach. For the autonomic control studies, DUOX2\textsuperscript{+/−} and DUOX2\textsuperscript{−/−} were treated with the muscarinic receptor antagonist atropine (1 μg/g; Sigma); the β-adrenergic receptor antagonist propranolol (1 μg/g; Sigma); the α\textsubscript{1} adrenergic receptor antagonist prazosin (1 μg/g; Sigma); and the ganglionic blocker hexamethonium (50 μg/g; Sigma). Continuous blood pressure was first recorded for one hour to establish baseline parameters, after which the mice were injected intraperitoneally (i.p.) with each drug. Recordings were continued for an additional hour. The peak blood pressure and heart rate responses to each drug were used to characterize respective pharmacological responses. These drugs were given between the daytime hours of 11:00 a.m. and 2:00 p.m., and the single injection of each drug was separated by a minimum of 24 hours.

Baroreflex sensitivity was also evaluated pharmacologically using phenylephrine (PE; 4 μg/kg; Sigma) and sodium nitroprusside (SNP; 10 μg/kg; Sigma). These drugs were given as a bolus intravenously (i.v. bolus) through a chronic, indwelling catheter inserted into the jugular vein. The venous catheter was inserted two days before injecting any drugs to allow for full recovery of baseline HR and MAP. Baseline MAP and HR
were measured for 30 minutes before the drugs were injected, and peak HR and MAP responses to the drugs were observed within one minute post-injection.

**T4 Enzyme-Linked ImmunoSorbent Assay**

Serum T4 levels in DUOX2\(^{+/−}\) and DUOX2\(^{−/−}\) animals at baseline and during thyroxine treatment were determined using a free T4 ELISA kit (Endocrine Technologies Incorporated; Newark, CA). Serum was collected during baseline and thyroxine treatment via a retro-orbital eye bleed. Diluents used in the protocol were prepared using a 1:4 serum to sample diluent mixture. The assay was performed using a single 96-microwell plate provided in the kit. First, 50 μl of the standards and diluted specimens were dispensed into the wells in duplicates. Next, 100 μl of the T4 HRP-Conjugate Reagent was added to each well and mixed with the sample for 30 seconds. The plate was then incubated at 37°C for one hour. The microwell wells were then completely washed using a wash buffer five times. Next, 100 μl of the TMB color reagent was added to each well, and the plate was incubated in the dark for 20 minutes at room temperature. The reaction was then stopped using 50 μl of 2N HCl and the plate read at an optical density of 450 nm. The serum T4 concentrations were determined by interpolation using a standard curve created using the provided standards.
**mRNA Expression Profile**

DUOX2 gene expression was determined qualitatively by *in situ* hybridization (ISH) and quantitatively by real-time PCR.

**In Situ Hybridization Probe**

A DUOX2 ISH probe was prepared and tested by Dot Blot. A Digoxigenin (DIG)-labeled RNA probe sequence was designed using BLAST and PrimerQuest and was based on the deleted sequence in the knockout mouse (Table 2). Serial dilutions of 1:1, 1:10, 1:100, 1:1000, and 1:10000 were prepared, and 1 μl of each dilution, including a β-actin control probe, was spotted onto an Immobilon-Ny+ membrane (Millipore, Billerica, MA). The blots were then completely dried for 10 minutes at 80°C. Next, ultraviolet cross-linking was performed using an energy setting of 12,000 μJoules/cm². The blot was then equilibrated in wash buffer for five minutes at room temperature before being agitated in blocking solution for one hour at room temperature. After incubation in the blocking solution, a dilute solution of Anti-DIG-Alkaline phosphatase (1:5000 in blocking solution) was prepared. The membrane was then placed and incubated in the anti-DIG-Alkaline phosphatase solution for a total of one hour at room temperature with agitation. The membrane was then washed in washing buffer for a total of 30 minutes. Next, the blot was incubated in detection buffer for five minutes followed by an incubation of 2-16 hours in staining solution (NBT/BCIP in detection buffer). After sufficient color intensity appeared in the control probe, the reaction was stopped by washing the membrane in sterile water for five minutes.
In Situ Hybridization

DUOX2°° and DUOX2° mice were placed under general anesthesia (ketamine, 117 mg/kg in combination with xylazine, 17 mg/kg, i.p.). A small incision was made in the throat area and the thyroid bluntly dissected from the surrounding fascia and trachea. Both lobes of the thyroid were excised and snap-frozen on dry ice.

Thyroid samples were mounted in optimal cutting temperature reagent (OCT), cryosectioned at 10 μm and mounted onto RNase-free microscope slides in triplicate for hybridization with either (1) no probe (control), (2) sense probe, or (3) antisense probe. Slides were incubated for 10 minutes at 65°C followed by room temperature (25°C) for 10 minutes. Tissues were then fixed at room temperature using a fresh preparation of 4% solution of paraformaldehyde (PFA). The slides were agitated in phosphate buffer saline (PBS) 3 times for 3 minutes each. Next, slides were incubated in acetylation solution at room temperature for 10 minutes. Acetylation solution was removed completely from the slides post-incubation by a series of three, three-minute washes with PBS. Slides were then placed in a chamber humidified with 50% formamide/5X SSC with approximately 500 μl of hybridization solution for one hour at room temperature. After the incubation with hybridization solution, 100 μl of the DUOX2 ISH RNA probe (sense or antisense) (400 ng/ml in hybridization solution) was placed on the slides, cover-slipped and incubated overnight at 65°C.

After this incubation, the slides were subjected to three hours of further incubation in 0.2X SSC at 65°C, with changes of solution every 45 minutes. The slides were then agitated for two sets of five minutes in Tris buffered saline (TBS). Slides were then subjected to incubation with blocking solution in a humidified chamber for 2 hours.
The blotting solution was blotted off and replaced with 250 µl of primary antibody (Alkaline Phosphatase-Anti-Digoxigenin/Fab fragments, Roche, Indianapolis, IN) for one hour at 4°C. Slides were washed with TBS three times for 10 minutes each followed by agitation in Buffer 3 for a period of 5 minutes. Slides were incubated with the staining solution (NBT/BCIP, Roche, Indianapolis, IN) in the dark until a purple color was observed. The staining solution was removed using TBS and slides counterstained with methyl green (Sigma) to observe histology, specifically the nuclei, of the thyroid. Staining was observed using a Leica Microsystems CTR6000 B light microscope at magnifications of 4X, 20X and 40X, and images were captured using a Q-Imaging Retiga 2000R camera attached to the microscope.

*Real-Time Polymerase Chain Reaction*

Thyroids of DUOX2+/− and DUOX2−/− mice were collected directly into TRizol reagent for immediate total RNA extraction. Tissue was homogenized using a Tissue Tearor homogenizer with RNase free disposable tips and incubated for 5 minutes at room temperature. Next, 0.2 ml of chloroform was added and allowed to incubate with the TRizol solution for a period of 3 minutes at room temperature. The solution was transferred into a 1.8 ml PCR tube and centrifuged at 12,000G for 15 minutes at 4°C. The aqueous phase was transferred to a new PCR tube and the following solutions added to the aqueous phase: 20 µg of RNase-free glycogen and 0.5 ml of isopropanol. The tube was then gently mixed and incubated at -80°C for 55 minutes. After incubation, the tube was centrifuged at 12,000G for 15 minutes at 4°C, the supernatant removed and the RNA pellet washed using 75% ethanol, and the pellet dried and re-suspended in 15 µl nuclease-
free water. RNA quality was tested using a Bioanalyzer; only RNA samples with a RNA integrity number (RIN) of 7.0 and higher were used for real-time PCR.

Single-stranded cDNA was prepared via reverse transcription with SuperScript III (Invitrogen, Carlsbad, CA). Briefly, 100 ng of total RNA was placed in a 0.5 ml PCR microcentrifuge tube along with 1 µl of random primers, 1 µl of dNTPs, and nanopure water up to a reaction volume of 13 µl. The mixture was heated to 65°C for five minutes. The tube was then briefly centrifuged and the following solutions from Invitrogen were added: 4 µl of 5X First-Strand Buffer, 1 µl of 0.1 M DDT, 1 µl of RNaseOUT, and 1 µl of SuperScript III RT (200 units/µl). The PCR tube was incubated at 25°C for 5 minutes, 50°C for 60 minutes and 70°C for 15 minutes.

cDNA equivalent to 12.5 ng of RNA was used for the real-time PCR plate. Real-time PCR was performed using Invitrogen’s Power SYBR Green PCR Master Mix. Briefly, each well on the PCR plate contained the following solutions for a total reaction volume of 25 µl: 12.5 µl of the SYBR master mix, 0.75 µl of the DUOX2 forward primer, 0.75 µl of the DUOX2 reverse primer and 10 µl of cDNA. The plate was centrifuged for 5 minutes at 6,000G and run using an Applied Biosystems 7500 Fast Real-Time PCR system.

Statistics

Results are expressed as mean ± SEM. Statistically significant differences in means, including changes in cardiovascular parameters, were analyzed by one-way ANOVA with a follow-up Bonferonni posttest. A student’s t-test was also performed where applicable. A value of p<0.05 was used to determine statistical significance.
Figure 2: Schematic of PCR primer design for genotyping DUOX2 mice based on the deleted region located at exons 26-28.

Table 1: PCR primer information for genotyping DUOX2 mice.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
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<tr>
<td>F</td>
<td>ACTGTATTCCTGACATGGCTGCT</td>
</tr>
<tr>
<td>R1</td>
<td>ACTTGGGATGGAAGGGACGGTA</td>
</tr>
<tr>
<td>R2</td>
<td>TTGCAGACCCGTGCCTACTGTAAGA</td>
</tr>
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</table>

Table 2: DUOX2 in situ hybridization probe information.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5' $\rightarrow$ 3')</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUOX2-610-SP76</td>
<td>CAGGTGCCTATGAAGGACAGAA</td>
<td>610 base pairs</td>
</tr>
<tr>
<td>(Sense)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DUOX2-610-T7</td>
<td>GGGACGGTAAGTGGGAAGATG</td>
<td>680 base pairs</td>
</tr>
<tr>
<td>(Antisense)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results

**DUOX2 null mice display hypotension and bradycardia compared to DUOX2 heterozygote controls.**

As shown in Figure 3, 24-hr mean arterial pressure (MAP) and heart rate (HR), which was measured via telemetry over a 5-day period, was significantly lower in DUOX2 null mice (MAP: 92.18±2.89 mmHg; HR: 471.8±16.0 bpm, n=11, p<0.05) compared to DUOX heterozygote controls (MAP: 103.20±1.37 mmHg; HR: 551.17±8.1 bpm, n=13). In regards to circadian rhythm, this hypotension and bradycardia were observed during periods of daytime as well as nighttime, with a much more pronounced “dip” in MAP and HR observed during the nighttime. This cardiovascular profile was independent of locomotor activity, as there was no significant difference observed between the two groups during any period of the day (Figure 3).

**Thyroid hormone supplementation does not eliminate the phenotypic cardiovascular differences in MAP and HR in DUOX2 null mice.**

Next, to test whether these changes in MAP and HR were due to thyroid hormone deficiency in DUOX2 null mice, the cardiovascular parameters of HR and MAP were recorded using radiotelemetry following supplementation of animals’ chow with thyroxine for 14 days. As shown in Figure 5, thyroid hormone supplementation raised both MAP and HR in DUOX2 null mice (from baseline: ΔMAP Day=12.58 mmHg, Night=18.42 mmHg; ΔHR Day=138 bpm, Night=130 bpm) and in DUOX2 control mice (from baseline: ΔMAP Day=16.54 mmHg, Night=18.89 mmHg; ΔHR Day=116 bpm, Night=125 bpm). However, the extent of the increase in both MAP and HR during thyroid hormone supplementation was comparable in both DUOX2 controls and DUOX2
null mice. In contrast, locomotor activity was significantly elevated during the nighttime in DUOX2+/− mice (10.4±1.97 a.u., n=11, p<0.05) compared to DUOX2+/− littermates (5.6±0.90 a.u., n=13) (Figure 4). Interestingly, the excessive drop, or “shift,” in MAP and HR observed in DUOX2−/− mice during the nighttime persisted during the thyroxine treatment period, further demonstrating that this unique circadian cardiovascular profile is not caused by thyroid hormone deficiency.

Thyroxine treatment increases serum levels of T4 in both DUOX2 heterozygote controls and DUOX2 null mice

In order to confirm that thyroxine supplementation did indeed increase serum T4 levels, a free T4 ELISA was performed. As anticipated, free T4 levels in DUOX2 control mice (9.3±2.2 ng/ml, n=4, p<0.05) were significantly higher than those in thyroid hormone-deficient DUOX2 null mice (0 ng/ml, n=3) at baseline (Figure 6). However, treatment with thyroid hormone elevated serum T4 levels to the same extent in both DUOX2 control (200.37±35.2 ng/ml, n=4) and DUOX2 null (214.57±18.6 ng/ml, n=3) mice (Figure 6).

Power spectral analysis demonstrates no change in either sympathetic or parasympathetic control of SBP and HR in DUOX2 null mice.

Since heart rate (HR) and blood pressure in DUOX2 null mice demonstrated a “dipper” circadian profile, we tested if this profile was due to a change in the autonomic control of heart rate and blood pressure variability. To determine sympathetic and parasympathetic regulation of blood pressure and HR, raw radiotelemetry data was subjected to power spectral analysis. In mice, the low frequency range (LF, 0.15-0.60 Hz) corresponds to the sympathetic component of blood pressure variability, whereas the high
frequency range (HF, 2.5-5.0 Hz) corresponds to the parasympathetic component of heart rate variability.\textsuperscript{42} The results of power spectral analysis of HR and systolic blood pressure (SBP) in DUOX2\textsuperscript{+/-} and DUOX2\textsuperscript{-/-} at baseline and during thyroxine treatment are presented in Figure 7. At baseline, DUOX2\textsuperscript{+/-} mice exhibited a significantly higher HF component of HR (13.93\pm1.22 msec\textsuperscript{2}, n=7, p<0.05) compared to DUOX2\textsuperscript{+/-} controls (8.23\pm0.84 msec\textsuperscript{2}, n=8), which resulted in a decreased LF/(HF+LF) ratio (DUOX2\textsuperscript{+/-}: 0.69\pm0.04; DUOX2\textsuperscript{-/-}: 0.56\pm0.04). However, there was no significant difference in autonomic control of blood pressure. Since sympathetic drive is not usually involved in beat-to-beat regulation of HR in non-diseased animals, autonomic modulation of blood pressure and heart rate was also assessed pharmacologically in a separate cohort of DUOX2\textsuperscript{+/-} and DUOX2\textsuperscript{-/-} animals. This was done using the ganglionic blocker hexamethonium (50mg/kg); the non-selective β-adrenergic receptor antagonist propranolol (4mg/kg); the α1-selective adrenergic receptor antagonist prazosin (1 mg/kg); and the muscarinic receptor antagonist atropine (2 mg/kg). As shown in Figure 8, the changes in MAP and HR evoked by each of these compounds were not different between DUOX2\textsuperscript{+/-} and DUOX2\textsuperscript{-/-} animals.

\textbf{DUOX2\textsuperscript{-/-} mice display augmented baroreflex control of blood pressure and heart rate.}

Spontaneous baroreflex sensitivity (BRS) was determined in DUOX2\textsuperscript{+/-} and DUOX2\textsuperscript{-/-} mice using the sequence method. In this analysis, BRS was calculated from spontaneously occurring sequences of four or more consecutive heartbeats during which MAP and HR pulse interval simultaneously increased ("up" sequence) or decreased ("down" sequence). Using the sequence method, the slope of the linear regression line, or gain, of the relationship between HR and MAP was calculated and used as an index of
BRS (Figure 9). DUOX2\textsuperscript{−/−} mice exhibited an increased baroreflex sensitivity baseline (5.14±0.97 msec/mmHg, \(n=7\); \(p<0.05\)) compared to DUOX2\textsuperscript{+/−} mice (2.26±0.33 msec/mmHg, \(n=8\)). This difference, however, was abolished by administration of thyroxine.

In another series of experiments, BRS was also assessed by the classical Oxford method, which entailed classical, vasoactive drug-evoked activation of the reflex using the \(\alpha_1\)-adrenergic receptor agonist phenylephrine (PE, 4\(\mu\)g/kg, i.v. bolus) and the potent nitric oxide donor and vasodilator sodium nitroprusside (SNP, 10\(\mu\)g/kg, i.v. bolus). As shown in Figure 10, i.v. bolus PE significantly elevated MAP in DUOX2\textsuperscript{−/−} (\(\Delta\text{MAP}=28.75±2.90\) mmHg, \(n=4\), \(p<0.05\)) compared to DUOX2\textsuperscript{+/−} controls (\(\Delta\text{MAP}=17.75±1.03\) mmHg, \(n=4\)) despite evoking a similar reflex bradycardia in both groups (DUOX2\textsuperscript{+/−}: \(\Delta\text{HR}=-146.50±26.72\) bpm; DUOX2\textsuperscript{−/−}: \(\Delta\text{HR}=-118.50±32.62\) bpm). Moreover, the hypotension and reflex tachycardia produced by i.v. bolus SNP were similar in both DUOX2\textsuperscript{+/−} and DUOX2\textsuperscript{−/−} animals.

**Real-time PCR and in situ hybridization confirm that DUOX2 null mice lack expression of DUOX2.**

The expression of DUOX2 mRNA in DUOX2 null mice was assessed via real-time PCR in the thyroid, and results were standardized against DUOX2 control mice (Figure 11). Compared to DUOX2 control mice, DUOX2 mRNA expression was completely abolished in the DUOX2 null mice.

**In situ** hybridization (ISH) was also performed to characterize DUOX2 gene expression in DUOX2 control vs. null mice. First, a DUOX2 mRNA probe was designed and tested (Figure 12) using a dot blot against \(\beta\)-actin. Compared to a \(\beta\)-actin control
probe, the DUOX2 probe exhibited very high expression at all dilution levels. Once this was confirmed, an ISH experiment was performed in which the DUOX2 mRNA probe was labeled with a colorimetric tag (purple color) conjugated to a Digoxigenin (DIG) antibody. Methyl green was used to counter-stain nuclei and to identify surrounding histology. In DUOX2 control mice, the DUOX2 sense probe properly yielded no labeling, whereas the antisense probe was localized primarily to the membranes of the colloid cells where thyroid hormone is synthesized (Figure 13 A-C). ISH of DUOX2 null mice showed no specific staining in any part of the thyroid (Figure 13 D-E), confirming the genotype of the DUOX2 null mice.
Figure 3: Baseline cardiovascular function and locomotor activity over a 5-day period in DUOX2\textsuperscript{+/−} (n=13) and DUOX2\textsuperscript{−/−} mice (n=11). DUOX2 \textsuperscript{−/−} mice exhibit significantly lower baseline MAP and HR during both the day and night in addition to a “dipper” circadian profile. Circadian data: 4-hr averages; Group data: 5-day averages during the “Day” (6 a.m. – 6 p.m.) and “Night” (6 p.m. – 6 a.m.) periods. Data presented as mean ± SEM. Mean arterial pressure (MAP), heart rate (HR), and activity (arbitrary units). *p<0.05 vs. DUOX2\textsuperscript{+/−} controls.
Figure 4: Effect of thyroid hormone supplementation on cardiovascular function and locomotor activity over a 5-day period in DUOX2−/− (n=8) and DUOX2+/+ (n=6) mice. Thyroxine treatment causes a significantly higher locomotor activity level in DUOX2−/− mice but does not eliminate differences in MAP and HR during the day and night or the circadian "dipper" profile. Circadian data: 4-hr averages; Group data: 5-day averages during the “Day” (6 a.m. – 6 p.m.) and “Night” (6 p.m. – 6 a.m.) periods. Data presented as mean ± SEM. Mean arterial pressure (MAP), heart rate (HR), and activity (arbitrary units). *p<0.05 vs. DUOX2+/+ controls.
Figure 5: Delta increases in MAP (top panel) and HR (bottom panel) after thyroxine treatment in DUOX2\textsuperscript{+/−} (n=8) and DUOX2\textsuperscript{−/−} (n=6). Thyroxine treatment causes a comparable increase in both MAP and HR in both DUOX2\textsuperscript{+/−} and DUOX2\textsuperscript{−/−} mice. Circadian data: 4-hr averages; Group data: 5-day averages for baseline and thyroxine treatment during the “Day” (6 a.m. – 6 p.m.) and “Night” (6 p.m. – 6 a.m.) periods. Data presented as mean ± SEM. Mean arterial pressure (MAP) and heart rate (HR).
Figure 6: Serum free T4 concentration in DUOX2$^{+/-}$ and DUOX2$^{-/-}$ mice at baseline (BL) and after thyroxine treatment (T4) periods (n=3-4/per group). Dietary supplementation with thyroxine eliminates the differences in T4 plasma levels observed between DUOX2$^{+/-}$ and DUOX2$^{-/-}$ mice at baseline. Data presented as mean ± SEM. *p<0.05 vs. BL DUOX2$^{+/-}$; †p<0.05 vs. both BL DUOX2$^{+/-}$ and BL DUOX2$^{-/-}$.
Figure 7: Power spectral analysis of heart rate and blood pressure variability in DUOX2<sup>+/−</sup> (n=8) and DUOX2<sup>−/−</sup> (n=7) mice before and after thyroxine treatment. DUOX2<sup>−/−</sup> mice exhibit a significantly lower LF/(LF+HF) ratio, corresponding to increased sympathetic control of HR. Data presented as mean ± SEM. Low frequency (LF) and high frequency (HF). *p<0.05 vs. DUOX2<sup>+/−</sup> controls.
Figure 8: Changes in MAP (top panel) and HR (bottom panel) evoked by i.p. hexamethonium, propranolol, prazosin and atropine in DUOX2<sup>−/−</sup> and DUOX2<sup>+/−</sup> mice. No significant difference in either sympathetic or parasympathetic control of MAP or HR was observed in DUOX2<sup>−/−</sup> and DUOX2<sup>+/−</sup>. Data presented as mean ± SEM.
Figure 9: Baroreflex sensitivity (BRS) in DUOX2^{+/-} (n=8) and DUOX2^{-/-} (n=7) mice as determined by the sequence method during periods of locomotor activity vs. inactivity. DUOX2^{-/-} null mice exhibit increased baroreflex sensitivity compared to DUOX2^{+/-} control mice. Data presented as mean ± SEM. *p<0.05 vs. DUOX2^{+/-} controls.
Figure 10: Peak changes in mean arterial pressure and heart rate evoked by pharmacological activation of the baroreflex with i.v., bolus sodium nitroprusside (SNP) and phenylephrine (PE) in DUOX2⁺/- (n=4) and DUOX2²⁻ (n=4) mice. PE evokes a significantly higher rise in MAP in DUOX2⁺/- mice compared to DUOX2²⁻ controls, demonstrating increased baroreflex sensitivity in DUOX2²⁻ mice. Data presented as mean ± SEM. *p<0.05 vs. DUOX2⁺/- controls.
Figure 11: DUOX2 gene expression is abolished in the thyroids of DUOX2−/− null mice as determined by real-time PCR. Data expressed represents fold-change in expression compared to DUOX2+/+ controls. DUOX2−/− mice exhibit completely lack DUOX2 gene expression in the thyroid, confirming their genotype as global DUOX2 knockouts. Data presented as mean ± SEM. *p<0.05 vs. DUOX2+/+ controls.
Figure 12: Dot blot quantification of the DUOX2 cRNA probe used for ISH (860 T7/SP6, 610 T7/SP6) performed in serial dilutions (1:1, 1:10, 1:100, 1:1000, 1:10000) and tested against a β-actin control probe. DUOX2 probes demonstrate staining comparable to the control probe in all dilutions. T7, Antisense Probe; SP6, Sense Probe.
Figure 13: *In situ* hybridization labeling of DUOX2 mRNA in DUOX2\(^{+/-}\) mouse thyroid using (A) no probe; (B) Sense Probe; and (C) Antisense Probe and in DUOX2\(^{-/-}\) mouse thyroid using (D) Sense Probe and (E) Antisense Probe. DUOX2\(^{+/-}\) mice exhibit specific staining localized to colloid cells, whereas DUOX2\(^{-/-}\) null mice exhibit no specific staining in the thyroid, confirming their genotype as global DUOX2 knockouts. 20X.
Discussion

The present studies have characterized the effects of DUOX2 gene deletion on the cardiovascular parameters of 24-hr conscious mean arterial pressure (MAP) and heart rate (HR). Our findings demonstrate that DUOX2 null mice, whose genotypes were confirmed using real-time PCR and in situ hybridization, exhibit baseline hypotension and bradycardia compared to DUOX2\textsuperscript{+/−} control littermates, neither of which is restored to control values following supplementation with thyroxine to establish equivalent serum T4 levels. Furthermore, the differences in both MAP and HR observed at baseline were independent of locomotor activity, which was somewhat surprising considering the lethargy commonly observed in hypothyroid patients.\textsuperscript{38} Based on these findings, we concluded that the baseline hypotension and bradycardia observed in DUOX2\textsuperscript{−/−} mice were not a result of the diminished thyroxine production due to the global deletion of DUOX2.

The present studies provide multiple lines of evidence demonstrating that elimination of the DUOX2 gene in the mouse not only results in the development of hypotension and bradycardia but also in an alteration of the circadian pattern of cardiovascular function. Specifically, DUOX2 null mice exhibit a characteristic dipper profile during both baseline and thyroxine treatment. Although this circadian profile could be attributed to increased parasympathetic drive\textsuperscript{43}, our findings support the concept that the dipper profile observed in DUOX2\textsuperscript{−/−} mice is not controlled by a change in autonomic control of heart rate and blood pressure variability and is not due to reduced locomotor activity. Specifically, two methods of determining autonomic tone in DUOX2 null were employed: 1) classical vasoactive administration of drugs that stimulate the
baroreflex and 2) power spectral analysis of spontaneous baroreflex sensitivity using 2,000 Hz telemetric recordings performed during both the baseline and thyroxine treatment periods. Surprisingly, two different results were observed. Specifically, no differences in the changes in either blood pressure or heart rate evoked by i.v. bolus atropine or propranolol were observed between groups. However, power spectral analysis of spontaneous baroreflex sensitivity demonstrated that DUOX2 null mice have a significantly higher sympathetic component of heart rate during baseline. One potential reason for this discrepancy could be that the sympathetic drive of HR typically does not impact beat-to-beat function. Alternatively, this discrepancy could be the method by which baroreflex sensitivity was assessed. Whereas the mice were virtually stress-free during acquisition of the telemetry recordings subsequently used for sequence method analysis of spontaneous baroreflex sensitivity, the animals were handled and subjected to more stress during the pharmacology studies.

In the present studies, we also examined whether the differences in cardiovascular function between DUOX2+/− and DUOX2+/+ mice were due, at least in part, to differences in baroreflex function. For this, baroreflex sensitivity (BRS) was assessed using two different methods. In the first method, spontaneous BRS was analyzed via the sequence method using high-frequency (2,000 Hz) blood pressure recordings obtained by telemetry. In the second method, baroreceptor reflex control of blood pressure and heart rate was quantified by classical vasoactive drug administration. In the spontaneous BRS study, the only significant difference observed was the "up" sequence gain at baseline in DUOX2+/− mice, which was significantly higher in these animals compared to DUOX+/− littermates. The "up" sequences correspond to when blood pressure and heart rate rise
and fall simultaneously, respectively. In the corresponding vasoactive drug study, PE elicited a significantly higher increase in MAP in DUOX2\textsuperscript{−/−} mice than in DUOX2\textsuperscript{+/−} mice, but no significant differences in HR were observed. As with the autonomic function studies, this discrepancy could be attributable to the handling of the mice prior to i.v. bolus injection of either PE or SNP as opposed to the “stress-free” state of the animals during the sequence method analysis.

In the present study, we also confirmed the genotype of DUOX2 null mice using real-time PCR and \textit{in situ} hybridization of thyroid tissue. DUOX2 control mice exhibited localization of DUOX2 to colloid cells in the thyroid, an area that is primarily responsible for production of thyroxine and DUOX2’s primary function.\textsuperscript{27} Furthermore, both real-time PCR and \textit{in situ} hybridization confirmed that DUOX2 mRNA is indeed absent in DUOX2 null thyroids, providing an explanation for the difference in serum T4 levels between DUOX2\textsuperscript{+/−} vs. DUOX2\textsuperscript{−/−} mice observed at baseline.

Since the dipper profile was not abolished upon supplementation of both DUOX2 control and DUOX2 null mice with thyroxine, there are three alternate explanations for this circadian profile. First, this difference could be due to a secondary function of DUOX2 in key cardiovascular control sites such as the SFO, PVN and RVLM. Studies examining DUOX2 expression in these regions are currently underway. Alternatively, results observed in separate studies demonstrate that overexpression of renin and/or angiotensin in rats can result in a “non-dipper” profile. Since DUOX2 has a major role in the production of ROS, it is possible that this shift can be due to diminished levels of key players in the renin-angiotensin system (RAS).\textsuperscript{44} Further studies testing circulating and brain levels of renin and angiotensin could help to determine if this profile is a result of
dysfunction in RAS activity. A third potential reason for this profile could be due to prenatal differences of DUOX2 null mice. It has been shown that stress experienced *in utero* can lead to adult-onset phenotypic differences in specific cardiovascular parameters including both HR and MAP.\(^{45-48}\) Further studies supplementing thyroxine to pregnant mothers to eliminate thyroxine-related stress could determine whether the prenatal environment is the cause of this circadian profile. The specific signaling pathway involved, however, will be the focus of future studies.

In summary, we have demonstrated that the elimination of the DUOX2 gene in mice not only results in a decrease in both MAP and HR but also an increased component of the sympathetic control of heart rate during baseline and a statistically lower sympathetic control of systolic blood pressure during thyroxine treatment, leading to the observed circadian profile characteristic of a classical "dipper". Furthermore, DUOX2 null mice demonstrate an increase in baroreflex sensitivity. Although supplementation with thyroxine restored MAP and HR in null mice to baseline control mice values, these differences do not appear to be entirely due to loss of thyroid hormone since thyroxine treatment did not fully abolish the phenotypic differences in cardiovascular function when compared to control mice also treated with thyroxine. This difference may be mediated by the function of DUOX2 in key areas of the brain responsible for control of cardiovascular, baroreflex and circadian parameters including the SFO, PVN, RVLM, NTS and SCN. Further studies examining ROS levels, renin, angiotensin-II and other key players in controlling blood pressure in DUOX2 null mice vs. DUOX2 control mice are underway to discern the exact mechanism by which DUOX2 regulates cardiovascular function. Moreover, real-time PCR can be employed to more precisely determine the
expression levels of DUOX2 in key cardiovascular and autonomic regulatory regions of
the brain, and in situ hybridization and immunohistochemistry studies will be needed to
determine which cell type (e.g., neurons, glia, astrocytes, etc.) DUOX2 is localized to at
these sites.
Literature Cited


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