

Overexpression of human Cu/Zn Superoxide Dismutase in Mice; The Effect of Increase Superoxide Scavenging on Autonomic Control of the Heart.

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OVEREXPRESSION OF HUMAN Cu/Zn SUPEROXIDE
DISMUTASE IN MICE: A MODEL TO STUDY THE EFFECT OF
INCREASED SUPEROXIDE SCAVENGING ON THE AUTONOMIC
CONTROL OF THE HEART.

by

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A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
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ABSTRACT

Dysregulation of the autonomic cardiovascular control is a complication of diseases including diabetes, hypertension, sleep apnea, and aging. A common factor in these conditions is an increase in reactive oxygen species (ROS) in neural, cardiac, and endothelial tissues. Cu/Zn superoxide dismutase (SOD1) is an intracellular anti-oxidant enzyme that catalyzes dismutation of the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2). Expression and function of this enzyme are diminished in pathologies that impair cardiovascular autonomic control. This study employed mice overexpressing a transgene for human SOD1 (hSOD1) to determine if its overexpression would alter autonomic regulation of BP, HR, and BRS in healthy animals, and if this animal line (C57B6SjL-Tg (SOD1)² Gur/J) could be used in future studies to determine if hSOD1 overexpression can preserve cardiac autonomic function in disease models. To accomplish this aim, using anesthetized SOD1 and C57 (control) mice, we recorded HR, and aortic depressor nerve (ADN) activity changes in response to pharmacologically-induced BP changes in order to measure baroreflex and baroreceptor sensitivity, respectively. In order to identify any alterations in central, efferent, and cardiac components of the baroreflex arc, we electrically stimulated the left ADN and left cervical vagus and compared the reductions in BP and HR between the C57 and SOD1 mice. Time- and frequency-domain analysis of heart rate variability (HRV) was performed using pulse pressure recordings prior to pharmacologic or surgical procedures. We found that hSOD1 overexpression in the SOD1 mouse line, in comparison to C57 controls did not significantly affect resting HR (C57: 558 ± 8 vs.

SOD1: 553 ± 13 beats-per-minute) or blood pressure (C57: 88.8 ± 2.9 vs. SOD1: 85.8 ± 2.1 mmHg). hSOD1 overexpression did not affect the decrease in average mean arterial pressure (MAP) following injection of sodium nitroprusside (SNP) (C57: 38.7 ± 1.4 vs. SOD1: 39.5 ± 1.3 mmHg) or increase in average MAP (C57: 135.8 ± 3.1 vs. SOD1: 136.6 ± 3.5 mmHg) following injection of phenylephrine (PE). BRS, as measured by the averaged regression lines for $\Delta\text{HR}/\Delta\text{MAP}$ for the SNP-induced tachycardic baroreflex (C57: 0.57 ± 0.06 bpm/mmHg, SOD1: 0.61 ± 0.08 bpm/mmHg) and the PE-induced bradycardic baroreflex (C57: -2.9 ± 0.57 bpm/mmHg, SOD1: -4.3 ± 0.84 bpm/mmHg) are not significantly different between C57 and SOD1. Baroreceptor activation showed a significant increase in gain (C57: 5.4 ± 0.3 vs. SOD1: 7.4 ± 0.5 %/mmHg, $P < 0.01$) in the SOD1 transgenic mice. Heart rate depression in response to electrical stimulation of the left ADN and cervical vagus was comparable between C57 and SOD1, though MAP reduction in response to ADN stimulation is slightly, but significantly increased at 50 Hz in SOD1 animals. Time-domain analysis of HRV did not reveal any significant difference in beat-to-beat variability between SOD1 and C57 (SDNN: C57: 2.78 ± 0.20, SOD1: 2.89 ± 0.27), although frequency-domain analysis uncovered a significant reduction in the low-frequency power component of the HRV power spectral distribution (C57: 1.19 ± 0.11, SOD1: 0.35 ± 0.06, $P < 0.001$). This study shows that although hSOD1 overexpression does not affect overall baroreflex function, it does potentiate baroreceptor sensitivity and brain stem control of arterial pressure, and reduces low-frequency beat-to-beat variations in HR, without affecting total HRV.

Dedicated to the memory of my brother, Mark Allen Hatcher, and the promise I
made to him.

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The time will come when diligent research over long periods will bring to light things which now lie hidden. A single lifetime, even though entirely devoted to the sky, would not be enough for the investigation of so vast a subject... And so this knowledge will be unfolded only through long successive ages. There will come a time when our descendants will be amazed that we did not know things that are so plain to them... Many discoveries are reserved for ages still to come, when memory of us will have been effaced.

Seneca, *Natural Questions*

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LIST OF ABBREVIATIONS

ABP	Arterial blood pressure
ACE	Angiotensin converting enzyme
ADN	Aortic depressor nerve
ANGII	Angiotensin II
ADNA	Aortic depressor nerve activity
ALS	Amyotrophic lateral sclerosis
ASICII	Acid-sensitive ion channel II
AVB	Atrioventricular block
BP	Blood pressure
BRS	Baroreflex sensitivity
cVLM	Carotid sinus nerve
DMNX	Dorsal motor nucleus of the vagus
DSS	Dextran sodium sulfate
ECG	Electrocardiogram
FFT	Fast fourier transform
G_{\max}	Maximal slope of gain

GPX	Glutathione peroxidase
H ₂ O ₂	Hydrogen peroxide
HF	Heart failure or high frequency power
HR	Heart rate
HRV	Heart rate variability
hSOD1	Human copper/zinc (Cu/Zn) superoxide dismutase
IBI	Inter-beat interval
LF	Low frequency power
LF/HF	Low frequency/high frequency ratio
MAP	Mean arterial pressure
MABP	Mean arterial blood pressure
mmHg	Millimeters of mercury
mnSOD	Manganese (or mitochondrial) superoxide disutase,
MPO	Myeloperoxidase
NA	Nucleus ambiguus
NADPH	Nicotine adenine dinucleotide phosphate
NOX	Nicotine adenine dinucleotide phosphate oxidase

NTS	Nucleus tractus solitarius
O ₂ ⁻ .	Superoxide anion
PAP	Pulse arterial pressure
PE	Phenylephrine
PVN	Paraventricular nucleus of the hypothalamus
PSNS	Parasympathetic nervous system
rVLM	Rostral ventrolateral medulla
ROS	Reactive oxygen species
SA	Sinus arrest
SDNN	Standard deviation of normal intervals
SE	Standard error
SNP	Sodium nitroprusside
SNS	Sympathetic nervous system
Tg-hSOD1	Transgenic human cu/zn superoxide dismutase

CHAPTER 1: INTRODUCTION

Reactive Oxygen Species (ROS) and Oxidative Stress.

It is estimated that metabolic processes utilizing molecular oxygen (O₂) arose approximately 2.9 billion years ago (Kim, 2012). The adaptation of early life toward the use of oxygen was a crucial step toward the development of complex, multicellular life, such as ourselves and most other forms of life on the planet. The transfer of electrons from organic molecules to oxygen provides a large release of free energy to meet the high-energy requirements of large, motile life forms (Catling, 2005). The drawback to the use of oxygen for metabolic processes is, that because of its reactivity, oxygen can accept unpaired electrons through both enzymatic and non-enzymatic reactions to form a family of toxic, highly reactive compounds known as reactive oxygen species (ROS) (Pham-Huy, 2008). ROS are usually very short lived, only lasting from a few nanoseconds to a few milliseconds, but can quickly react with and damage DNA, proteins, and lipids. ROS attack on DNA can cause lesions to the DNA that lead to mutations or fatal damage, lipids may be peroxidized to malondialdehyde (MDA), a potent cytotoxin and mutagen. ROS damage to protein can lead to loss of function or possibly protein aggregation. Most ROS are formed in the mitochondria as an unavoidable byproduct of ATP synthesis, and are usually tightly regulated by endogenous antioxidant enzymes that can convert ROS into less harmful compounds, although they are rapidly oxidized themselves, meaning that there is a fairly high rate of synthesis and turnover for these enzymes in order for the cell to keep ahead of ROS generation. The most important antioxidant enzymes produced include various types of

superoxide dismutases (SOD1, SOD2, SOD3), catalase (CAT), glutathione reductase (GRx), and glutathione peroxidase (GPx). SOD enzymes are the first line of defense against ROS, catalyzing the dismutation (reduction) of the superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2). Hydrogen peroxide is subsequently converted into water or oxygen by the actions of catalase or GPx.

ROS Play Important Roles in Cell Signaling.

As dangerous as these compounds appear to be, ROS are critical to cellular signaling (Forman, 2010; Fukai, 2011) and defense against infections (Yang, 2013; Nathan, 2013), and are often produced in a controlled manner to serve these functions. The Angiotensin II signal transduction pathway, for example, is dependent on superoxide anions generated by NADPH oxidase (NOX) enzymes. Vascular tone is also maintained by production of nitric oxide ($\cdot NO$), which is in turn negatively regulated by $O_2^{\cdot-}$. These two signaling mechanisms play crucial roles in the regulation of arterial pressure and arterial baroreflex response (Datla, 2010; Hirooka, 2008; Li YL, 2011b, 2013; Zhang, 2009; Zimmerman, 2004).

Oxidative Stress in Disease States

Increased markers of oxidative stress in the heart, blood vessels, peripheral nervous system, and brainstem are a notable feature in conditions as diverse as hypertension (Braga, 2011; Chan, 2014; Datla, 2010; Hirooka, 2008), sleep apnea, (Zhang, 2012), diabetes (Figuerola-Romero, 2008; Li YL, 2013; Maritim, 2003), and aging (Gemma, 2007; Schoneich, 1999). A common contributor to the increase in

oxidative stress seen in these conditions is the $O_2^{\cdot-}$ -dependent angiotensin II (AngII) signaling pathway (Braga, 2011; Li, YL, 2011, 2013; Wang, 2002). AngII is a peptide hormone that has potent effects on the cardiovascular and renal systems, leading to an increased sympathetic outflow from medullary control centers, systemic vasoconstriction, and sodium reabsorption by the proximal tubules of the kidneys, promoting an increase in blood fluid volume (Montani, 2004). These responses collectively increase blood pressure. If the renin-angiotensin-aldosterone system is working properly, AngII signaling will only remain active long enough to return arterial pressure to a healthy set point. In the conditions listed above, AngII levels in the blood remain elevated long after the physiologic requirement for it is passed, contributing to hypertension and a persistent elevation in NOX-dependent $O_2^{\cdot-}$ synthesis as part of the AngII signal transduction pathway, continuously elevating oxidative stress in the brain and blood vessels. Over time this can lead to permanent damage to the nervous system and blood vessels (Cai, 2000; Friedman, 2011).

Rationale and Aims

Because reactive oxygen species, including the superoxide anion, despite contributing to various pathologies, also figure prominently into important cellular signaling functions, overexpression of antioxidant enzymes have the potential to be either helpful, by reducing oxidative stress, or harmful by disrupting normal physiology. Studies have shown SOD1 overexpression to be either helpful (Kinouchi, 1991; Xu, 2004; Wang, 1998) or harmful (Rando, 1999; Bar-Peled, 1996; Gahtan, 1998). Prior studies have suggested mechanisms by which increased SOD1 expression, or $O_2^{\cdot-}$

scavenging by the SOD1 mimetic tempol can prevent inhibition of nitric oxide synthase by nitric oxide (Brennan, 2002) improve baroreflex function in spontaneously hypertensive rats (Guimaraes, 2012), increase the sensitivity of baroreceptor neurons damaged by diabetes (Li YL, 2011), normalizes carotid chemoreceptor activity in heart failure rabbits (Ding, 2009), and decreases activity in sympathetic brain stem nuclei in sino-aortic denervated rats, leading to a decrease in blood pressure and heart rate (Wei, 2009). These investigations support the possibility that increased $O_2^{\cdot-}$ may treat or prevent deficits in cardiovascular function, including baroreflexes and chemoreflexes. However, there are some studies which demonstrate that SOD1 overexpression may cause abnormal morphology in spinal motor neurons (Del Canto, 1995, Jaarmsa, 2000) or interfere with long-term potentiation in the hippocampus (Gahtan, 1998). The latter studies cause concern that SOD1 overexpression can have deleterious effects in neuronal tissues.

With the interests of validating a SOD1 overexpressing mouse line (for use in studying possible protective effects of enhanced $O_2^{\cdot-}$ scavenging against cardiac autonomic neuropathy in diabetes, sleep apnea, and aging, we selected an established hSOD overexpressing line (C57B6SJL-Tg (SOD1)² Gur/J Jackson catalog # 002297, Gurney, 1994), hereafter referred to as SOD1. These mice were derived from the C57BL/6J strain (Jackson catalog # 000664), therefore C57BL/6J (hereafter referred to as C57) were employed as controls. Two phases of study were planned in order to determine if hSOD1 overexpression lead to any deficits in the autonomic control of the

heart. The first phase was designed to evaluate overall baroreflex sensitivity and basic cardiovascular parameters. These objectives were achieved by:

1. Measurement of heart rate (HR) and mean arterial pressure (MAP) in isoflurane – anesthetized SOD1 and C57 mice, in order to determine if hSOD overexpression directly affected these fundamental measures of cardiovascular function.
2. Using pharmacologic manipulations of arterial pressure in order to assess the baroreflex control of heart rate.
3. Direct recording of aortic baroreceptor neuron activity changes in response to induced low-to-high blood pressure ramps in order to evaluate any changes in arterial baroreceptor function.

The second phase of the study was intended to assess function of the central (brainstem) component of the baroreflex response, as well as the target organ (heart), and to compare beat-to-beat variability of heart rate between the two animal lines as a measure of autonomic control of heart rate. These objectives were achieved by:

1. Measure HR and AP responses to electrical stimulation of the ADN as a measure of central component function.
2. Measure HR response to electrical stimulation of the left cervical vagus nerve as a test of heart rate response to vagal stimulation (cardiovagagal response).
3. Perform heart rate variability (HRV) time-domain analysis based on pulse intervals as a measure of autonomic control of beat-to-beat HR changes.

4. Perform HRV frequency domain analysis in order to detect any possible changes in power spectral frequency distribution, which may signal changes in sympathetic, parasympathetic, or humoral controls of heart rate.

Results of this study will help us determine whether the SOD1 strain of transgenic mice (C57B6SjL-Tg (SOD1)² Gur/J) is a suitable base for investigations on the effect of constitutive, long-term down-modulation of superoxide radicals in the prevention or treatment of cardiac autonomic neuropathies associated with diabetes, sleep apnea and aging. A desirable model would show no significant changes in autonomic regulation of the heart rate compared to non-transgenic (C57) controls in disease-free animals. Significant alterations in any of the studied parameters would indicate the SOD1 overexpression can independently affect components of the autonomic nervous system. Such findings would be interesting to investigate further as they may identify mechanisms of cardiovascular regulation in which superoxide anions play a significant role. However, since findings of significant alterations in autonomic cardiovascular control may be due to pathological changes in the hSOD1 overexpressing mice, use of the mice in disease studies would be contraindicated until the source and nature of these changes are identified.

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CHAPTER 2: AUTONOMIC CONTROL OF THE HEART IN HUMAN SOD1 TRANSGENIC MICE: BAROREFLEX SENSITIVITY AND BARORECEPTOR ACTIVATION

Introduction

Cardiac autonomic control in disease

Control of the cardiovascular system by the autonomic nervous system is a critical homeostatic function that is compromised in multiple disease conditions such as diabetes (Olshan, 1983; Maser, 2005; Vinik, 2003), hypertension (Moreira, 1992), sleep apnea (Narkiewicz, 2001)., and aging (Kuo, 1999; Saint Martin, 2015; Fauvel, 2007; Monahan, 2007). One of the autonomic functions impacted is baroreflex control of heart rate (HR). Barosensitivity (BRS) is a measure of the strength of baroreflex control of heart rate in response to changes in blood pressure, and is an important index of cardiac autonomic function, and several clinical conditions are strongly associated with an attenuated baroreflex mediated control of heart rate, including hypertension (Lai, 2006), vasovagal syncope (Ford, 1999) and heart failure. (Thames, 1993), and is considered as an independent risk factor for cardiac failure and sudden death (Osterziel, 1995; Mortara, 1997; Okada, 2010).

Oxidative Stress and Autonomic Dysfunction

Both sympathetic and parasympathetic components of the baroreflex response are both positively and negatively regulated by a complex of components including the aortic and carotid baroreceptors, the nucleus tractus solitarius (NTS), paraventricular nucleus of the hypothalamus (PVN), nucleus ambiguus (NA), caudal ventrolateral

medulla (CVLM) and rostral ventrolateral medulla (RVLM) (Machado, 2001; Dampney, 1994, 2002, 2005). An increase in levels of reactive oxygen species (ROS) in the critical neural components of the baroreflex arc including the carotid and aortic baroreceptors (Zhang L, 2009; Peng, 2012; Li YL, 2013; Ding, 2009), Nucleus tractus solitarius (NTS) (Hirooka, 2008; Nozoe, 2007; Tsai, 2013), Paraventricular nucleus of the hypothalamus (PVN) (Yuan, 2012), and rostral ventrolateral medulla (RVLM) (Kishi, 2004; Gao, 2004, 2007) are seen in conditions such as diabetes, sleep apnea, hypertension, and heart failure.

Antioxidant Therapy in Treating Autonomic Dysfunction

Numerous lines of research suggest that antioxidant therapy, whether applied locally (Li Z, 1996; Zhang L, 2009; Peng, 2012) or systemically (Kawada, 2015; Gouty, 2003; Wei, 2009), can restore normal baroreflex function in some of these disease states, ostensibly by decreasing levels of ROS species. However, reactive oxygen species, including the superoxide radical, play critical roles in regulating the firing properties of neurons (Forman, 2010). For instance, the ANGII signaling pathway is dependent on superoxide anions generated by the actions of NADPH oxidase (Hirooka, 2008; Braga, 2011). Indeed, some studies have noted that superoxide scavenging can affect central regulation of heart rate and blood pressure in healthy animals as well as disease models (Kawada, 2015; Li Z, 1996; Campese, 2004). Before we can consider any potential benefits of SOD1 overexpression for the preservation of normal BRS in disease states, we need to consider whether or not the intensified superoxide

scavenging found in SOD1 overexpressing animals can directly affect components of the baroreflex loop in healthy animals.

Deleterious Effects of SOD1 Overexpression

Conversely, other studies have suggested that SOD1 overexpression can be detrimental to neuronal tissues. Several studies (Jaarsma, 2000; Dal Canto & Gurney, 1995) found evidence of axonal degeneration and death of motor neurons in mice overexpressing hSOD1. Other investigators have found evidence of increased lipid peroxidation (Fullerton, 1998; Amstad, 1991), increased sensitivity to kainic acid excitotoxicity (Bar-Peled, 1996), and impaired recovery following nerve injury (Kotulska, 2006) in hSOD1 transgenic mice. Some evidence suggests that SOD1 expression is protective up to a certain level, after which further increases in expression contribute to peroxide formation and deleterious sequela for the tissues (McCord, 2005, 2008). SOD1 overexpression has also been investigated as a contributor to the pathology of Down syndrome, a condition in which SOD1 overexpression is well documented (Brooksbank & Balazs, 1984; Capone, 2001; Epstein, 1987).

Materials and Methods

Animals

Mice (C57BL/6j, 3-4 mo, n= 16) were used as controls for the transgenic Cu/Zn SOD mice (C57B6SJL-Tg (SOD1)² Gur/J, Jackson catalog # 002297 n= 16).

Procedures were approved by the University of Central Florida Animal Care and Use Committee and followed the guidelines established by the National Institutes of health. Diligent efforts were made to conduct the experiments humanely and to minimize the numbers of animals used.

Surgical Procedure

Mice were anesthetized with 3% isoflurane inhalation and maintained with 1% in a 95% O₂ and 5% CO₂ through a tracheal tube. Depth of anesthesia was monitored by eye blink, withdrawal reflexes (toe-pinch), and fluctuations of arterial blood pressure. The tips of plastic catheters (polyethylene-50) were tapered to ~0.3mm diameter, the right femoral artery and left femoral vein were exposed, and the tapered ends of the two catheters were filled with heparinized saline and inserted into the femoral artery and vein. Measurement of blood pressure was through the right femoral artery. Vasoactive drugs were infused into the femoral vein using a microinfusion pump. These mice were used for the baroreflex control of HR and aortic depressor nerve (ADN) recordings which were conducted while the animals were in the anesthetized state.

Baroreflex Sensitivity

The blood pressure catheter was connected to a blood pressure transducer (MIT0699, AD instruments). The transducer tip was positioned near the level of the heart. Arterial blood pressure (ABP) was measured using the Powerlab Data Acquisition System (PowerLab/8 SP). Mean arterial pressure (MAP) and heart rate were (HR) were derived from the ABP values in Chart 5 software (AD instruments). Microinfusions of phenylephrine (PE) or sodium nitroprusside (SNP) were averaged over 5 s at the end of 60 s sustained microinfusion of these vasoactive drugs for each dose. Before the end of 60 s sustained microinfusion of PE or SNP at varying doses (10-30 μ l SNP, 6-15 μ l PE, 0.1 μ g/ μ L each), before the HR and MABP responses had reached a plateau. The maximal HR responses relative to HR baseline level (Δ HR) to ABP change relative to the ABP baseline level (Δ MABP) induced by microinfusion of PE or SNP were averaged over 5 s at the end of 60 s sustained microinfusion of these vasoactive drugs for each dose. Application of the various doses of PE (or SNP) was in random sequence. When compared with rats (Soukhova-O'Hare, 2006; Yan, 2008), we found that more time was required to reestablish a steady baseline in the return to pre-stimulus values in mice. In our experiments, 45 minutes were used before the next microinfusion. During this interval, blood pressure and HR had already returned to their original baseline levels. We also noted that injections of PE and SNP in the same mouse frequently led to unstable ABP in some animals. Thus, SP and SNP were infused in different groups of mice during different days.

The ratio of the maximal change in HR over the Change in MABP was then calculated and averaged at each dose for each drug in each animal group. The

averaged ratio of HR change over MABP change ($\Delta\text{HR}/\Delta\text{MABP}$) was used as an indicator or estimate for baroreflex sensitivity. Dose-dependent curves of $\Delta\text{HR}/\Delta\text{MABP}$ as functions of PE and SNP concentration were plotted for C57 and SOD1 mice. In addition, curves of ΔHR - ΔMABP relationship were plotted to show the maximal HR responses induced by MABP changes after the responses had reached a steady plateau. Baroreflex sensitivity was also assessed during PE and SNP infusion at 0.4 $\mu\text{g}/\text{min}$. Changes in HR (ΔHR) were measured and averaged over 0.5 s every 5-mmHg increase of MABP ($\Delta\text{MABP} = 5 \text{ mmHg}$). ΔHR was then plotted as a function of ΔMABP to show the transient HR responses as MABP changes.

Baroreceptor Afferent Function

The left aortic depressor nerve (ADN) was identified in the cervical region using a dissecting microscope. The left ADN was carefully isolated from surrounding connective tissues with fine glass tools to avoid injury of the nerve. Then, the left ADN was placed on miniaturized bipolar platinum electrodes (0.12 mm outer diameter). The nerve and electrodes were soaked in mineral oil. Aortic depressor nerve activity (ADNA) was amplified (10,000) with band-pass filters set between 300 and 1000 Hz by an AC amplifier (model 1800, A-M systems, Sequim, WA, USA). The ADNA, integrated ADNA, phasic arterial pressure (PAP), HR, ECG, and body temperature were all recorded and simultaneously displayed on different channels of the PowerLab System. Chart 5.2 Software and SigmaPlot 9.0 were used for data acquisition, analysis, and presentation. The ADNA for analysis had a signal-noise ratio above 10.1

The ADNA signal occurred as rhythmic burst that exhibited cardiac cycles and were synchronized with Arterial PAP. ADNA signal was integrated using a 10-ms time constant to obtain the integrated ADNA (Int.ADNA). The “ADNA Silent” or the “Noise Level” between ADNA bursts is shown in the small boxes in the Int. ADNA signal of Fig. 2. The averaged ADNA value from 30 “ADNA Silent” intervals was used to determine the noise level for Int. ADNA. The averaged noise level was subtracted from original Int. ADNA signal to obtain the corrected Int. ADNA with the averaged noise level of 0uV.s. The corrected ADNA and MAP were used to construct baroreceptor afferent function curves. For simplicity, we used the Int. ADNA for corrected Int. ADNA in the text below. The baroreceptor function curve was calculated as the rising phase of PE-induced AP Change starting from the Nadir or SNP –induced fall in AP to the Maximum of the AP. R waves of the ECG signal were used to automatically define cardiac cycles by the Chart 5.2 Macro function (arrows in Fig. 2). The baroreceptor function curve was fitted by plotting the percent (%) change on the mean Int. ADNA per cardiac cycle relative to the Int. ADNA baseline value before drug administration using a sigmoid logistic function (Kent., et. al. 1972). The logistic function for Int. ADNA used the mathematical expression:

$$Y=P1/\{1 + \exp[P2(X-P3)]\}+P4,$$

Where:

X=MAP, Y= Int. ADNA (% Baseline), P1=maximum – minimum Int. ADNA (range), P2=Slope coefficient, P3= MAP at 50% of the Int. ADNA range (P_{mid}), P4=maximum Int. ADNA.

The P_{th} and P_{sat} were calculated from the 3rd derivative of the logistic function, and they were expressed as $P_{th} = P_3 - (1.317/P_2)$.

The maximum slope or gain (G_{max}) was calculated at P_{mid} from the 1st derivative of the logistic function $G_{max} = -P_1 - P_2/4$.

Approximately 200-500 data points measured over 30- 50 s were used to construct a baroreceptor function curve using sigma plot software.

The squared correlation coefficient r^2 was used to determine the goodness of the curve fitting.

Data Analysis

Data are expressed as means \pm SE, unless stated otherwise. Differences between groups were determined using two-tailed t-tests (for paired and unpaired data) with statistical significance set at $P < 0.05$. To compare the differences of dose-dependent curves or baroreflex function curves between C57 and SOD1 mice, two-way repeated measures ANOVA followed by Newman-Keuls post hoc tests were used.

Results

Baseline and SNP/PE Induced Changes in MAP and HR

Under isoflurane anesthesia, mean arterial pressures (MAP) were not significantly different between the two experimental groups ($P > 0.05$) (Table 1). SNP induced minimums and PE induced maximums for MAP were comparable for both C57 and SOD1 ($P > 0.05$), allowing us to investigate baroreflex response over a similar range of blood pressure changes. hSOD1 overexpression does not significantly alter baseline MAP and HR, nor does it change MAP and HR response to SNP or PE infusion. The lack of effect hSOD1 overexpression has on HR and BP in healthy animals supports the applicability of using these animals to study the effect of increased antioxidant capacity in mediating cardiovascular derangement in disease state models such as sleep apnea, diabetes, and aging.

Baroreflex Sensitivity Measurements

Figure 1 shows raw recordings of heart rate changes (ΔHR) in response to sequential injections of sodium nitroprusside (SNP) and phenylephrine (PE). SNP infusion (lt. gray box) induces a reduction in blood pressure and a concomitant increase in HR driven by the baroreflex response (tachycardic phase). PE infusion (dk. Gray box) results in an increase in blood pressure that drives a baroreflex-mediated reduction in heart rate (bradycardic phase).

Separate regression lines ($\Delta HR / \Delta MAP$) were fitted for both tachycardic and bradycardic phase data for C57 and SOD1 animals ($n=8$). The slopes of regression,

when averaged (Figure 2A, 2B) show identical baroreflex slopes for the tachycardic (SNP) phase between the C57 (-0.57 ± 0.06 Bpm/mmHg) and SOD1 (-0.60 ± 0.08). The bradycardic (SNP) phase generated averaged regression lines of -2.9 ± 0.57 bpm / mmHg for C57 vs. -4.3 ± 0.84 bpm / mmHg for SOD animals. The difference in slopes did not reach significance ($P > 0.2$), even though the plotted lines are visibly dissimilar. Overexpression of hSOD1 in these mice did not significantly enhance or decrease overall baroreflex control of heart rate.

Aortic Depressor Nerve Recordings

The recordings show that ADNA decreases in response to hypotension induced by infusion of SNP, and increases in response to hypertension brought on by the infusion of PE. Fig.4A shows recordings of ADN activity increasing or decreasing in response to changes in blood pressure in representative C57 and SOD1 mice. The sigmoid logistic function curve was used to fit these representative relationships between the Int. ADNA and MAP in these two mice as shown in fig. 4B. The averaged parameters of the logistic function curves (Table 2) show that the SOD1 animals had a significantly larger ADNA range (P1) and maximal ADNA response (P4) compared to C57 ($P < 0.02$). The maximal gain of the ADNA response (G_{max}) was also significantly greater in SOD1 vs. C57 ($P < 0.01$). The plots of the sigmoid logistic function of the averaged int. ADNA vs. MAP for C57 and SOD1 mice is shown in Fig. 5.

Discussion

Blood Pressure and Heart Rate in Anesthetized Mice

Superoxide radicals have a profound effect in the modulation of neural activity in the brain stem (Hirooka, 2008; Zimmerman & Davisson, 2004), and elevated levels of ROS are strongly linked to hypertension (Datla, 2010; Chan SH & Chan JY, 2014; Hirooka, 2008). One of our concerns in using the SOD1 mouse line is that hSOD1 overexpression would alter basal HR and MAP. Previously, hSOD1 overexpression in the paraventricular nucleus of spontaneously hypertensive rats was found to reduce sympathetic activity and attenuate hypertension (Yuan, 2012), and endothelial specific catalase overexpression caused a significant reduction in blood pressure in mice (Suvorava & Kojda, 2009). Systemic administration of tempol, a SOD mimic (Muscoli, 2003) has previously been found to reduce MAP and HR in urethane anesthetized rats (Wei, 2009). The same study noted reduced spontaneous discharge rate of neurons in the paraventricular nucleus of the hypothalamus (PVN) and the rostral ventrolateral medulla (RVLM), two critical nuclei involved in sympathetic regulation of the heart and circulatory system. A similar study performed on normotensive WKY and spontaneously hypertensive (SHR) rats also demonstrated reduced HR and MAP during tempol administration, as well as decreased splanchnic nerve activity (Kawada, 2015). Kawada's study also determined that the reduction in blood pressure was not caused by changes in peripheral vascular tone, although a significant reduction was noted in the SHR animals, suggesting that the superoxide radical scavenging by tempol is capable of affecting central nervous system components of the autonomic control of blood pressure in healthy animals. In contrast, our study determined that SOD

overexpression had no significant effect on MAP and HR in healthy mice under isoflurane anesthesia. This is in agreement with a previous published report (Wang, 2002) showing blood pressure equivalence between C57bl/6J mice and 6-TgN(SOD1)3Cje mice with a 3-fold overexpression of hSOD1 (Jackson Catalog#: 002629). This mouse line was developed by the laboratory of Dr. Mark Gurney (Gurney, 1994) concurrently with the mice used in our study, and express an identical hSOD1 transgene.

Baroreflex Control of Heart Rate is not Significantly Affected by hSOD1 Overexpression.

Previous studies have shown that redox species modulate the activity of baroreceptor neurons (Zhang L, 2009; Peng, 2012; Li YL, 2013; Ding, 2009; Peng, 2012), as well as the NTS (Hirooka, 2008; Nozoe, 2007; Tsai, 2013), and sympathetic brain stem nuclei such as the PVN (Yuan, 2012), and RVLM (Kishi, 2004; Gao, 2004, 2007), and the intrinsic cardiac ganglia (Whyte, 2009) as well. Perturbations in the function of any of the components of the baroreflex loop can alter baroreflex sensitivity and function. Indeed, it is well established that increased ROS levels in these components can reduce baroreflex sensitivity and response (Li Z, 1996; Tsai, 2013; Chan SH & Chan JY, 2014). The effect of chronic antioxidant supplementation including overexpression of SOD1 on baroreflex function in healthy animals is less well documented. Li Z (1996) found that exogenous SOD or catalase applied to the carotid sinus caused a small, but significant increase in baroreceptor activity between the pressures of 60 to 80 mmHg, but did not increase the maximal baroreceptor activation. However, this study did not measure HR response to blood pressure ramps (Δ HR /

Δ MAP), so it is uncertain if local application of SOD or catalase to the carotid baroreceptors would have had an appreciable effect on the baroreflex control of heart rate. Guimaraes (2012) investigated the effects of NADPH-derived superoxide anion reductions by I.V Tiron (a superoxide anion scavenger, Ledenev, 1986) or Apocyanin (NADPH oxidase inhibitor, Petronio, 2013) on BRS in WKY and SHR rats, and found that the acute application of these agents had no significant effect on BRS in healthy animals, although they improved BRS in the hypertensive animals. Unfortunately, this still does not give any indication as to what effects long-term superoxide anion scavenging could have on BRS. The results of our investigation suggest that moderate (~3.5 fold, Gurney, 1994) hSOD1 overexpression does not significantly impact baroreflex sensitivity in healthy animals (Fig.2).

Baroreceptor Afferent Function is Enhanced by Overexpression of Human SOD1.

As shown in fig.5, our investigation found that hSOD1-overexpressing mice showed a significantly increased baroreceptor activation slope and gain (5.4 ± 0.3 vs. 7.4 ± 0.5 %/mmHg) compared to C57 controls. This was unexpected, but not unprecedented. Li Z (1996) reported that carotid sinus nerve activation in response to blood pressure ramps was enhanced by application of SOD or catalase to the carotid sinus in healthy rabbits. In contrast, another study (Li YL & Zheng H, 2011) showed that application of tempol or apocyanin to isolated nodose ganglia neurons had no effect on resting membrane potential, input resistance, or number of action potentials induced by a 50-pA current injection, nor did transient overexpression of mitochondrial SOD (mnSOD) in the nodose ganglia of Sprague-Dawley rats (Tu, 2012) in a similar study by

the same group. In our study, as well as the 1996 study (Li Z, 1996), the activity spikes recorded in the baroreceptor fibers of the aortic depressor nerve (ADN) or carotid sinus nerve (CSN) were triggered by activation of stretch receptors in the aortic arch by artificially induced increases in arterial pressure, whereas other studies (Li YL & Zheng H, 2011; Tu, 2012) used whole-cell patch clamp techniques, and action potentials in individual neurons were stimulated by current injection. The behavior of single, isolated neurons will not be the same as clusters of nerves or nerve fibers being activated by blood pressure elevation. Individual baroreceptor activation shows little activity at first, until an activation threshold is reached, and is then followed by a nearly linear, proportional rise in activity as arterial pressure increases, up to a saturation point, at which further increases in arterial pressure fail to yield any further increase in firing rate of individual neurons. When recording bundled baroreceptor afferent fibers in the aortic depressor ADN or carotid sinus nerve (CSN), we will also see a more gradual threshold as multiple fibers are recruited at different activation thresholds, and begin to sum up during the mid-part of the activation curve, until saturation, leading to a more sigmoid pattern (Eckberg, 2004). Thus, the lack of effect ROS scavenging had on firing rate observed by Li and Tu (Li YL & Zheng H, 2011; Tu, 2012) of nodose ganglia cells does not necessarily conflict with the results showing increased baroreceptor activation in the current study.

Proposed Mechanisms for hSOD1 Overexpression Enhancement of Baroreceptor Function.

Increased Arterial Compliance

Nonetheless, the discrepancies between these studies bear further consideration. Dr. Li's group clearly demonstrated that acute treatment with antioxidants had no measurable effect on the electrophysiology of nodose ganglia cell bodies, leaving the question as to why we see increased activation in the current study, as well as the 1996 study (Li Z, 1996). One possibility is that increased antioxidant levels are enhancing mechanosensitivity of the baroreceptor nerve endings in the aortic arch and carotid bifurcation. We did not have the opportunity to investigate this possibility, but it could help explain why we see enhanced response of baroreceptor fiber bundles to pressure ramps, but not increased sensitivity of individual ganglia neurons to current injection. SOD1 is known to support nitrous oxide (NO) mediated vasorelaxation (Fukai, 2011), which improves arterial compliance (Didion, 2002a, 2002b; Lynch, 1997). A low degree of vascular compliance is associated with hypertension and decreased baroreflex sensitivity, while a high degree of vascular compliance is linked to improved baroreflex sensitivity (Monahan, 2001, 2007; Michas, 2012). There is a wealth of research that supports the hypothesis that antioxidant supplementation can improve large artery compliance (Shargorodsky, 2010; Ulker, 2003). Based upon current research, it is possible that our hSOD1 transgenic mice have a higher than normal degree of arterial compliance, which may enhance baroreceptor function.

Increased Expression or Function of Ion Channels involved with Supporting Mechanotransductive Properties of Arterial Baroreceptors.

It is also possible that SOD1 can directly affect the mechanosensory properties of the aortic baroreceptor, although an exact mechanism is uncertain. There is considerable evidence that ROS have an inhibitory effect on expression of ASIC2 (Acid-Sensitive Ion Channel) that is critical to mechanotransduction of arterial pressure changes by barosensitive neurons (Abboud, 2010; Lu, 2009). ASIC2 expression is downregulated in SHR rats (Lu, 2007; Snitsarev, 2005), which show a similar diminution of baroreceptor activation to ASIC2 null mice (Lu, 2009). Application of the superoxide mimetic tempol to nodose ganglia cells in either the ASIC2 null mice or SHR rats has been shown to restore baroreflex activation of baroreceptor neurons to near normal levels (Abboud, 2010). Hyperpolarization-activated cyclic nucleotide (HCN) channels are also strongly linked to mechanoreception in arterial baroreceptors, are shown to be downregulated in type 1 diabetes mellitus, and are associated with reduced excitability of baroreceptor neurons that can be rescued with tempol (Li YL, 2013). Although such associations to the enhanced baroreceptor activation in the current study are highly speculative, they are feasible and open to investigation at a later date.

In this study, we demonstrated that the overexpression of human SOD1 does not detectably affect arterial pressure or heart rate in healthy animals. This suggests that the C57B6SjL-Tg (SOD1)² Gur/J mouse line (Jackson catalog # 002297, Gurney, 1994) could serve as a useful model for the investigation of the potential therapeutic value of systemically enhanced antioxidant activity in ameliorating cardiovascular autonomic dysfunction often seen in diabetes, sleep apnea, aging, and other conditions.

The study did reveal significantly enhanced baroreceptor activation in response in blood pressure ramps. While we did not anticipate this finding, there are other studies in the literature that find increased baroreceptor sensitivity following antioxidant application to baroreceptor neurons and cardiovascular nuclei.

Conflict of Interest

None declared.

Acknowledgements

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Tables

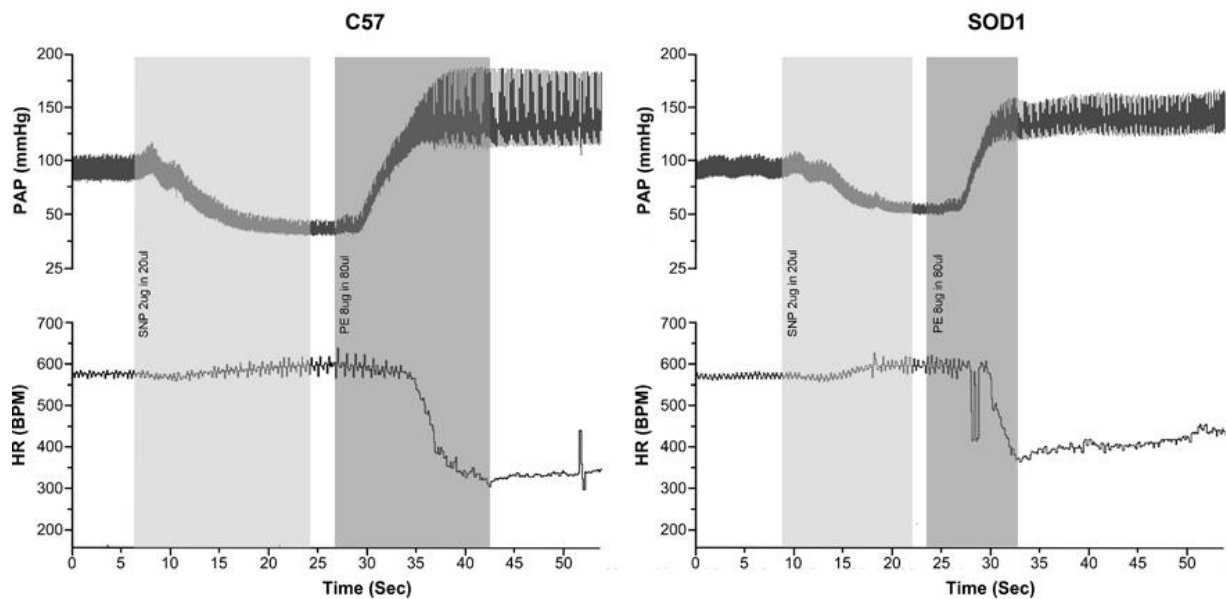
Table 1: Heart Rate, Blood Pressure, and Arterial Pressure Changes Following SNP/PE Infusion

Animal Group	Average HR (BPM)	MAP (mmHg)	SNP MAP	PE MAP
C57 (n=8)	558 ± 8	88.8 ± 2.9	38.7 ± 1.4	135.8 ± 3.1
SOD1 (n=8)	553 ± 13	85.8 ± 2.1	39.5 ± 1.3	136.6 ± 3.5

There are no significant differences in Baseline heart rate (HR) and mean arterial pressure (MAP) between C57 and SOD mice. Depression in arterial pressure following exposure to SNP, or increase in arterial pressure after PE infusion, is also the same between the two groups of animals.

Table 2: Parameters defining the baroreceptor afferent function curve (ADNA% baseline) in C57 and SOD mice.

Animal Group	R ²	P ₁ (%)	P ₂	P ₃ (mmHg)	P ₄ (%)	G _{max} (%/mmHg)	P _{th} (mmHg)	P _{sat} (mmHg)
C57 (n=8)	0.95 ±0.01	-307 ±19	0.07 ±0.006	94 ±3	314 ±18	5.4 ±0.3	74 ±3	114 ±4
SOD (n=8)	0.95 ±0.01	-434 ±44	0.07 ±0.004	94 ±3	436 ±37	7.4 ±0.5	73 ±2	114 ±4
<i>P</i> value	N.S	<i>P</i> < 0.02	N.S	N.S	<i>P</i> < 0.02	<i>P</i> < 0.01	N.S	N.S



Figures

Figure 1: Original recordings of heart rate responses to PAP changes induced by the sequential administration of SNP/PE in representative C57 and SOD animals

A Tachycardic phase was induced by a baroreflex-mediated increase in heart rate in response to decreased blood pressure due to SNP infusion. A bradycardic phase was induced by a baroreflex-mediated decrease in heart rate in response to increased blood pressure caused by injection of PE. During SNP application, MAP and HR changes from the baseline of MAP to the nadir of MAP were measured as shown in the light gray box. During PE application, MAP and HR changes were measure from the peak of the HR to the nadir of the HR as shown in the dark gray box.

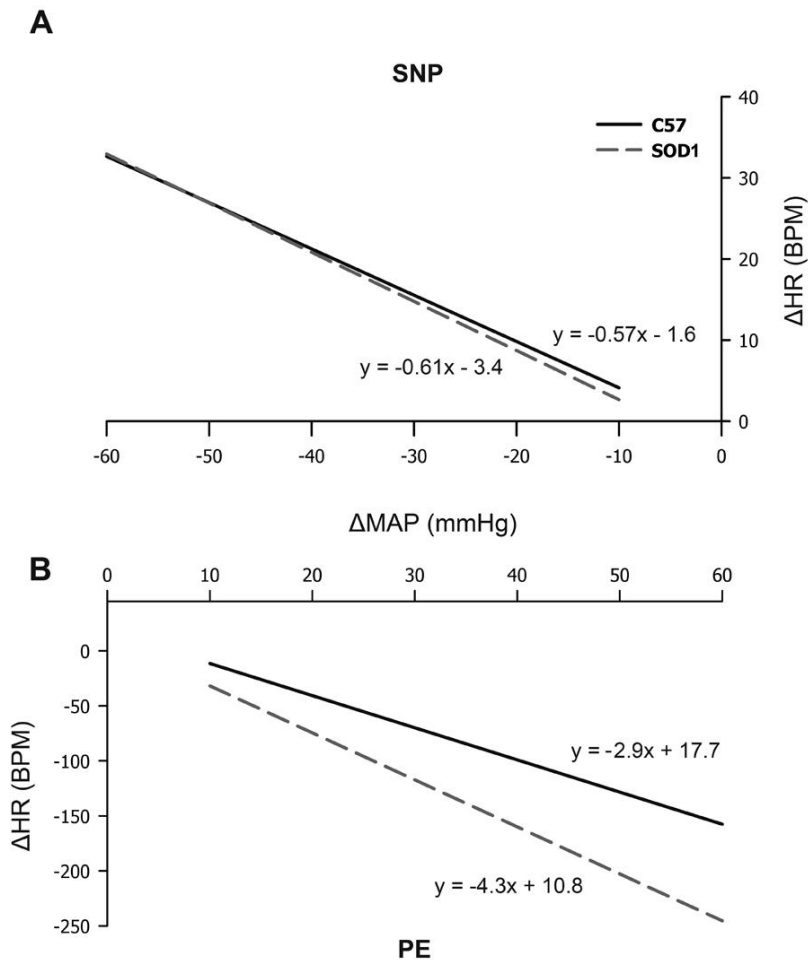


Figure 2: Baroreflex sensitivity

(A) The averaged regression lines for $\Delta\text{HR}/\Delta\text{MAP}$ for the SNP-induced tachycardic baroreflex for C57 (n=8) and SOD (n=8). Regression lines for tachycardic baroreflex response are identical between the two groups (C57: 0.57 ± 0.06 bpm/mmHg, SOD1: 0.61 ± 0.08 bpm/mmHg). (B) The averaged regression lines for $\Delta\text{HR}/\Delta\text{MAP}$ for the PE-induced bradycardic baroreflex for C57 (n=8) and SOD (n=8). Though visibly distinct, the slope of the regression line for SOD mice was not significantly different than that of C57 (C57: -2.9 ± 0.57 bpm/mmHg, SOD1: -4.3 ± 0.84 bpm/mmHg, $P > 0.05$).

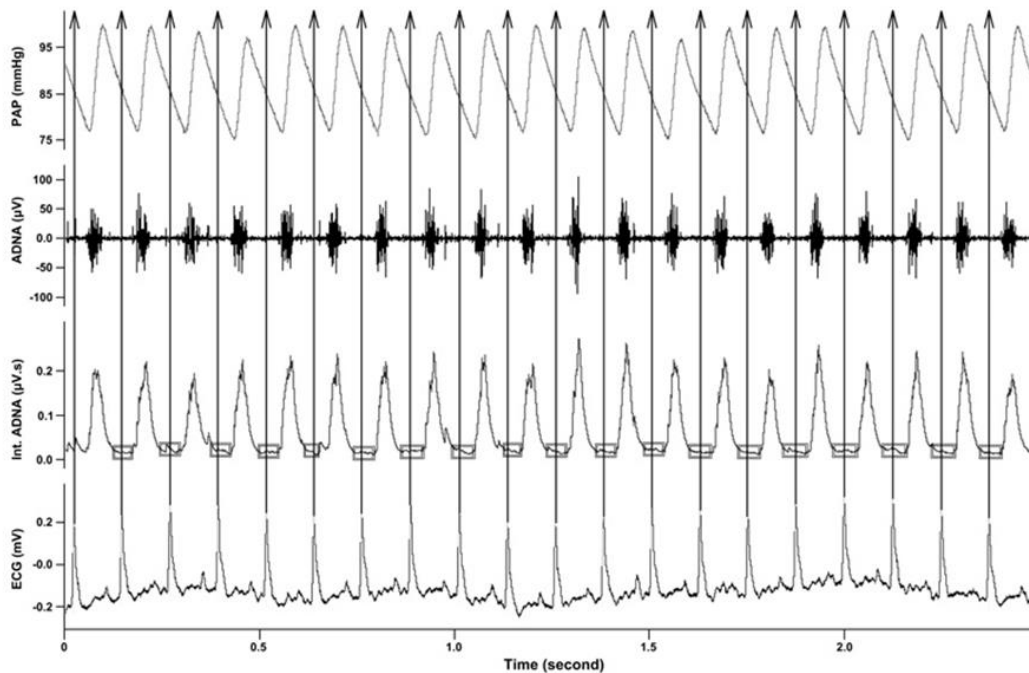


Figure 3: Original recording of PAP, ADNA (Raw ADNA), integrated ADNA (int. ADNA) and ECG in aC57 mouse.

Trace 1: PAP. Trace 2: ADNA occurred as rhythmic bursts that exhibited cardiac rhythmic patterns and was synchronous with the arterial pulse pressure. Note: ADNA increased prior to AP increases. This is because the catheter for blood pressure measurement was inserted into the femoral artery. Trace 3: ADNA signal was integrated using a 10-ms time constant to obtain the Int. ADNA curve. The small boxes in the int. ADNA trace enclose the intervals between ADNA bursts where signal noise can be measured. Trace 4: ECG. The R-waves of the ECG signal were used to separate ADNA firing intervals automatically by chart 5.2 (arrows).

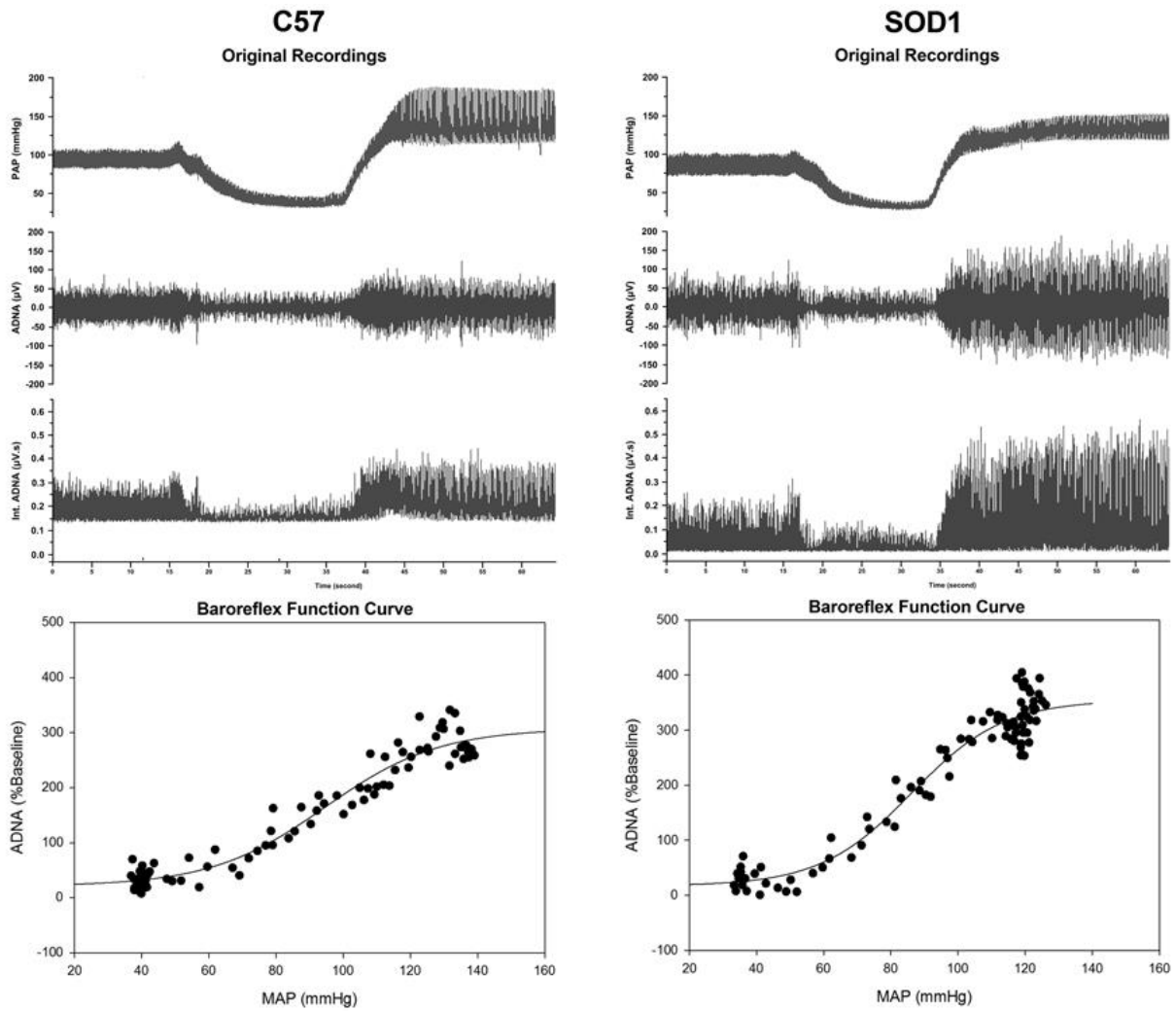


Figure 4: Original recordings showing arterial pulse arterial pressure (PAP) and ADNA responses to Sequential i.v. injections of SNP/PE in C57 and SOD mice, Respectively

(Int. ADNA: Integrated ADNA).

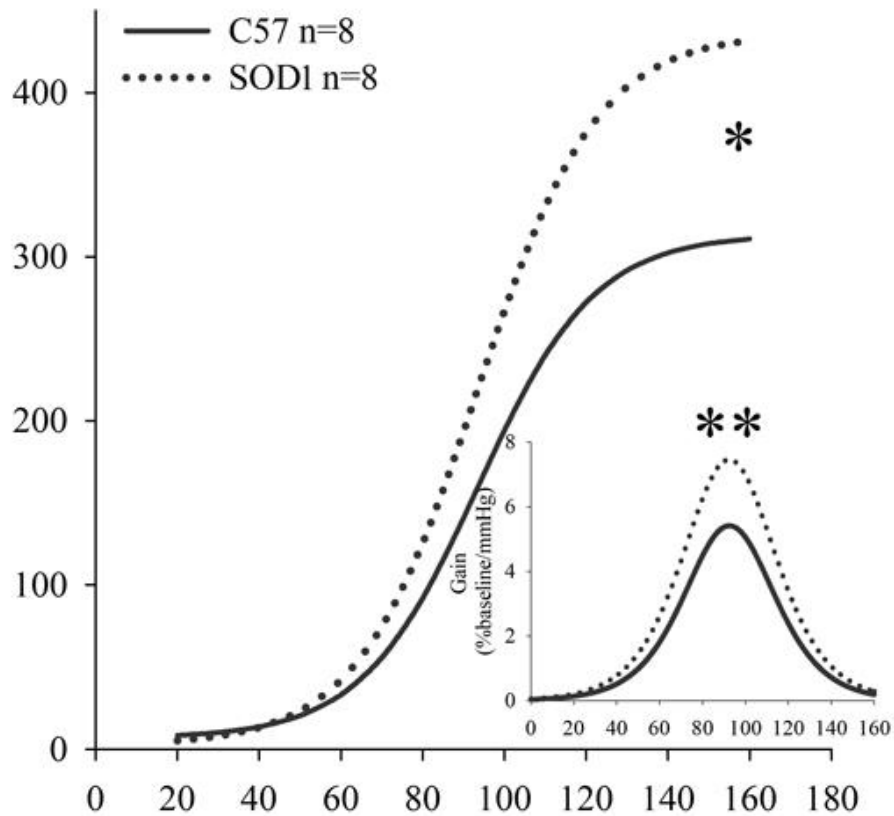


Figure 5: Baroreceptor Afferent Function.

Baroreceptor function curves for C57 (n=8) and SOD (n=8) groups were reconstructed using the averaged parameters of sigmoid logistic function curves (see Table 2). The baroreceptor discharge function curve for SOD mice is significantly higher for SOD mice compared to C57 (* $P < 0.05$) and the gain of the baroreceptor afferent function curve was significantly higher for the SOD mice than for the C57 mice (** $P < 0.01$).

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CHAPTER 3: AUTONOMIC CONTROL OF THE HEART IN HUMAN SOD1 TRANSGENIC MICE: CENTRAL COMPONENTS, CARDIOVAGAL RESPONSE, AND HEART RATE VARIABILITY

Introduction

Neural Components of Baroreflex Control of Heart Rate

Arterial baroreflexes exert potent control of heart rate, functioning on a beat-to-beat basis. The cardiovascular autonomic nervous system dynamically regulates heart rate (HR) and blood pressure (BP) to adapt them to changing physiological conditions and maintain optimal tissue perfusion and oxygenation. As with any such homeostatic system, the status of blood pressure and oxygenation needs to be detected, and this information relayed to a control center that can interpret changes that deviate from a set-point, form an appropriate response and relay those signals to brainstem nuclei controlling target organ response in order to return arterial pressure and tissue oxygen saturation to the set point. 1) Barosensitive nerve endings in the aortic arch and carotid sinus detect changes in blood pressure. Information about these changes is relayed via the aortic depressor nerve (ADN) and the glossopharyngeal nerve to the nucleus tractus solitarius (NTS). This constitutes the *afferent* limb of the baroreflex arc. 2) Baroreceptor information is received by NTS, which processes the inputs and conditionally relays the information to parasympathetic and sympathetic brain stem nuclei that regulate heart rate (Parasympathetic: nucleus ambiguus (NA), dorsal motor nucleus of the vagus (DMNX). Sympathetic: paraventricular nucleus of the hypothalamus (PVN) caudal ventrolateral medulla (cVLM) and rostral ventrolateral medulla (rVLM). These nuclei represent the *central components* of arterial baroreflex control. 3) The parasympathetic

central nuclei communicate directly with the heart through efferent fibers of the cardiac vagus nerve. Sympathetic nuclei send projections down in to the spinal cord to contact spinal interneurons which then contact ganglia neurons in the sympathetic ganglia chain, some of which project to heart and blood vessels (Pilowsky & Goodchild, 2002; Guyenet, 2006; Andreson, 2004; Kara, 2003). Finally, the heart itself has a network of intracardiac ganglia receiving input from the parasympathetic and sympathetic nerve fibers. These ganglia modulate central autonomic control and shape the responses of heart rate, contractility, and conduction rates (Adams & Cuevas, 2004). Combined, these constitute the *efferent* component of the baroreflex arc.

Tests of Central and Efferent Component Function

This study investigates the effects of the overexpression of a human Cu/Zn superoxide dismutase transgene (tg-hSOD1) on the central and efferent components of the baroreflex arc using the SOD1 mouse line (C57B6SJL-Tg (SOD1)² Gur/J, Jackson catalog # 002297, Gurney, 1994, originally published as N1029) overexpressing tg-hSOD1. Because oxidative species, including superoxide, function as signaling molecules that modulate neural activity (Hirooka, 2008; Zimmerman & Davisson, 2004) and vascular tone (Snetkov, 2011), we had to consider it a possibility that hSOD1 overexpression could alter central, efferent, and target organ function. Previously, we established that the slopes of the baroreflex regression lines in response to hypertensive and hypotensive challenges in SOD1 and C57 controls were comparable, but that the gain of the baroreceptor response (afferent response) was significantly greater in the SOD1 mice (C57: 5.4 %/mmHg \pm 0.3 vs. SOD1: 7.4 \pm 0.5 %/mmHg, $P <$

0.05). This demonstrates that although baroreflex response measured as $\Delta\text{HR}/\Delta\text{MAP}$ is not significantly changed by tg-hSOD1 overexpression, function of individual components within the baroreflex arc may have been altered. As a broad test of the function of the central components (brainstem nuclei) of the baroreflex arc, we applied electrical stimulation stimuli [square wave pulses (3 μA : 1 ms) at 2, 5, 10, 20, 30, 40, 50, and 60Hz for 20 s] to the ADN and measured ΔHR and ΔMAP (mean arterial pressure). ΔHR in response to ADN stimulation is mediated not only by the brainstem nuclei, but also by conduction of the brainstem autonomic impulses by the efferent fibers of the cardiac vagus nerve, and by the cardiovagal response mediated by the intracardiac nervous system and conduction fibers. In order to evaluate efferent conduction and cardiac response, we applied an electrical stimulus to the left cervical vagus nerve [square wave pulses (3 μA : 1 ms) at 2, 5, 10, 20, and 30 Hz for 20 s] and recorded ΔHR

Heart Rate Variability: Time and Frequency Domain

Autonomic control of heart rate, including baroreflex modulations, affect the heart rate on a beat-by-beat basis, subtly shortening and lengthening the time between beats. These continuous shifts in the inter-beat interval (IBI) are known as heart rate variability (HRV). The dominant influence on HRV is the parasympathetic nervous system (PSNS), whose effects on HR have lower latency and faster recovery than sympathetic nervous system (SNS) influences (Spear, 1979). In this study we examine the time and frequency domains of HRV, deriving the standard deviation of normal intervals (SDNN) from the time-domain analysis as a measure of total variability from all sources (PSNS, SNS, intrinsic cardiac, and humoral) (Nolan J, 1998). We use SDNN from C57 animals

as a benchmark for comparing HRV in SOD1 animals. Impairment of sympathetic or parasympathetic controls will result in a lower SDNN value. Using a fast-fourier transform to convert time-domain HRV data (recorded for at least 5 minutes) in a frequency domain reveals patterns of IBI variations as a distribution of frequencies. The more frequently the variations occur at a particular frequency, the higher the power of that frequency component (Malik 1996). For frequency analysis, total power in normalized units (Nu) can be used as an estimate of HRV from all inputs. More contentious is the use of power distribution (PSD) in the low frequency (LF) range and high frequency (HF) ranges as markers of the relative strengths of sympathetic and parasympathetic influence on the HRV (Eckberg, 1997; Reyes del Paso, 2013). In this investigation, we will not use LF power, HF power, or LF:HF ratios to draw conclusions on the balance of regulatory power between the PSNS and SNS, but rather to use gross changes in the spectral distribution as an indicator of a change in autonomic function.

Materials and Methods

Animals

Mice (C57BL/6j, 3-4 mo, n= 10 were used as controls for the transgenic Cu/Zn SOD mice (C57B6SjL-Tg (SOD1)² Gur/J, Jackson catalog # 002297 n= 10.

Procedures were approved by the University of Central Florida Animal Care and Use Committee and followed the guidelines established by the National Institutes of health. Diligent efforts were made to conduct the experiments humanely and to minimize the numbers of animals used.

Surgical Procedure

Mice were anesthetized with 3% isoflurane inhalation and maintained with 1% isoflurane in a mixture of 95% O₂ and 5% CO₂ through a tracheal tube. Depth of anesthesia was monitored by eye blink, withdrawal reflexes (toe-pinch), and fluctuations of arterial blood pressure. The tips of plastic catheters (polyethylene-50) were tapered to ~0.3mm diameter, the right femoral artery and left femoral vein were exposed, and the tapered ends of the two catheters were filled with heparinized saline and inserted into the femoral artery and vein. Measurement of blood pressure was through the right femoral artery. These mice were used for assessment of the cardiovascular responses to ADN or vagal nerve stimulation, all of which were conducted while the animals were in the anesthetized state.

Blood Pressure and Heart Rate Recordings

The blood pressure catheter was connected to a blood pressure transducer (MIT0699, AD instruments). The transducer tip was positioned near the level of the heart. Arterial blood pressure (ABP) was measured using the Powerlab Data Acquisition System (PowerLab/8 SP). Using a 2,000 Hz sampling rate, Mean arterial blood pressure (MABP) and heart rate were (HR) were derived from the ABP values in Chart 5 software (AD instruments).

Heart Rate Variability Analysis

Using the Chart 5 Software (AD instruments) Heart Rate Variability (HRV) plugin, we generated a beat-by-beat Systolic BP time series derived from the carotid BP waveforms sampled at an equidistant interval of 0.05S (20 Hz) to calculate the pulse interval (PI) of systolic peaks. The beat-by-beat Pulse interval (PI) was determined by measuring dt between the maxima of systolic peaks. A 5-minute section of stable ABP was selected at the beginning of each experiment for C57 (n=10) and SOD1 (n=10) mice. Each section was visualized manually using the HRV plugins data analysis tool to select for a period free of erratic fluctuations in HR or ABP. Peak detection thresholds were set manually for each animal to reduce artifacts. Manual short-artifact removal was available in software, and was used as necessary. These steps allow for detection and quantification of “normal” beat intervals (NN). Bin size for spike histogram sampling was set to 10ms. Delta NN (difference of consecutive NN) was set to detect consecutive interval distances > 6ms, reflecting cardiac parasympathetic activity. From normalized interbeat interval (time-domain indexed) data, we derived the mean of

the interbeat intervals, and the standard deviation of NN (SDNN). SDNN is a widely accepted measure of autonomic variability from all sources, with higher values indicating greater variability. PI analysis of HRV is accepted as a substitute for ECG-derived beat intervals, and is commonly used for HRV and blood pressure variability (BPV) analysis from blood-pressure telemetry data (Baudrie, 2007)

A fast-fourier transformation was used to convert time domain data from the NN variability to the frequency domain to create a power spectral distribution of HRV. We used a 1,024-point series for the FFT algorithm. With the 0.05s sampling rate (20Hz), each spectral component band was a harmonic of $20/1,024$ Hz, or about 0.019 Hz. Window overlap was set to 75%. Power spectra was divided along the x-axis into a frequency range from 0 to 5 Hz, and categorized into 3 major divisions. Very Low Frequency (VLF), Low Frequency (LF), and High Frequency (HF) as such: $VLF < 0.04$ Hz $< LF < 1.5$ Hz $< HF < 5$ Hz. The low frequency domain is associated with sympathetic control of HR, although is also reflects parasympathetic and humoral influences (Malpas, 2002; Reyes del Paso, 2013). The HF domain is more strongly associated with parasympathetic control, and includes heart rate variances driven by respiratory sinus arrhythmia mediated by vagal influence (Piepoli, 1997). Divisions of the power spectrum follow the recommendations of Thireau (2007) for frequency-domain analysis of HRV in mice.

HR and BP Responses to Stimulation of the Left ADN

The left aortic depressor nerve (ADN) was identified in the cervical region using a dissecting microscope. The left ADN was carefully isolated from surrounding

connective tissues with fine glass tools to avoid injury of the nerve. Then, the left ADN was placed on miniaturized bipolar platinum electrodes (0.12 mm outer diameter). The nerve and electrodes were soaked in mineral oil. The left ADN was then crushed at a point caudal to the electrodes. The baseline values of MAP and HR were measured 30 s prior to stimulation. The nerve was stimulated with rectangular pulses that were delivered to the electrode at frequencies of at 2, 5, 10, 20, 30, 40, 50, and 60Hz from a Grass S48 Stimulator (Grass Instrument Co., West Warwick, RI, USA) through an isolation unit (Grass, Model PSIU 6). The output current from the isolation unit was rectangular pulses (3 μ A, 1-ms duration). The train stimulus was 20 s, and the inter-train stimulus was 3–5 min. Responses to each frequency of stimulation were measured at least twice in each experiment with the order of changes of frequency reversed during the second round of stimulation. The responses were repeatable. The maximal or peak amplitudes of the HR responses to electrical stimulation of the left ADN were measured. In addition, the time course of the HR responses in percent (%) baseline value during stimulation, which were sampled and averaged every 0.5 s interval, was assessed. The stimulation-induced changes in HR were abolished after crushing the left ADN cranial to the electrodes, confirming that the responses were reflex in nature.

HR and BP Responses to Electrical Stimulation of the Left Cervical Vagus Nerve

A cervical midline incision was performed, and the trachea was cannulated with a catheter (polyethylene-50) to facilitate ventilation in spontaneously breathing mice. The left cervical vagus nerve was carefully dissected free from surrounding structures, and was cut just caudal to the nodose ganglion. After the vagal nerve was cut, the caudal

cut end was placed on a pair of bipolar platinum hook electrodes and electrically stimulated with a Grass Stimulator (S48). The stimuli [square wave pulses (30 μ A: 1 ms) at 2, 5, 10, 20, and 30, Hz for 30 s] were delivered with an isolation unit (ISU 6). Maximal HR and blood pressure responses to electrical stimulation of the cervical vagal nerve were measured. The Δ HR and Δ MABP were plotted as a function of the stimulation frequency.

After the onset of electrical stimulation at low frequencies, HR decreased, initially in a gradual manner and then at a more precipitous rate. We calculated the period needed for HR responses to drop sharply from the onset of electrical stimulation.

Statistical Analysis

Data are expressed as means \pm SE, unless stated otherwise. Differences between groups was determined using two-tailed t-tests (for paired and unpaired data) with statistical significance set at $P < 0.05$. To compare the differences of ADN and Vagus nerve stimulation curves for HR and MAP between C57 and SOD 1 mice, two-way repeated measures ANOVA followed by Newman-Keuls post hoc tests were used.

Results

Heart Rate and Blood Pressure Responses to Electrical Stimulation of the Aortic Depressor Nerve

Electrical stimulation of the left aortic depressor nerve (ADN) evoked frequency dependent decreases in HR and MAP in both SOD1 and C57 mice (Fig. 6). There was no difference in the HR depression in SOD1 mice compared to C57 over the course of electrical stimulation (Fig. 7). For BP depression in response to electrical stimulation of the ADN, there was a slightly, but significantly ($P < 0.05$) greater decrease in MAP in SOD1 mice ($\Delta\text{MAP}: -71 \pm 5$) than in C57 ($\Delta\text{MAP}: -57 \pm 5$) at 50 Hz (Fig. 8)

Heart Rate Response to Electrical Stimulation of the Left Cervical Vagus Nerve

Electrical stimulation of the left cervical vagus nerve evoked frequency dependent decreases in HR in both SOD1 and C57 mice (Fig. 9). The plot for $\Delta\text{HR}/\text{Hz}$ appears shallower for SOD1 animals, but the difference between SOD1 and C57 for each of the test frequencies did not reach significance (student t-test, $P > 0.05$).

Time-Domain Heart Rate Variability Analysis

Major findings for time-domain analysis of HRV are shown in table 3. The standard deviation of normal intervals (SDNN) is similar between SOD1 (2.69 ± 0.25) and C57 (3.11 ± 0.32) (Fig. 12A). The maximal normal interval is significantly ($P < 0.05$) longer in C57 mice (149.54 ± 4.18 ms) than for SOD1 (137.66 ± 4.08 ms). The shorter maximal NN for SOD1 agrees with the slightly, but not significantly higher heart rate in the SOD1 animals, (SOD1: 465 ± 14 , C57: 431 ± 11).

Frequency Domain Analysis of Heart Rate Variability.

5- minute pulse arterial pressure recording were subjected to a fast-fourier transform, yielding power spectral distribution graphs for C57 and SOD1 animals (figures 11A and 11B) Data for frequency domain analysis of HRV is found on table 4. The high-frequency (HF), very low frequency (VLF), and total spectral powers, were comparable between SOD1 and C57 animals ($P > 0.05$). The low-frequency (LF) power was significantly lower in SOD1 compared to C57 (SOD1: $0.91 \pm 0.20 \text{ ms}^2$, C57: $2.43 \pm 0.55 \text{ ms}^2$, $P < 0.01$). Consequently, the LF/HF ratio was significantly lower in SOD1 as well, compared to C57 (SOD1: 0.35 ± 0.06 , C57: 1.19 ± 0.11 , $P < 0.01$).

Discussion

Human SOD1 Overexpression Does not Alter HR response to ADN stimulation, but Slightly Increased Reduction in Arterial Pressure.

Aortic depressor nerve stimulation tests the function of the central components of baroreflex in the brain stem, including the nucleus tractus solitarius (NTS), nucleus ambiguus (NA), dorsal motor nucleus of the vagus nerve (DMNX), paraventricular nucleus of the hypothalamus (PVN), caudal ventrolateral medulla (cVLM) and rostral ventrolateral medulla (rVLM) (Pilowsky & Goodchild, 2002; Guyenet, 2006; Andreson, 2004; Kara, 2003). Baroreflex induced reductions in HR are mediated almost entirely by the parasympathetic nervous system (PSNS) through an increase in vagal output to the heart (Mendolowitz, 1999). ADN afferent fibers transmit impulses from baroreceptors to the NTS, which integrates the impulses, and in turn signal motor neurons in the DMNX and NA which send projections through the vagus nerve to the heart (Pilowsky & Goodchild, 2002). If any of these components are damaged, or their function altered, HR response to ADN stimulation would reflect those changes (Gu, 2008). The lack of any significant difference in HR reduction between SOD1 and C57 animals suggests that hSOD1 overexpression does not affect the overall function of the central and efferent (vagal) components.

There was a very slight, but significant potentiation of MAP decrease in response to ADN stimulation at 50 Hz in SOD1 mice compared to the C57 animals. There was no significant difference at 2, 5, 10, 20, 30, 40, or 60 Hz, although the line for MAP reduction in SOD1 appears to show a continuation of increasing MAP reduction from 40 – 60 Hz, whereas C57 shows MAP reduction levels off from 30 – 60 Hz. (Fig. 8). The

Δ MAP / Frequency (Hz) line for SOD1 showed a lower trend compared to C57 from 40 to 60 Hz, but 50Hz was the only point at which the difference was statistically significant. Over the range of frequencies from 2, 5, 10, 20, and 30 Hz, MAP reductions were similar between both C57 and SOD1 animals, suggesting that in the C57 animals, vasorelaxation or reduction of cardiac contractility had reached a maximum, and that SOD1 animals had a higher capacity for relaxation of the resistance vessels or reduction of contractile force in the heart. The literature does provide some support for SOD1 overexpression increasing arterial relaxation and compliance by reducing nitric oxide synthase (NOS) downregulation by superoxide, or by maintaining higher elasticity in the arterial collagen (d'Uscio, 2010; Seals, 2014; Shargorodsky, 2010). Another possibility would be SOD1-mediated inhibition of central and arterial ANGII signaling (Yuan, 2012), which is dependent on O₂⁻ (Wang, 2002) ANGII signaling maintains a higher constant sympathetic output in the sympathetic brainstem nuclei such as the rVLM or PVN (Gao, 2004). Further research would be needed to resolve this question

hSOD1 Overexpression does not Affect HR Reduction in Response to Stimulation of the Left Cervical Vagus Nerve.

The vagal nerve stimulation study examines the responsiveness of HR to vagal control. We found no significant difference in HR depression in response to vagal nerve stimulation between the SOD1 animals and C57 controls. This finding suggests that hSOD1 overexpression in mice does not disrupts cardiac response to PSNS controls.

HRV Time Domain Analysis Shows No Change in Heart Rate Variability in Mice Overexpressing hSOD1.

The standard deviation of normal intervals (SDNN) is an accepted measure of total heart rate variability. We found no significant difference in SDNN between the SOD1 and C57 mice, providing evidence that heart rate variability is not negatively impacted by overexpression of hSOD1. There was a slight decrease in the distance between maximum normal intervals in SOD1 animals, which may be due to a slightly, but not significantly higher HR measured in the SOD1 mice (more heart beats per minute of necessity reduces the intervals between heart beats).

Frequency-Domain Analysis of HRV Shows Decreases in LF-Power and LF:HF Ratio.

By converting the time-domain sequence of normal intervals into the frequency domain using a fast-fourier transform (FFT), a power-spectral distribution (Fig. 11) is generated which beat-to-beat autonomic control of heart rate as a distribution of frequencies. The high frequency (HF) component (1.5 – 5 Hz in mice) is largely driven by PSNS influence, which has a more rapid effect on HR (Spear, 1979). This includes influence of respiratory sinus arrhythmia as well as beat-to-beat baroreflex changes (Piepoli, 1997). LF (0.04 – 1.5 Hz in mice) is more complex, and is suspected be mostly formed from PSNS contribution, and PSNS interaction with SNS (Malpas, 2002; Reyes del Paso, 2013). Prior assertions that LF power correlated strongly to SNS control of heart rate have been rejected (Eckberg, 1997; Reyes del Paso, 2013). Total spectral power and HF power were comparable between the two groups (Table. 4) though in SOD1 mice, we found that LF spectral power was markedly decreased compared to C57. This finding is difficult to interpret due to the ongoing debate regarding the relative

contributions of sympathetic, parasympathetic, humoral, and mechanical contributions to the LF spectrum of HRV. Parasympathetic influence is thought to be the largest contributor to LF power, though functionally we were not able to find evidence of a PSNS deficit in HR control through baroreflex studies (Fig. 2), or electrical stimulation of the ADN (Fig. 7) and left cervical vagus (Fig. 10). Time-domain analysis of HRV also shows that overall heart rate variability is quite comparable between the C57 and SOD1 animals (Table 3, Fig. 12A). However, the decrease in LF spectral power is significant, and demonstrable in each individual power spectral histogram (Fig. 11B is a representative example). These findings indicate that autonomic control of HR is altered by overexpression of hSOD1, but is not detectable by baroreflex analysis, ADN or vagal stimulation, or by time-domain HRV analysis. Reduction in LF power has been linked to an increased risk of sudden death in chronic heart failure patients (Galiniere M., 2000), but its significance in this study is undetermined.

One possible explanation for LF power reduction is SOD1 inhibition of superoxide-ANGII signaling. Studies with mice lacking ANGII- type 1a (AT1a) receptors show a significant decrease in the LF component of the HRV power spectra (Chen Y., 2005) similar to what we observed in the SOD1 mice, although the AT1a- deficient mice (At1a -/-) showed a similar decrease in the HF power. Nonetheless, as superoxide scavenging by tempol or overexpression of SOD1 has been shown to inhibit ANGII sympathoexcitatory activity (Campese, 2004, 2005; Gao, 2004) and reverse baroreflex inhibition caused by ANGII (Guimaraes, 2012; Li & Zheng, 2011; Li 2011,2013), we must consider ANGII suppression as a potential mechanism for later study. ANGII also

increases vascular tone by acting both directly on the vascular smooth muscle as well as by increasing sympathetic outflow (Gao, 2004; Nguyen, 2011) which allows us to hypothesize that SOD1-mediated ANGII suppression may contribute to the mildly enhanced MAP decrease seen during ADN stimulation (Fig. 8). This is a target for future studies.

Characterization of Cardiac Autonomic Control in SOD1 Mice by Time and Frequency Domain HRV Analysis, Afferent Stimulation, and Efferent Stimulation.

In this study, our goal was to determine if the overexpression of tg- hSOD1 in mice altered autonomic control of the heart rate. This objective was pursued requisite to developing the SOD1 mouse as a model for studying the effects of constitutively increased superoxide scavenging in murine disease models of sleep apnea, diabetes, and aging. These studies will help elucidate the role of the superoxide anion in the progression of cardiovascular autonomic dysfunction, and evaluate the effectiveness of therapies aimed at reducing superoxide concentration in halting or reversing the progression of cardiac autonomic neuropathies. The results of this investigation demonstrate that the central and cardiovagal components involved in baroreflex control of heart rate in SOD1 mice appear to be intact if viewed solely from the results of ADN stimulation (central component), vagal stimulation (cardiovagal), and time-domain analysis of HRV. However, frequency-domain analysis of HRV in the SOD1 animals revealed a significant reduction in the power of the LF component of the HRV spectra. LF power is derived from multiple sources, including mostly vagal modulation of HR, and to a lesser degree sympathetic and humoral modifiers of HR, as well as other unidentified contributors (Malpas, 2002; Reyes del Paso, 2013). MAP reduction in

response to electrical stimulation of the left ADN was also slightly enhanced in the SOD1 mice, but only significantly at 50 Hz (Fig. 8). These findings indicate that the SOD1 mice are an imperfect model for use in to studies SOD1 overexpression as a mechanism to decrease superoxide-mediated oxidative stress and preserve cardiac autonomic function in disease. Use of the SOD1 mice for these investigations is possible, but further studies are recommended to assess: 1) Tissue-specific hSOD1 expression. 2) neuroanatomical differences between SOD1 and C57 mice, including baroreceptors in the aortic arch, cardiac ganglia, and parasympathetic/sympathetic innervation of the heart. 3) Measurements of sympathetic nerve activity, such as muscle sympathetic nerve activity (MSNA) or renal sympathetic nerve activity (RSNA) at baseline and in response to AP challenges. 4) Measurement of markers of oxidative stress in the baroreceptor afferents, cardiac brain stem nuclei, and cardiac ganglia. 5) EKG analysis to look for arrhythmias or conduction abnormalities. 6) Echocardiography to assess cardiac contractility and ejection fractions. 7) Circulating levels of epinephrine and angiotensin II.

Conflict of Interest

None declared.

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Tables

Table 3: Summary of Time Domain HRV Measurements

Mouse		Maximum NN (ms)	Minimum NN (ms)	Range NN (ms)	Mean NN (ms)	Avg HR (BPM)	SDNN
C57, n = 10	Mean	149.54	131.35	18.19	139.85	431	3.11
C57, n = 10	Std. Error	4.18	2.60	2.29	3.34	11	0.32
SOD1, n = 10	Mean	137.66	123.84	13.83	130.02	465	2.68
SOD1, n = 10	Std. Error	4.08	3.62	1.32	4.01	14	0.25
P=Value		0.047	0.134	0.087	0.100	0.089	0.326

Heart rate variability parameters were nearly identical between SOD1 mice and C57 controls. The Maximum Normal-to-Normal Interval (Maximum NN) was significantly ($P < 0.05$) shorter in SOD1 mice (137.66 ± 4.08 ms) compared to C57 controls (149.57 ± 4.18 ms). This is most likely due to the faster, but not significantly faster heart rate in the SOD1 mice (C57; 431 ± 11 bpm, SOD1; 465 ± 14 bpm, $P > 0.05$)

Table 4: Summary of Frequency Domain HRV Measurements

Mouse		Total Power (ms²)	VLF (<0.4HZ)	LF (0.04-1.5Hz)	HF(1.5-5Hz)	LF/HF Ratio
C57, n = 10	Mean	6.76	1.83	2.43	1.98	1.19
C57, n = 10	Std. Error	1.21	0.46	0.55	0.36	0.11
SOD1, n = 10	Mean	5.46	1.84	0.91	2.64	0.35
SOD1, n = 10	Std. Error	1.05	0.72	0.20	0.40	0.06
P=Value		0.451	0.993	0.008	0.240	0.000003

VLF, HF, and Total spectral power were comparable between the SOD1 mice and C57 controls. LF power was significantly reduced in SOD1 compared to C57 ($P < 0.01$) and the LF/HF ratio was also significantly reduced due to the decrease in LF power ($P < 0.01$).

Figures

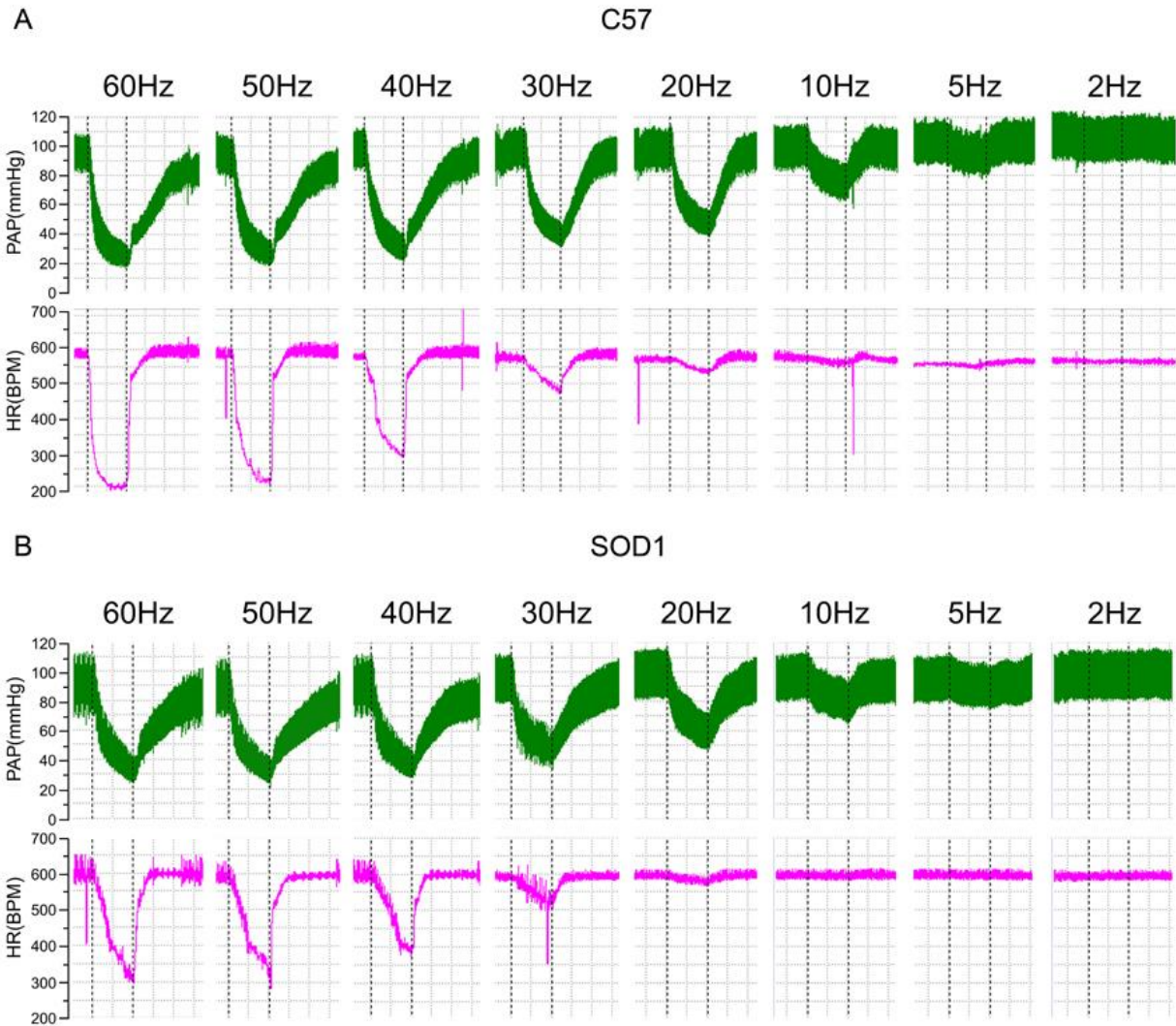


Figure 6: Original recordings showing HR and MAP responses to electrical stimulation of the left ADN at 2 – 60 Hz, 30 μ A, 1 ms in representative C57 and SOD1.

Dashed lines represent 20 s stimulation intervals. (A) C57 mice. (B) SOD1 mice

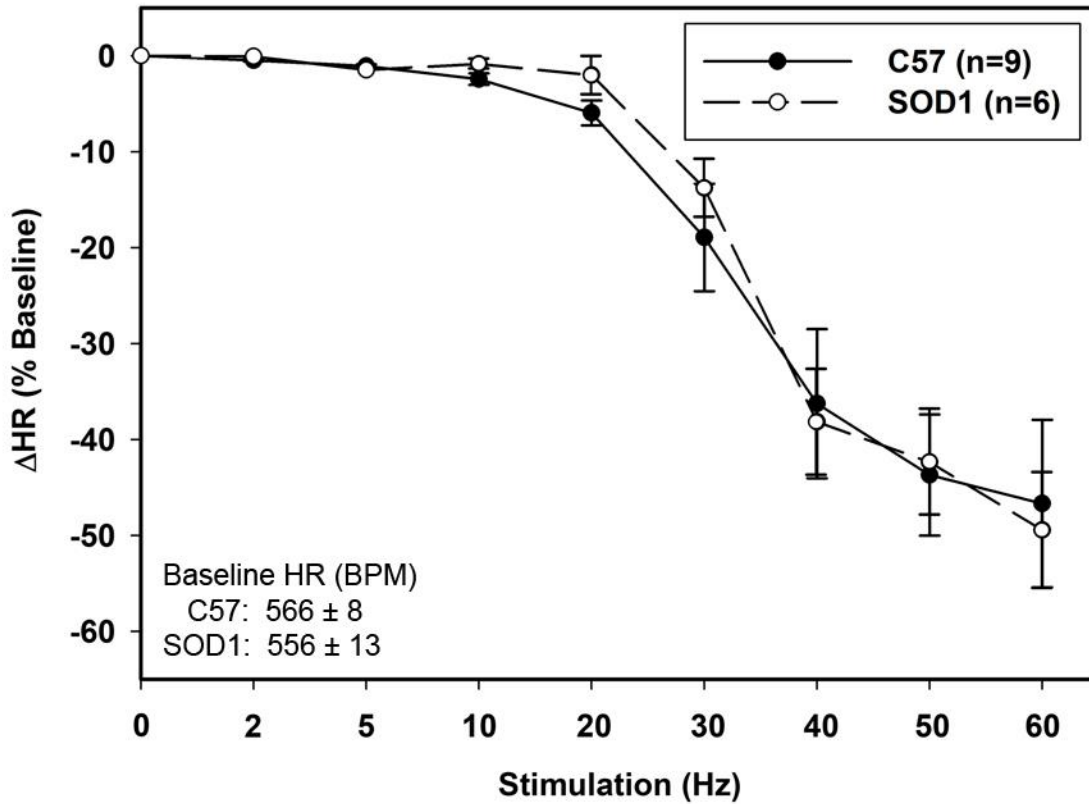


Figure 7: HR response to ADN afferent stimulation.

Heart rate responses are plotted as a function of electrical stimulation frequency. Overexpression of hSOD1 did not significantly affect HR responses to electrical stimulation across the range of frequencies applied (2-Way ANOVA , $P > 0.05$).

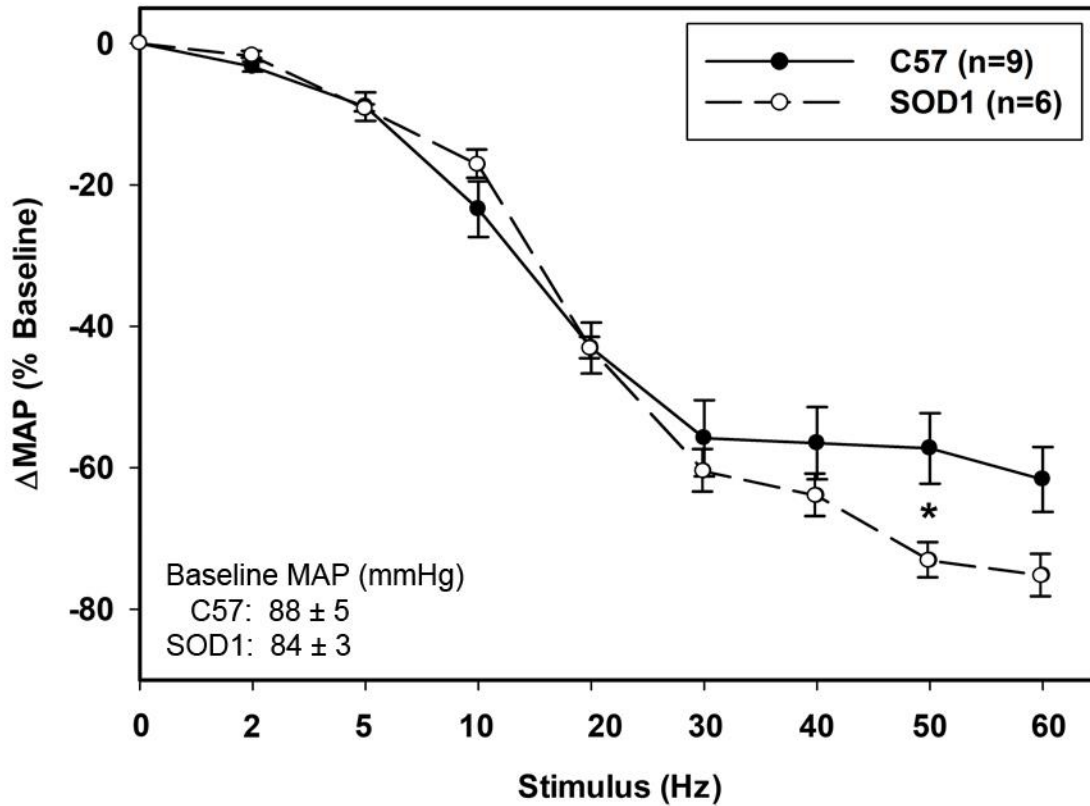


Figure 8: MAP response to ADN afferent stimulation.

MAP responses are plotted as a function of electrical stimulation frequency.

Overexpression of hSOD1 did not significantly affect HR responses to electrical stimulation across the range of frequencies applied ($P > 0.05$), up through 40 Hz. MAP reduction was significantly greater ($P < 0.05$) in SOD1 compared to C57 at 50 Hz, but not at 60Hz. Statistical significance measured by 2-Way ANOVA.

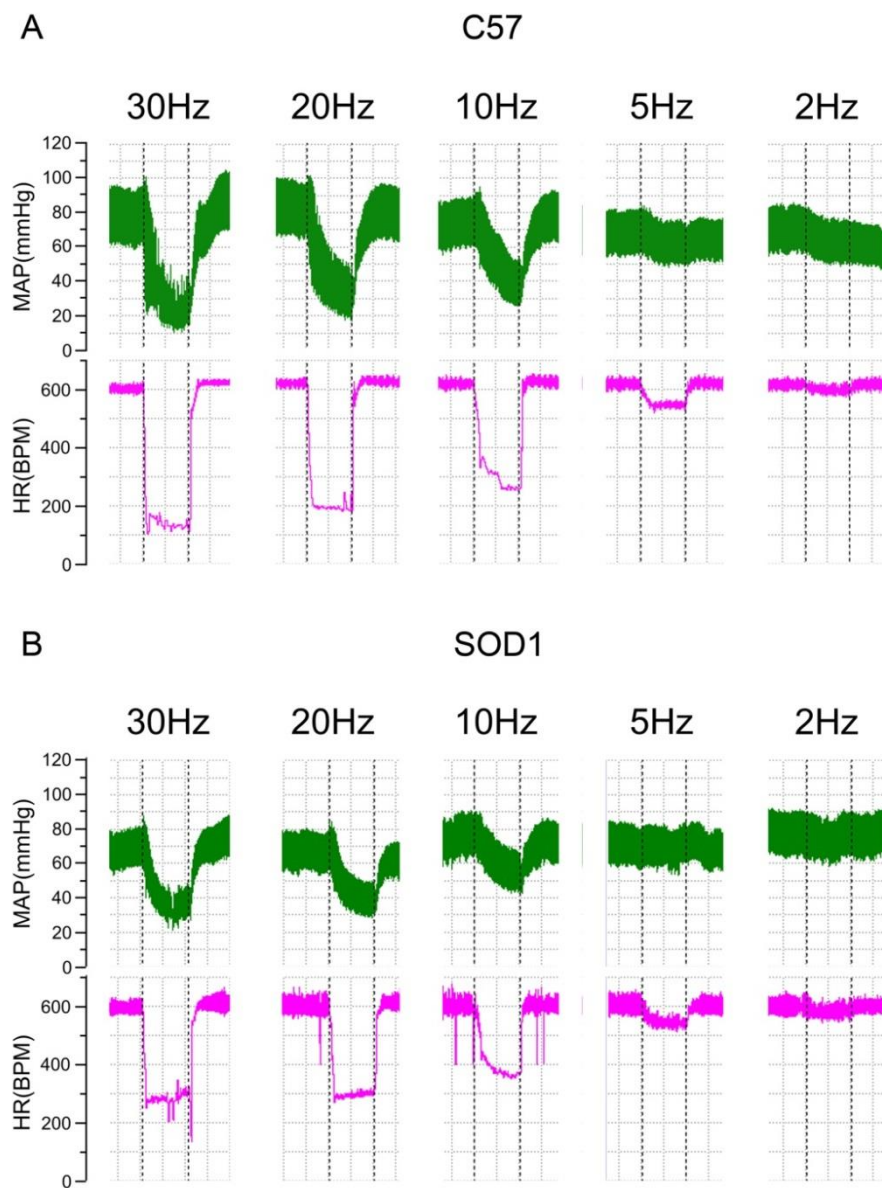


Figure 9: Original recordings showing HR and MAP responses to electrical stimulation of the left cervical vagus at 2 – 30 Hz, 30 μ A, 1 ms in representative C57 and SOD1.

Dashed lines represent 20 s stimulation intervals. (A) C57 mice. (B) SOD1 Mice.

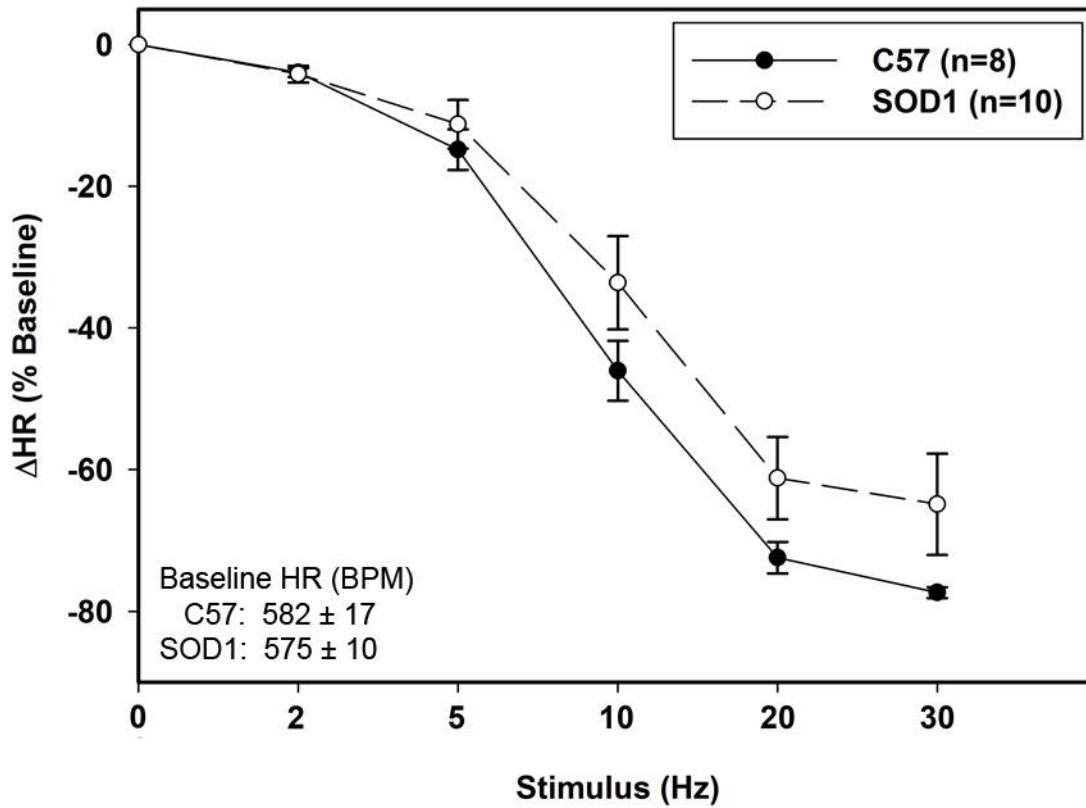


Figure 10: HR response to efferent stimulation.

MAP responses are plotted as a function of electrical stimulation frequency of the left cervical vagus nerve. Overexpression of hSOD1 did not significantly affect HR responses to electrical stimulation across the range of frequencies applied. (2-Way ANOVA, $P > 0.05$).

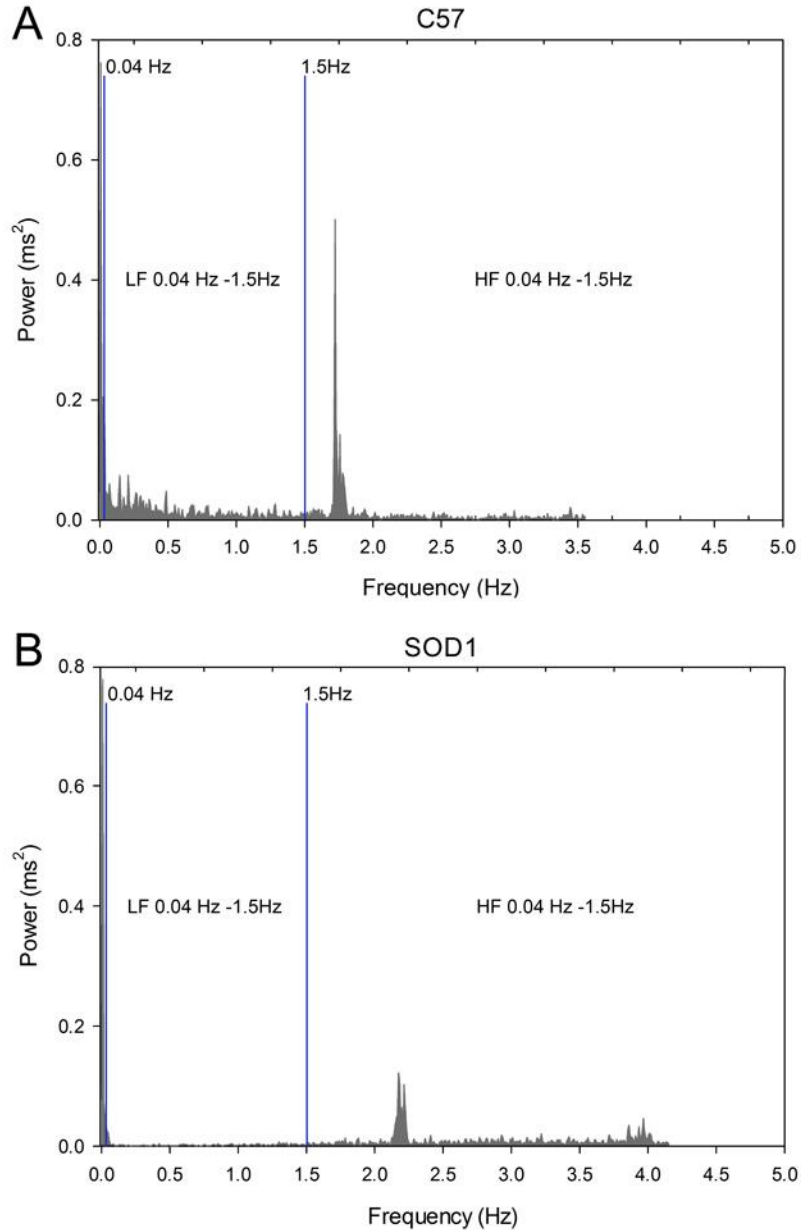


Figure 11: Representative HRV Power Spectra.

Power spectral distributions for C57 (A) and SOD1 (B) mice. There is a visible decrease in density of the power spectra in the LF range (0.04 – 1.5 Hz) in SOD1 mice compared to the WT C57 mice.

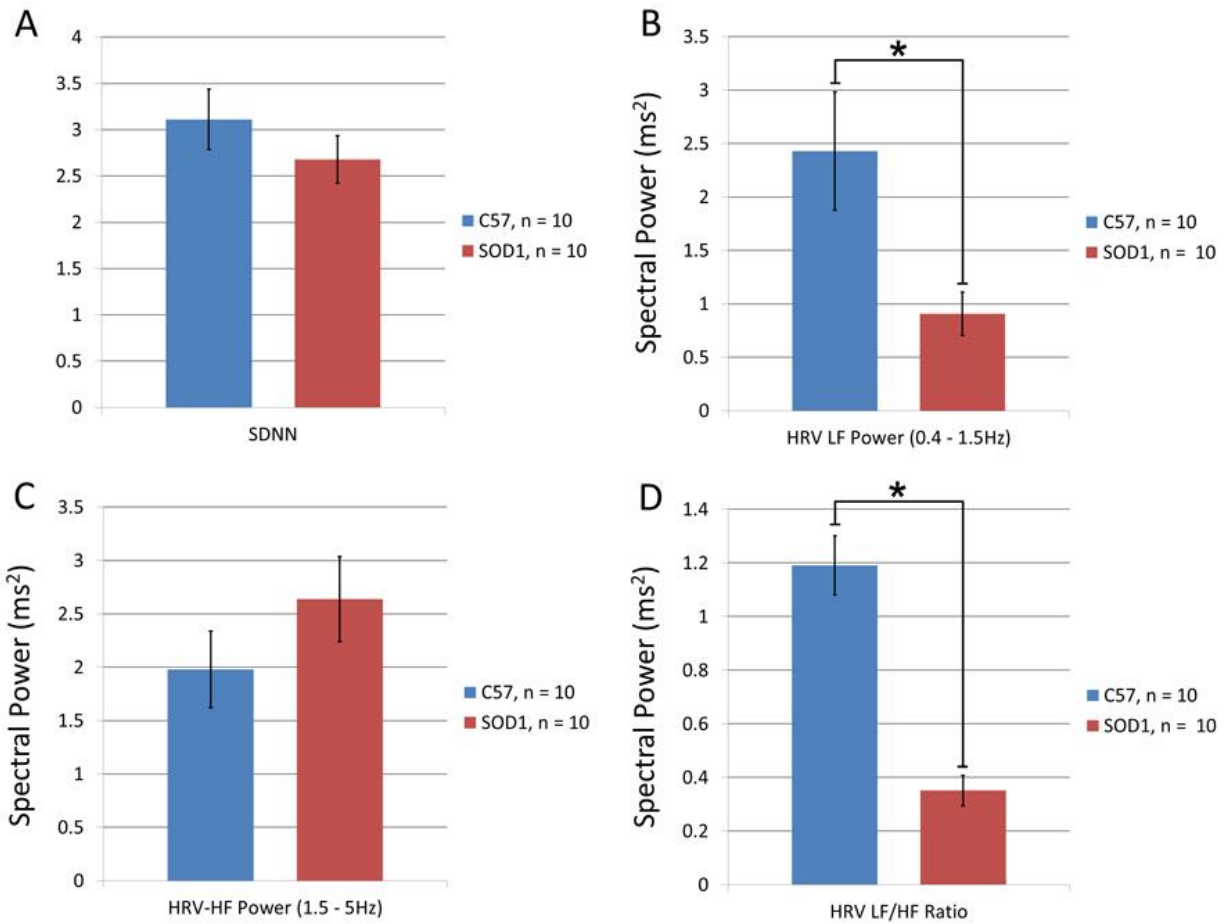


Figure 12: Heart Rate Variability Analysis from Pulse Pressure Waveforms.

(A) Standard deviation of the interbeat interval (SDNN) is comparable between SOD1 (2.89 ± 0.27) animals and C57 controls (2.78 ± 0.20) ($P > 0.05$). (B) The low frequency (LF; 0.04 – 1.5 Hz) component of the power spectral distribution of HRV was significantly lower in SOD1 mice ($0.91 \pm 0.20 \text{ ms}^2$) than in C57 WT ($2.43 \pm 0.55 \text{ ms}^2$) ($P < 0.01$). (C) The high frequency (HF; 1.5 – 5 Hz) component of the power spectral distribution of HRV was not significantly different between the SOD1 ($2.64 \pm 0.40 \text{ ms}^2$) and C57 control animals (1.98 ± 0.36) ($P > 0.05$). (D) The LF:HF ratio is significantly and markedly smaller in SOD1 (0.35 ± 0.06) animals compared to C57 controls (1.19 ± 0.11) ($P < 0.001$). * $P < 0.001$

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CHAPTER 4: DISCUSSION

Origin of C57B6SJL-Tg (SOD1)² Gur/J Mouse Line used in Current Study

Sleep apnea, diabetes, and aging are all risk factors for the development of hypertension, and differing degrees of cardiac autonomic dysfunction, including baroreflex impairment (which is also seen with hypertension). A large and still growing body of research has shown that interventions based on increasing available antioxidants can be largely effective in reducing blood pressure, increasing vascular compliance and function, and improving baroreflex-mediated control of heart rate (Gouty, 2003; Guimaraes, 2012; Kawada, 2015; Nozoe, 2007; Shargorodsky, 2010; Yuan, 2012). However, because ROS have a wide variety of necessary biological functions, it is important to evaluate the effect of high-dose antioxidant therapy on cardiac autonomic function in the absence of any other disease state. The current study uses a transgenic mouse model based on the C57bl/6j mouse line which has been engineered to express human Cu/ZnSOD (SOD1) at a level roughly 3.5 over normal murine SOD1 expression. This transgenic mouse line (C57B6SJL-Tg (SOD1)² Gur/J, Jackson catalog # 002297, Gurney, 1994, originally published as N1029) was developed in 1994 as a gene-dosage control for a mouse model of amyotrophic lateral sclerosis (ALS) overexpressing a SOD1-G93A mutation.

Prior Characterization of C57B6SJL-Tg (SOD1)² Gur/J mice

The SOD1 mouse line used in this study, as well as other lines used in Gurney's original experience, showed no signs of ALS-like symptoms. Gurney's lab also performed anatomical assessment of neural tissue, and found that the mice

overexpressing hSOD1 at levels similar to the G93A strain showed very subtle changes in the anterior portion of the anterior horn (mild swelling of motor fibers and vacuolization of dendrites), but were free of any ALS-like symptoms (Dal Canto & Gurney, 1995). Jaarmsa (2000) performed a similar study with Gurney's N1029 mouse line, finding again that there were mild neurodegenerative changes measured a 30 to 40 weeks of age. Functionally, however, there were no signs of impaired motor performance until 58 weeks of age (rotating rod test). Tg-hSOD1 mice in the current experiment were between 12-16 weeks of age, well before the window in which histological and motor function changes are observed.

Studies Demonstrating Negative Outcomes of hSOD1 Overexpression in Rodents

Aside from this paper, there have been few other studies that have investigated the effect of systemic SOD1 overexpression on cardiac autonomic regulation. Thireau (2008) used a hSOD1 transgenic mouse line based on the FVB mouse line. Activity of hSOD1 in Thireau's line, as measured by enzymatic activity, was 4- fold for the brainstem, and 9-fold for cardiac tissue. Thireau's study was conducted with the intent of looking for pathologies associated with SOD1 overexpression, such as are found in Down's syndrome (Capone, 2001; Groner, 1994a) and ALS (Groner, 1994b; Jaarmsa, 2000). Thireau's study found a reduction of heart rate (measured as R-R intervals) in the transgenic animal compared to controls ($109 \pm 2\text{ms}$ vs. $100 \pm 1\text{ms}$, $P < 0.01$). Heart rate variability (HRV) measurements also revealed that the tg-hSOD1 mice had an increased HRV showed a spectral power for both high frequency (parasympathetic) and low-frequency (sympathetic) that was significantly higher during both light and dark

cycles. However, the LF / HF ratio was significantly lower in the transgenic animals, indicating a parasympathetic dominance in heart-rate control. Other effects noted included increased incidence of atrioventricular blocks (AVB) and sinus arrests (SA) in tgSOD1 mice. Cholinesterase activities in the hearts of the tg-hSOD1 mice were also decreased, which the authors attempted to link to the increased vagal tone and instances of arrhythmias, which had some support by the significantly stronger effect atropine had on decreasing HRV in tg-hSOD1 mice compared to FVB controls. Our study found no evidence of decreased resting heart rate, and the hypertensive baroreflex-mediated depression of heart rate, which is vagally mediated, was not significantly increased in our SOD1 mice. Disagreement between our results could be due to several factors, including age of mice at time of experiment (the present study used mice 3-4 months old, Thireau's mice were 12 months old), and different levels of SOD1 activity in the target tissues (Thireau reported 7661 ± 1941 UA/mg protein in tg-hSOD1 in the brainstem, Dal Canto (1995) measured an equivalent of 3700 UA/mg protein in cortical tissues in Gurney's N1029 mouse line, used in current study).

Although SOD1 has been well-studied as an important protective enzyme that combats the deleterious effects of oxidative stress in cells, paradoxically, some studies have shown that overexpression of hSOD in mice has elicited a broad spectrum of negative effects. These include a decrease in hippocampal long-term potentiation (Gahtan, 1998), impaired uptake of serotonin into blood platelets, resulting in low levels of serotonin in the blood (Schickler, 1989), neuromuscular abnormalities in the tongue (Avraham, 1988). These effects are similar to those seen in trisomy 21 (Down

syndrome) (Gröner, 1994a), in which SOD1 is overexpressed due to gene duplication (Brooksbank & Balazs, 1984; Capone, 2001; Epstein, 1987), which is why transgenic mice overexpressing hSOD1 are sometimes used as models for Down syndrome (Epstein, 1987; Gröner, 1994a; Reeves, 1995; Gahtan, 1998; Schickler, 1989). Rando (1998) observed dystrophy of muscle tissue (necrotic fibers, fat and fiber infiltration into interstitial tissue) in hSOD1 overexpressing mice (Epstein, 1987) with increased levels of serum creatine phosphokinase and markers of lipid peroxidation by 2 months of age. Rando's findings were contradicted by another study in which SOD1 transgenic strains showed no signs of the dystrophic changes or elevated serum creatine levels (Almer, 1999), even in mice with nearly double the hSOD1 expression of Rando's line. Rando, in a 1998 letter to the American Neurologic association, noted expanded on his previous paper noting dystrophic changes in muscle tissue in tgSOD1 mice (Rando, 1998), finding that the disease severity was variable between different strains of tg SOD1 mice. Hemizygous tg-hSOD1 strains, with lower, but still elevated expression of SOD1, did not show the pathological changes seen in the homozygous strains (Rando, 1999).

Studies Demonstrating Beneficial or Neutral Outcomes of hSOD1 Overexpression in Mice

Other studies showed clearly beneficial effects of hSOD1 overexpression in mice, including enhanced angiogenesis (Marikovsky, 2002; Kotulska, 2006). Kruidenier (2003) reported that hSOD1 overexpression in mice (Gurney, 1994, N1029) showed significant anti-inflammatory properties in acute and chronic murine dextran sodium sulfate (DSS) induced colitis, as indicated by lower activity of neutrophilic myeloperoxidase (MPO), as well as increased survival in the transgenic animals. Xu

(2004) reported the mice overexpressing hSOD1 showed increased resistance to oxidative stress and apoptosis of cortical neurons after exposure to chronic intermittent hypoxia than non-transgenic mice. hSOD1 overexpression has also been shown to protect against mitochondrial cytochrome C release and subsequent apoptosis in focal cerebral ischemia models of stroke (Fujimura, 2000). In a model of cardiac ischemia, transgenic mice overexpressing hSOD1 are also reported to show a lower rate of superoxide radical generation following coronary artery ligation, as well as a 2-fold increase in recovery of contractility and a 2.2 fold reduction in infarct size (Wang, 1998).

The Good and Bad of SOD1 Overexpression

There is still considerable debate whether the net impact of SOD1 overexpression is positive or negative. Based on the studies above, it would appear that there can be both beneficial as well as deleterious effects. Although SOD1 can greatly reduce the damage that superoxide anions can inflict upon cells, overexpression can lead to increased concentrations of the product of the dismutation of the superoxide radical: Hydrogen peroxide (H_2O_2) (Schmidt, 1995; Amstad, 1991). It has been proposed that the antioxidant benefits of SOD1 expression are biphasic, with low expression of the enzyme permitting accumulation of superoxide anion and downstream ROS, while increasing expression of SOD1 clears the superoxide anion and reduces oxidative stress, but only until the point that H_2O_2 production exceeds the capacity of catalase or glutathione peroxidase to clear it (Xing, 2002). Xing did examine activities of glutathione peroxidase and catalase in tghSOD1 mice (both homozygous and heterozygous), and found that they did not increase in step with the elevated H_2O_2

concentrations found in the transgenic mice. It has also been postulated that SOD1 can generate H_2O_2 independently of its actions as a $O_2^{\cdot-}$ dismutase by acting as a superoxide reductase or superoxide oxidase (Liochev & Fridovich, 2000), depending on the electron donor and electron receptor participating in the reduction or oxidation of the Cu active site.

Most of the studies showing increased peroxides were conducted with in vitro cell cultures (Xing, 2002, Schmidt, 1995, Amstad, 1991) in which the partial pressure of oxygen is greater than for in vivo conditions (20 % O_2 in vitro vs. 11 % - 2 % O_2 in vivo, depending on tissue perfusion) (Sullivan, 2006). It is fairly well established that superoxide anion generation increases with increasing oxygen tensions (Orsi & Leese, 2001; D'Agostino, 2007). It is possible that the supraphysiologic oxygen tension in in vitro experiments is driving elevated superoxide anion generation, yielding more substrate for overexpressed SOD1, and contributing toward increased intracellular H_2O_2 seen in the in vitro experiments.

Use of tg-hSOD1 Mice: Testing Role of ROS in Derangement of Autonomic Cardiac Regulation

The central aim of this study was to evaluate suitability of the C57B6SjL-Tg (SOD1)² Gur/J mouse line (Jackson catalog # 002297, Gurney, 1994, N1029) in determining if enhanced superoxide anion scavenging can preserve normal cardiovascular autonomic response in disease states whose pathologies are strongly linked to increased oxidative stress; Specifically, diabetes (Maritim, 2003; Figueroa-Romero, 2008), sleep apnea (Zhang & Veasey, 2012; Yamauchi & Kimura, 2008), and

aging (Schoneich, 1999; Gemma, 2007). Our investigation determined that the C57B6SJL-Tg (SOD1)² Gur/J mouse line did not show significant changes in resting heart rate, blood pressure, or baroreflex control of heart rate compared to wild-type (C57BL/6j) mice. Heart rate responses to electrical stimulation of the left ADN and left cervical vagus were also similar between the SOD1 and C57 mice, as was time-domain analysis of heart rate variability measures as the SDNN. Our experiments uncovered three notable differences between hSOD1 overexpressing mice and C57 WT controls.

Increased baroreceptor sensitivity in SOD1 mice

Our investigation determined that the C57B6SJL-Tg (SOD1)² Gur/J mouse line did not show significant changes in resting heart rate, blood pressure, or baroreflex control of heart rate compared to wild-type (C57BL/6j) mice. We did discover that baroreceptor gain was significantly ($P < 0.01$) increased in the tg-hSOD1 animals compared to the controls (Fig. 5). This increase in baroreceptor function was unexpected, and the exact mechanism is as of yet undetermined. Li (1996) saw a similar effect of SOD1 enhancement of carotid sinus nerve activation in response to blood pressure ramps in healthy rabbits. It is possible that the tg-hSOD1 mice have a higher degree of large-artery compliance (Fukai, 2011; Didion, 2002a, 2002b; Lynch, 1997), which may contribute to greater stretch of the aortic arch and increased activation of stretch receptors (Monahan, 2001, 2007; Michas, 2012). Alternatively, hSOD1 overexpression may improve the function of ASIC or HCN channels, which are integral to mechanoreception in aortic arch baroreceptors, and are inhibited by ROS (Abboud, 2010; Lu, 2009; Li YL, 2013). This question deserves further study, and may

be informative as to the role of ROS in modulating mechanotransduction of blood pressure increases. Since overall baroreflex-mediated reduction of heart rate in response to hypertension was not significantly affected ($P > 0.2$) (Fig.2), it would appear that the increased signaling from the aortic baroreceptors is attenuated by other components of the baroreflex loop, such as the NTS, NA, or within the heart itself (Thireau, 2008). Whether this is a product of normal physiologic compensation or something of a more pathologic nature is undetermined. Studies of baroreceptor activation in aging tg-hSOD1 mice, or tg-hSOD1 mice exposed to chronic intermittent hypoxia (sleep apnea model) would be warranted to investigate whether the increased baroreceptor sensitivity in healthy animals would translate to normalization of sensitivity in disease states.

hSOD1 enhancement of MAP depression following ADN stimulation

We found a slight, but significant increase in MAP reduction in response to ADN stimulation at 50 Hz in the SOD1 mice compared to C57 (Fig. 8). The $\Delta\text{MAP} / \text{Frequency (Hz)}$ line for SOD1 showed a lower trend compared to C57 from 40 to 60 Hz, but 50Hz was the only point at which the difference was statistically significant. Over the range of frequencies from 2, 5, 10, 20, and 30 Hz, MAP reductions were similar between both C57 and SOD1 animals. In contrast to PSNS-dominant control of HR, baroreflex-driven changes in AP are predominately effected by the sympathetic nervous system (SNS). The MAP response to the stimuli is almost entirely sympathetically mediated (Guyenet, 2006). Vascular tone is maintained by a constant, baseline sympathetic outflow (Guyenet, 2006) generated by the rVLM and PVN neurons sending

projections to sympathetic preganglionic neurons (SPGNs) in the spinal column, which in turn project to sympathetic ganglia neurons (SGNs) that innervate the vasculature. Increase in sympathetic tone leads to vasoconstriction and an increase in AP, while withdrawal of sympathetic tone promotes vasodilation and a decrease in AP. Humoral factors, such as angiotensin II (ANGII), adrenalin, and NO also influence vascular tone (Guyenet, PG., 2006) and must be considered while interpreting these findings. Since the SNS directly controls release of adrenalin and elevates ANGII levels in the blood, these humoral factors reflect function of the SNS as well. Though the differences in MAP decreases between 30 – 60 Hz in the SOD1 and C57 animals are not dramatic, it suggests the possibility that hSOD1 overexpression may have a mild effect on one or more of the components of the sympathetic baroreflex.

Superoxide scavenging in the PVN or rVLM is known to reduce ANGII mediated activity in these sympathetic nuclei (Campese, 2004, 2005; Gao, 2004). Because ANGII also increases vascular tone directly via a superoxide dependent signaling pathway, superoxide scavenging also increases large artery compliance (Fukai, 2011; Didion, 2002a, 2002b; Lynch, 1997; d'Uscio, 2010; Seals, 2014; Shargorodsky, 2010) by blocking ANGII signaling, and uncoupling nitric oxide synthase (NOS) inhibition from NO. Another possibility would be SOD1-mediated inhibition of central and arterial ANGII signaling (Yuan, 2012), which is dependent on O_2^- (Wang, 2002) ANGII signaling maintains a higher constant sympathetic output in the sympathetic brainstem nuclei such as the rVLM or PVN (Gao, 2004).

Reduced LF spectral power in SOD1 Mice

Frequency-domain analysis of heart rate variability has been used, historically, to attempt to measure the balance of sympathetic and parasympathetic controls of beat-to-beat variability of heart rate (Pagani, 1984, 1986). In healthy animals, there is a great deal of variability in the instantaneous heart rate due to autonomic attempts at maintaining homeostasis of tissue perfusion and oxygenation (Malik, 1996). The parasympathetic nervous system influence on HRV is fast acting, with low latency of onset and rapid withdrawal (Spear, 1979). The sympathetic influences have a higher latency of onset, and longer residual effects. The power spectral distribution (PSD) generated by fast-fourier transform of the time-domain to the frequency domain is divided into 3 main bands: the very-low frequency (VLF) band from 0.0 - 0.04 Hz, the low frequency (LF) band between 0.04 – 1.5 Hz, and the high frequency (HF) band between 1.5 – 5.0 Hz in mice (Thireau, 2008). Historically, parasympathetic modulations of instantaneous heart rate were assigned to the HF band, and sympathetic modulations to the LF band. The ratio of the low-frequency to high-frequency spectral powers LF:HF ratio was considered to be a fair measure of sympathovagal balance (Pagani, 1984, 1986). This type of spectral power analysis is no longer considered a valid measure of sympathetic: parasympathetic balance (Eckberg, 1997; Reyes del Paso, 2013) but it is well established that changes in the PSD are seen with autonomic blockade (sympathetic, parasympathetic, or total) (Thireau, 2008) and in a range of diseases that reduce heart rate variability by impairing autonomic controls (Malik, 1996). In this analysis, we used spectral analysis simply as

a means to reveal any changes to autonomic heart rate control attributable to tg-hSOD1 overexpression, which were not apparent in our other experiments.

We found that HF spectral power was unchanged in SOD1 mice, but that the LF component was significantly and dramatically reduced. Due to the complexity of neural and hormonal controls in the LF power band, and the lack of scientific consensus, a precise interpretation is unlikely, and without further research on this line, the cause will remain unknown. However, we can postulate a possible target for further investigation. It has been reported that treatment with angiotensin converting enzyme inhibitors (ACE inhibitors. (Souza, 2004; Dasilva) lowers the LF power of HRV, although these findings were in human patients undergoing coronary bypass, and aging rats, respectively. However, taken together with our prior observation of an increased MAP depression following ADN stimulation in SOD1 animals, we can find a common thread to a possible down-regulation of the renin – angiotensin system. The dependence of ANGII on superoxide as a signaling molecule (Wang, 2002; Zimmerman, 2004; Li & Zheng, 2011; Li YL 2011, 2013) makes it a feasible explanation both the increased MAP sensitivity to ADN stimulation and the decreased LF power seen in these studies. It has already been established that scavenging of O_2^- by SOD mimetic or hSOD overexpression ameliorates ANGII-induced increases in sympathetic brainstem activity and promotes relaxation of the resistance blood vessels (d'Uscio, 2010; Seals, 2014; Shargorodsky, 2010; Yuan, 2012; Wang, 2002). Experiments probing HR, BP, and HRV responses following injection of ANGII and ANGII inhibitors in the SOD1 and C57 mice could provide further evidence to establish this link.

Conclusions

Suitability of the use of the C57B6SjL-Tg (SOD1)² Gur/J Mouse strain to study contributions of superoxide to the pathogenesis of cardiac autonomic neuropathies (CAN), as well as studied to determine if chronic, elevated superoxide scavenging can be beneficial in preventing or treating CAN is still not fully determined. Basic investigation of resting HR, BP, and baroreflex sensitivity does not reveal any differences in autonomic control of the heart rate between C57 controls and SOD1 mice. Th-hSOD overexpression –induced changes in the baroreflex arc were only revealed by direct measurement of aortic depressor nerve response to arterial pressure increases, MAP response to aortic depressor nerve stimulation, and by obtaining a spectral power distribution of heart rate variability. These tests uncovered an increased baroreceptor sensitivity to BP increases, enhanced MAP depression in response to ADN stimulation, and a decrease in low-frequency spectral power. These three changes have speculative ties to a reduction in ANGII signaling, but further investigations will be needed to determine if this is a factor. If ANGII signal pathway is found to have been disrupted in the SOD1 animals, and the bulk of the literature supports this hypothesis, then the C57B6SjL-Tg (SOD1)² Gur/J may prove useful in the study of ANGII-O₂⁻ signaling pathways in the pathogenesis of CAN in models of diabetes, sleep apnea, and aging.

Anatomical studies to examine morphological changes in baroreceptor nerve terminals would also be indicated to rule out remodeling of the baroreceptor terminals as a source of increased baroreceptor activity. RNA and protein expression studies of tg-hSOD1 expression in the target tissues (neural, cardiac, vascular) are also indicated

in order to quantify tissue-specific overexpression in these animals. Histochemical analysis of ROS markers will give us a better understanding on whether oxidative stress is reduced or enhanced (Rando, 1998, 1999) in the tissues. As we are investigating the effect overexpressed tg-hSOD1 on the cardiovascular system, echocardiography and electrocardiography should be performed to give us a more complete physiologic profile of these animals.

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