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Research Paper

Increased mitochondrial superoxide in the brain, but not periphery, sensitizes mice to angiotensin II-mediated hypertension

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ABSTRACT

Angiotensin II (AngII) elicits the production of superoxide ($O_2^{\bullet-}$) from mitochondria in numerous cell types within peripheral organs and in the brain suggesting a role for mitochondrial-produced $O_2^{\bullet-}$ in the pathogenesis of hypertension. However, it remains unclear if mitochondrial $O_2^{\bullet-}$ is causal in the development of AngII-induced hypertension, or if mitochondrial $O_2^{\bullet-}$ in the absence of elevated AngII is sufficient to increase blood pressure. Further, the tissue specific (*i.e.* central versus peripheral) redox regulation of AngII hypertension remains elusive. Herein, we hypothesized that increased mitochondrial $O_2^{\bullet-}$ in the absence of pro-hypertensive stimuli, such as AngII, elevates baseline systemic mean arterial pressure (MAP), and that AngII-mediated hypertension is exacerbated in animals with increased mitochondrial $O_2^{\bullet-}$ levels. To address this hypothesis, we generated novel inducible knock-down mouse models of manganese superoxide dismutase (MnSOD), the $O_2^{\bullet-}$ scavenging antioxidant enzyme specifically localized to mitochondria, targeted to either the brain subfornical organ (SFO) or peripheral tissues. Contrary to our hypothesis, knock-down of MnSOD either in the SFO or in peripheral tissues was not sufficient to alter baseline systemic MAP. Interestingly, when mice were challenged with chronic, peripheral infusion of AngII, only the MnSOD knock-down confined to the SFO, and not the periphery, demonstrated an increased sensitization and potentiated hypertension. In complementary experiments, over-expressing MnSOD in the SFO significantly decreased blood pressure in response to chronic AngII. Overall, these studies indicate that mitochondrial $O_2^{\bullet-}$ in the brain SFO works in concert with other AngII-dependent factors to drive an increase in MAP, as elevated mitochondrial $O_2^{\bullet-}$ alone, either in the SFO or peripheral tissues, failed to raise baseline blood pressure.

1. Introduction

Reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$) have been implicated in the pathogenesis of angiotensin II (AngII)-mediated hypertension [1,2]. While the downstream reactions of these ROS are distinctly unique, the derivation of these ROS primarily begins with a one electron transfer to oxygen forming $O_2^{\bullet-}$, followed by the dismutation to H_2O_2 or the rapid reaction with nitric oxide ($NO^{\bullet}$) to form $ONOO^-$ [3]. Thus, the fundamental understanding of how $O_2^{\bullet-}$ is involved in AngII-mediated hypertension is at the crux of deciphering the redox-regulation of this disease.

Superoxide may be produced from various subcellular sources in response to AngII, but mitochondria have been demonstrated to be a primary source in various cell types [2,4–9]. For example, we have demonstrated that AngII increases mitochondrial localized $O_2^{\bullet-}$ in cultured neurons [7,9]. This increase in mitochondrial $O_2^{\bullet-}$ contributes to the AngII-induced inhibition of outward K^+ current as over-expressing manganese superoxide dismutase (MnSOD), the mitochondrial-targeted $O_2^{\bullet-}$ scavenging antioxidant enzyme, attenuates this AngII-induced neuronal response [7]. Additionally, it has been demonstrated that over-expression of MnSOD in the subfornical organ (SFO), a well-established AngII-sensitive cardiovascular control region in the brain [10,11], in mice attenuates the acute pressor response to centrally administered AngII [10]. Likewise, whole body over-expression of MnSOD or systemic infusion of the mitochondrial-targeted antioxidant MitoTempol has also been shown to attenuate chronic AngII-mediated hypertension in mice [8]. Conversely, heterozygous MnSOD mice become more hypertensive compared to wild-type when aged or challenged with high salt [12]. Overall, these previous studies are highly suggestive that mitochondrial $O_2^{\bullet-}$ contributes to the pathogenesis of hypertension; however, it remains unclear if increased

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mitochondrial $O_2^{\bullet-}$ in the absence of pro-hypertensive stimuli is sufficient to increase systemic blood pressure. Further, because whole body over-expression models of MnSOD or systemic MnSOD heterozygote mice have been utilized, it remains uncertain which cell types or organs are responsible in mediating changes in blood pressure.

Due to these gaps in knowledge, we set out to examine the tissue-specific effects of increased mitochondrial $O_2^{\bullet-}$ on basal and AngII-challenged hemodynamics. With observations from our lab and others suggesting mitochondrial $O_2^{\bullet-}$ is a critical signaling molecule in virtually all cell types, we hypothesized that both central and peripheral increases in mitochondrial $O_2^{\bullet-}$ elevate baseline systemic MAP, and moreover sensitize mice to AngII-mediated hypertension. To test this hypothesis, we utilized a homozygous conditional MnSOD knock-down mouse (loxP derived; $MnSOD^{L/L}$) combined with two strategies for targeting cre-recombinase to generate peripheral or brain SFO MnSOD knock-down animals. While we observed significant knock-down of MnSOD in both models, contrary to our hypothesis, neither model demonstrated any change in MAP at baseline. However, when challenged with AngII only the brain SFO MnSOD knock-down animals displayed a potentiated hypertensive response, thus suggesting mitochondrial $O_2^{\bullet-}$ in the SFO plays a preferential role over the periphery in sensitizing to AngII-mediated hypertension.

2. Materials and methods

2.1. Mice

All experiments were performed using 8–12 week-old male mice of a C57BL/6 background. Wild-type mice (*i.e.* C57BL/6NHsd) were purchased from Harlan Laboratories/Envigo (Indianapolis, IN). Mice possessing loxP elements flanking (*i.e.* floxed) exon 3 of the MnSOD gene locus (*i.e.* B6.Cg-Sod2^{tm1Lox}; shorthand $MnSOD^{L/L}$) have been previously described [13]. Mice possessing a conditionally-expressed tamoxifen-inducible cre-recombinase targeted to the ubiquitously expressed ROSA26 gene locus (*i.e.* B6.129-Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$/J; shorthand ROSA-Cre$^{+/+}$) have been previously described and were purchased from Jackson Laboratories (Bar Harbor, ME) [14]. $MnSOD^{L/L}$ and ROSA-Cre$^{+/+}$ mice were backcrossed to the F3 generation to allow for 100% usable progeny of either $MnSOD^{L/L}$ ROSA-Cre$^{+/-}$ (inducible knock-down) or $MnSOD^{L/L}$ ROSA-Cre$^{-/-}$ (control) genotypes. Mice were given access to standard chow (Teklad Laboratory Diet #7012, Harlan Laboratories, Madison, WI) and water *ad libitum*. For all survival surgical procedures, mice were anesthetized using 0.5–2% isoflurane supplemented with 1 l/min oxygen. Bupivacaine (0.5% solution) was used as post-surgical anesthetic, and mice were monitored daily post-operation for signs of illness or infection. Mice were euthanized by pentobarbital overdose (150 mg/kg, Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) administered intraperitoneally. All procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

2.2. Knock-down and over-expression of MnSOD

Protocol 1: For peripheral knock-down of MnSOD, $MnSOD^{L/L}$ ROSA-Cre$^{+/-\text{ and }-/-}$ mice were treated daily with tamoxifen (Sigma-Aldrich #T5648, St. Louis, MO) for 5 days. Tamoxifen was resuspended at 20 mg/mL in sunflower seed oil (Sigma-Aldrich #S5007, St. Louis, MO) and injected at 150 mg/kg intraperitoneally per mouse per day. While the ROSA26 promoter is expressed in all cell types, negligible central nervous recombination has been observed in this model after tamoxifen administration [15], thus allowing for an efficient peripheral knock-down of MnSOD. Protocol 2: For knock-down of MnSOD in the brain SFO, $MnSOD^{L/L}$ mice were administered adenovirus (4×10^7 plague-forming units (PFU)) encoding either cre-recombinase (AdCre; The University of Iowa Viral Vector Core Facility, Iowa City, IA) or control adenovirus (AdGFP or AdEmpty, collectively AdControl; The University of Iowa Viral Vector Core Facility, Iowa City, IA) by a one-time intracerebroventricular (ICV) injection. Stereotactic coordinates, as previously described [16], where used for ICV injections. Protocol 3: For over-expression of MnSOD in the SFO, wild-type mice were administered adenovirus (4×10^7 PFU) encoding human MnSOD (AdMnSOD, ViraQuest Inc., North Liberty, IA) or AdControl by a one-time ICV injection.

2.3. Mean arterial pressure recording

Induction of hypertension was performed by implantation of subcutaneous osmotic minipumps (Alzet #1002, Durect Corporation, Cupertino, CA) delivering AngII (400 ng/kg/min; Sigma #A9525, St. Louis, MO) until complete emptying of the pumps (approximately 3 weeks) [17]. Telemetric recording of mouse hemodynamics has been previously described in detail [17]. Briefly, blood pressure recordings were performed using intra-carotid arterial catheters (PA-C10, Data Sciences International, Minneapolis, MN) attached to radio telemeters for direct measurement of mean arterial pressure and heart rate in conscious unrestrained animals. Hemodynamic recordings were performed for 20 s every minute for the same 2-h time period daily for the duration of the experiment. Averages of mean arterial pressure were calculated daily over the 2-h period when the mice displayed minimal activity.

2.4. Tissue isolation

Analysis of tissues for knock-down studies occurred 14 days after gene recombination (*i.e.* 14 days after the last tamoxifen injection for peripheral tissues or virus administration for SFO) to allow endogenous MnSOD protein to degrade, while tissue analysis in over-expression studies was undertaken 4 days after AdMnSOD injection to allow production of exogenous MnSOD protein. At time of euthanasia, blood was removed from the tissues by 0.9% saline solution perfused via peristaltic pump through the left ventricle of the heart. Following saline perfusion, tissues were either isolated for use in assays requiring live cells or further perfused using a 4% paraformaldehyde solution to fix tissues prior to isolation and analysis.

2.5. Laser-capture microdissection and quantitative reverse transcription real-time PCR (qRT-PCR)

Freshly isolated and non-fixed brains were snap frozen using dry ice and mounted in blocks using OCT freeze media (Thermo Fisher Scientific #23-730-571, Waltham, MA). Brains were cut by cryostat to the level of the SFO and 50 μm sections were placed upon RNase-free frame-foiled polyethylene terephthalate slides (North Central Instruments #11505190, Plymouth, MN). The SFO region was imaged, mapped, and dissected on a Leica LMD7000 laser microdissection microscope. Collected tissue was immediately placed into TRIzol Reagent (Thermo Fisher Scientific #15596-018, Waltham, MA), and total RNA was extracted via manufacturer's instructions. Concentration of RNA was determined spectrophotometrically using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and cDNA was obtained using the high capacity cDNA archive kit (Applied Biosystems #4368813, Grand Island, NY). SYBR green qRT-PCR for MnSOD was performed on a BioRad iCycler iQ Real-Time PCR Detection System (BioRad, Hercules, California). A threshold in the linear range of PCR amplification was selected and the cycle threshold (Ct) determined. Levels of transcripts were then normalized to the 18S loading control and compared relative to the control sample using the $2^{-\Delta\Delta Ct}$ method. Primers specific to the coding region of either mouse or human MnSOD were used. Primer sequences were as follows: 18S forward, 5′-GCCCGAAGCGTTTACTTTGA-3′; 18S reverse, 5′-TCATGGCCTCAGTTCCGAA-3′; mouse MnSOD forward, 5′-GCTCTGGCCAAGGGAGATGT-3′; mouse MnSOD reverse, 5′-