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Nicotinamide Riboside and the Aortic Response to Angiotensin II Infusion in Mice

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ABSTRACT

Damage to vascular cells of the aorta drives vascular dysfunction and disease. Nicotinamide adenine dinucleotide (NAD^+) is a cellular metabolite critical to cellular health, but NAD^+ levels decline during oxidative insults and aging. The NAD^+ precursor, nicotinamide riboside (NR) can augment NAD^+ levels. Herein, I determined whether orally administrated NR could protect the aorta of middle-aged mice from acute (3-day) and sustained (28-day) angiotensin II (Ang II) infusion-induced damage. I demonstrate, for the first time, that Ang II infusion can induce early death of aortic endothelial cells and smooth muscle cells, that this early death response was associated with DNA strand breakage, and that NR abrogated both cell death and DNA degradation. In addition, NR blunted DNA oxidation, proinflammatory signaling, and vascular cell senescence, during sustained delivery of Ang II. Thus, orally administered NR can protect the aorta from damage imposed by Ang II, a finding with clinical implications.

KEYWORDS:

NAD^+, nicotinamide riboside, angiotensin II, aorta, endothelial cells, vascular smooth muscle cells, cell death, DNA damage, inflammation, senescence

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8-oxo-dG-	8-oxo-2'-deoxyguanosine
AAA-	abdominal aortic aneurysm
ADP-	adenosine diphosphate
ADPR-	adenosine diphosphate–ribose
AIF-	apoptosis inducing factor
ANG II-	angiotensin II
AT1R-	angiotensin II type 1 receptor
AT2R-	angiotensin II type 2 receptor
ATF4-	activating transcription factor 4
ATP-	adenosine triphosphate
BP-	blood pressure
cADPR-	cyclic ADP ribose
CD38-	cluster of differentiation 38
CHF-	chronic heart failure
CRP-	c-reactive protein
DBP-	diastolic blood pressure
EC-	endothelial cell
ECM-	extracellular matrix
EPC-	endothelial progenitor cell
ETC-	electron transport chain
EthD-III-	Ethidium Homodimer-III
GADPH-	glyceraldehyde 3-phosphate dehydrogenase
H_2O_2-	hydrogen peroxide
HFD-	high fat diet
HMGB1-	high mobility group box 1
KO-	knockout
KYN-	kynurenine
MPT-	mitochondrial permeability transition
NA-	nicotinic acid

NAAD-	nicotinic acid adenine dinucleotide
NAADP-	nicotinic acid adenine dinucleotide phosphate
NAD^+-	nicotinamide adenine dinucleotide
NADH-	nicotinamide adenine dinucleotide hydride
NADP-	nicotinamide adenine dinucleotide phosphate
NADPH-	nicotinamide adenine dinucleotide phosphate oxidase
NAM-	nicotinamide
NAMN-	nicotinic acid mononucleotide
NAMPT-	nicotinamide phosphoribosyltransferase
NMN-	nicotinamide mononucleotide
NMNAT-	nicotinamide mononucleotide adenylyltransferase
NO-	nitric oxide
Nox-	nadph oxidase
NR-	nicotinamide riboside
NRK-	nicotinamide riboside kinase
PAR-	poly (ADP-ribose)
PARG-	poly (ADP-ribose) glycohydrolase
PARP-	poly (ADP-ribose) polymerase
RAGE-	receptor for advanced glycation end products
RAS-	renin-angiotensin system
RIPK-	receptor interacting protein kinase
ROS-	reactive oxygen species
SBP-	systolic blood pressure
SIRT-	sirtuin
TRAIL-	tnf-related apoptosis inducing ligand
Trp-	tryptophan
VCAM-1-	vascular cell adhesion molecule-1
VSMC-	vascular smooth muscle cell
vWF-	von willebrand factor

1 INTRODUCTION

This introduction will begin by briefly introducing cardiovascular disease, the aorta and its cellular composition. Nicotinamide adenine dinucleotide (NAD^+) may promote vascular cell health and its biosynthesis and various cellular roles will be introduced. Subsequently, proteins that consume NAD^+ will be comprehensively reviewed, highlighting their roles in NAD^+ homeostasis, cellular health and pathology. I will then discuss the decline in NAD^+ seen during oxidative stress and aging. Furthermore, nicotinamide riboside (NR) supplementation will be introduced as a means to counteract this decline. Finally, angiotensin II (Ang II) infusion will be introduced as a model of age/pathology-related vascular oxidative stress, and the potential for this to benefited by NR administration will be introduced.

1.1 Cardiovascular disease, the aorta and its cellular constituents

Cardiovascular disease remains the commonest cause of death globally[1]. Critical to cardiovascular health is the maintenance of blood vessel structure and function. The aorta is the largest vessel in the body, responsible for the distribution of blood to the downstream systemic circulation[2]. Due to its proximity to the heart, the aorta is exposed to unique biophysical stresses and is susceptible to aneurysms, atherosclerosis and stiffening[3,4]. Dysfunction in cells that comprise the aorta has been implicated in these conditions [5,6].

The aorta is lined by a single layer of endothelial cells (ECs), which together with underlying extra cellular matrix (ECM) and scattered cells, constitutes the tunica intima[7].

These endothelial cells control the permeability of the vessel wall, secrete vasculoprotective compounds, and crosstalk with vascular smooth muscle cells (VSMCs) to alter vessel dynamics[7].VSMCs are found in the tunica media. This is the thickest region of the aortic wall, which is also made of ECM including distinct layers of elastin. VSMCs contract and relax to alter blood flow, response to vessel injury and secrete ECM during development and disease. The outermost layer of the aorta is the tunica adventitia. This layer is comprised of loose connective tissue and a number of cell types including fibroblasts and progenitor cells[8]. The adventitia anchors vessels to organs and plays a role in tissue repair and inflammation[8].

Damage to ECs and VSMCs within the aortic wall compromises their function and is considered a driver of vascular disease[5,6]. Biophysically, aortic cell damage can entail the breakage of DNA strands and the fragmentation of DNA as part of the cell death cascade.[9,10]. DNA damage can be induced by oxidative stress which is considered a key feature of cardiovascular disease.[11,12]. Thus, strategies that allow ECs and VSMCs to resist damage, including oxidative damage and DNA degradation, may be critical to reducing aortic disease and its complications. One such strategy is the provision of a NAD^+ precursor to augment the cellular levels of NAD^+, a metabolite that promotes cell health and function, including resistance to oxidative stress[13]. I discuss this strategy in the following sections, introducing NAD^+, its biosynthesis, cellular roles and homeostasis.

1.2 NAD$^+$: Routes of synthesis and cellular functions

1.2.1 NAD$^+$: A brief history

NAD$^+$ (nicotinamide adenine dinucleotide) is necessary to sustain life and accordingly, so are it's biosynthetic precursors. In the absence of adequate dietary NAD$^+$ precursor intake, a condition termed Pellagra develops, which was epidemic in United States during the 20th Century[14]. Research determined that niacin, now known as a precursor to NAD$^+$, could cure Pellagra. This eradicated the disease[14]. In parallel with this, NAD$^+$ was identified and demonstrated to be involved in cellular redox reactions[15]. Hence, NAD$^+$ precursor intake has been known as an important aspect of human health and nutrition. However, in recent decades, NAD$^+$ has been identified as a metabolite with several cellular functions; whose levels decline during age and disease, impacting cellular health[16]. Thus, supplementation with NAD$^+$ precursors has been begun to be investigated as a strategy to maintain cellular NAD$^+$ levels during aging and disease[16].

1.2.2 NAD$^+$ synthesis through dietary tryptophan

Tryptophan (Trp) is an essential amino acid, with several biological roles beyond protein synthesis[17]. Trp is a precursor for serotonin and melatonin, but is primarily catabolized in the liver through the kynurenine (KYN) pathway, which can yield NAD$^+$[17]. This occurs through an 8-step pathway, termed the *de-novo pathway*, because the nicotinamide base is made de novo and not recycled, as is the case in other NAD$^+$

synthetic pathways. These latter pathways are termed *salvage pathways.* Interestingly, Trp will only yield NAD^+ if there is enough substrate to saturate the activity of enzymes directing kynurenine pathway intermediates through alternate routes[18]. For this reason, and because of the pleotropic biological functions of Trp, it is estimated that 60 mg of Trp is equivalent to only 1 mg of the NAD^+ precursor niacin[19]. Therefore, Trp is not an ideal precursor to NAD^+ and cannot sustain NAD^+ requirements[20]. In addition, the KYN pathway should not be augmented as its intermediates can induce pathology[21].

1.2.3 NAD^+ synthesis through dietary salvage pathway intermediates

When NAD^+ is consumed by cellular proteins, nicotinamide (NAM) is formed as a byproduct and can be recycled to NAD^+ through several salvage pathway intermediates[20]. These intermediates, and NAD^+ itself, are naturally present in whole food and can be obtained through the diet to generate intracellular NAD^+ [22,23,24]. Several early animal studies have shed light on the absorption of these precursors (Fig. 1).

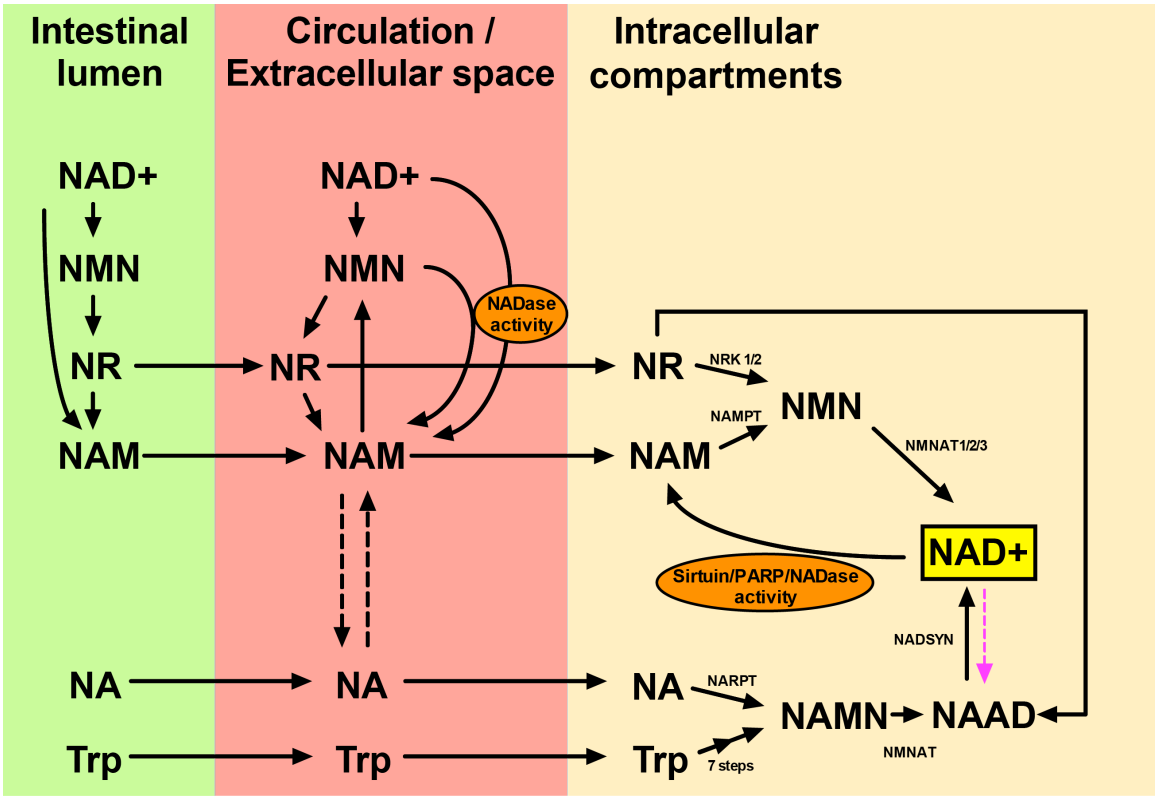
Studies suggest that NAD^+ and most of its salvage precursors are converted to NAM in the small intestine before absorption[25]. In the small intestine, NAD^+ is converted to NAM directly[26] or indirectly[25] through sequential conversion to nicotinamide mononucleotide (NMN), NR, and then NAM. This indirect pathway gives insight into the intestinal absorption of these NAD^+ precursors (NMN, NR, NAM). NMN appears to be rapidly converted to NR, which accumulates and is subsequently converted to NAM[25]. NAM is then the major intestinal uptake product, and accordingly, when NAM itself is injected into the small intestine it is absorbed intact[25]. However, when NR is administered orally, some is also directly absorbed and utilized to generate tissue

NAD^{+}[27]. Nicotinic acid (NA) appears to be the only salvage intermediate not substantially absorbed as NAM. Instead, when injected in the small intestine, NA remains as is and is absorbed largely intact.

Understanding the absorption of these precursors is important as they appear to elevate NAD^{+} and NAD^{+} breakdown products to different degrees[28]. This may be due to minor but relevant absorption of non-NAM/NA NAD^{+} precursors, as has been seen with NR[27]. As well as tissue-specific utilization of absorbed precursors[27,29,28].

Figure 1.1 NAD^+ precursor uptake and metabolism.

This figure illustrates the absorption and metabolism of NAD^+ precursors from the intestine to the inside of a cell. As precursors are absorbed from the intestinal lumen to the circulation, intestinal cells do perform some minor intracellular metabolism, which is not highlighted in this figure. Also not shown is intra- to extra-cellular precursor transport and transport from the circulation to the intestinal lumen. Dashed lines indicate less elucidated pathways, which may be due to intracellular precursor metabolism and secretion. The purple dashed line indicates a pathway that has been hypothesized but not directly proven. Abbreviations used are: NAD^+, nicotinamide adenine dinucleotide; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; NAM, nicotinamide; NA, nicotinic acid; Trp, tryptophan; NAMN, nicotinic acid mononucleotide; NAAD, nicotinic acid adenine dinucleotide; NRK1/2, nicotinamide riboside kinase 1/2; NAMPT, nicotinamide phosphoribosyltransferase; NMNAT1/2/3, nicotinamide mononucleotide adenylyltransferase 1/2/3; NARPT, nicotinate phosphoribosyltransferase; NADSYN, NAD^+ synthase.


Intestinal lumen
Circulation / Extracellular space
Intracellular compartments
NAD+
NMN
NR
NAM
NA
Trp
NADase activity
NRK 1/2
NAMPT
NMNAT1/2/3
Sirtuin/PARP/NADase activity
NADSYN
NARPT
NAMN
NAAD
NMNAT
7 steps

1.2.4 NAD^+ synthesis and salvage at the cellular level

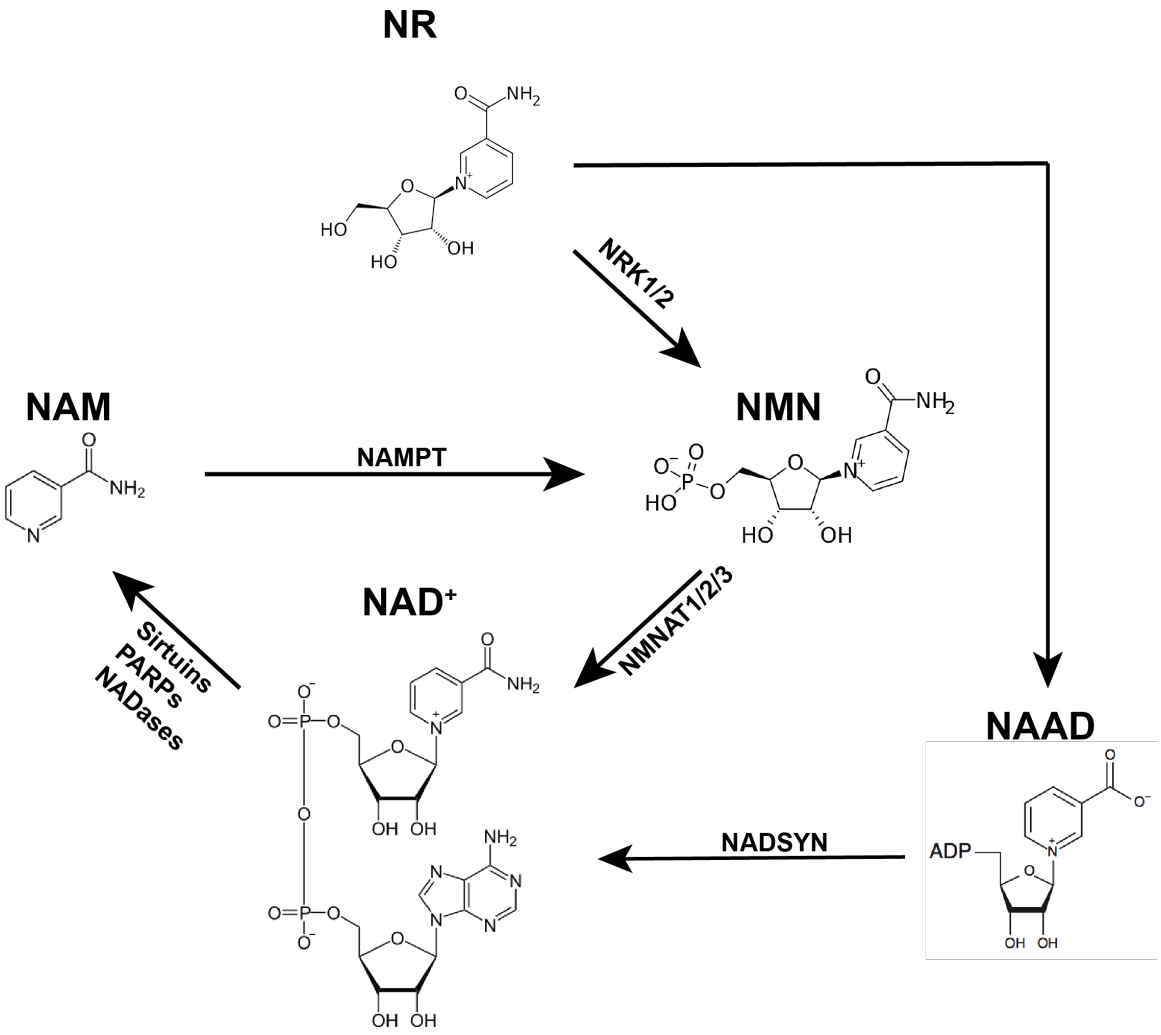
In this section, I will outline the handling and processing of NAD^+ precursors, specifically, intracellular and extracellular transport and processing. NAM, NA and Trp can all be directly transported across the cell membrane for utilization[30]. Intracellularly, NAM requires the least processing, undergoing conversion to NMN by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT) and subsequent adenylation by nicotinamide mononucleotide adenylyltransferase (NMNAT1/2/3) to form NAD^+[20]. NA on the other hand, goes through the 3-step Preiss-Handler pathway, from NA to nicotinic acid mononucleotide (NAMN) to nicotinic acid adenine dinucleotide (NAAD) and finally NAD^+[20]. Interestingly, the Trp 8-step de-novo pathway, which also occurs intracellularly, converges with the 3-step Preiss-Hanlder pathway at NAAD (step 7)[31]. Not all precursors can cross the cell membrane, as is the case with NMN[20]. Extracellular NMN must first be converted to NR, which can then be transported across the cell membrane by nucleoside transporters[29]. NAD^+ is also largely imported into the cell by conversion to NMN and subsequently NR[20]. Once across the cell membrane, nicotinamide riboside kinases (NRK1/2) convert NR back to NMN, which is subsequently converted to NAD^+ by NMNAT1/2/3[20]. Interestingly, NAMPT also exists extracellularly, where it can convert NAM to NMN[32].

Importantly, when NAD^+ is utilized by sirtuins, poly (ADP-ribose) polymerases (PARPs) or NADases, it is cleaved to form NAM and ADP-ribose (Adenosine diphosphate ribose)[20]. This NAM can be salvaged back to NAD^+ by NAMPT and NMNAT1/2/3[31]. This salvage pathway is believed to be critically important for cellular NAD^+ homeostasis as blood levels of NAD^+ precursors are significantly lower than those

required to elevate NAD^+ *in vitro*[20].

Figure 1.2 NAD^+ synthesis from NR and through the salvage pathway.

This figure depicts the chemical structure of NAD^+ and intermediates arising during NR- and salvage-derived NAD^+ synthesis. Classically, NR is converted to NMN and subsequently NAD^+. Recently however, it has been identified that NR can directly form NAAD, which can then be converted to NAD^+. When NAD^+ is consumed, NAM is formed as a byproduct. NAM can then be salvaged back to NAD^+ through conversion to NMN and subsequently NAD^+. Abbreviations used are: NAD^+, nicotinamide adenine dinucleotide; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; NAM, nicotinamide; NAAD, nicotinic acid adenine dinucleotide; NRK1/2, nicotinamide riboside kinase 1/2; NAMPT, nicotinamide phosphoribosyltransferase; NMNAT1/2/3, nicotinamide mononucleotide adenylyltransferase 1/2/3; NADSYN, NAD^+ synthase.


NR
NRK1/2
NAM
NAMPT
NMN
NAD+
NMNAT1/2/3
Sirtuins
PARPs
NADases
NAAD
NADSYN
ADP

1.2.5 Importance of NAD^+ in redox metabolism

NAD^+ is traditionally known for its role in cellular energy metabolism[33]. Cytoplasmic conversion of glucose to pyruvate in glycolysis requires NAD^+. Specifically, GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) reduces NAD^+ to NADH (nicotinamide adenine dinucleotide hydride), converting the glycolytic intermediate glyceraldehyde 3-phosphate to 1-3-biphosphoglycerate. Cytosolic NAD^+ can also be reduced to NADH during the formation of acetyl-CoA from pyruvate. The resulting NADH from these reactions can be converted back to NAD^+ in the cytoplasm by the lactate forming action of lactate dehydrogenase. Cytosolic NADH can also be shuttled to the mitochondrion where another pool of NADH exists from the local reduction of NAD^+ by several steps in the tricarboxylic acid cycle. In the mitochondria, Complex I of the electron transport chain (ETC) oxidizes NADH back to NAD^+, gaining 2 electrons in the process. The gain and transfer of these electrons among ETC complexes facilitates the pumping of protons from the mitochondrial matrix to the intermembrane space, creating a proton gradient. Protons flowing down this gradient through ATP (adenosine triphosphate) synthase, power oxidative phosphorylation of ADP (adenosine diphosphate) to form ATP. Here in lies the importance of NAD^+ in cell and organism viability as a key player in ATP generation.

1.2.6 Importance of NAD^+ in $NADP^+$/NADPH homeostasis

$NADP^+$/NADPH (nicotinamide adenine dinucleotide phosphate/nicotinamide adenine dinucleotide phosphate hydride) are also derived from NAD^+ and have distinct

cellular roles. Addition of a phosphate group to NAD^+ by NAD^+ kinase forms $NADP^+$, which is rapidly dehydrogenated to NADPH[34]. Hence, NADPH is present in higher concentrations than $NADP^+$ and both are maintained at much lower levels than cellular NAD^+[35]. Reduction of NADPH to $NADP^+$ is important for forming antioxidants, powering detoxifying enzymes and for NADPH oxidase activity[31]. It is also utilized for the synthesis of DNA, fatty acids, steroids and forming of the calcium mobilizer NAADP (nicotinic acid adenine dinucleotide phosphate)[36].

1.2.7 Importance of NAD^+ as a co-substrate for proteins

The previous sections highlighted NAD^+'s role in energy metabolism. Here I will briefly introduce NAD^+'s role as a co-substrate for enzymatic reactions mediated by cellular proteins. Each protein class and their functions will be more thoroughly reviewed in section 1.2.

There are 3 classes of cellular proteins that consume NAD^+ for their activity - *sirtuins, PARPs and NADases*. Sirtuins and PARPs are both important for cellular health, but PARP-1 has a lower K_m and larger V_{max} for NAD^+ than sirtuins[37]. Thus, PARP-1 activity is ordinarily not limited by NAD^+ availability, but sirtuin activity is particularly sensitive[37].

This phenomenon becomes particularly important during conditions of oxidative stress that induce DNA damage and therefore activate PARP. PARP is a DNA damage repair protein, that cleaves NAD^+, liberating the ADP-ribose (ADPR) moiety to place it at sites of DNA damage[38]. Importantly, this process creates chains of poly ADP-ribose (PAR) to recruit DNA damage repair protein. A consequence however, is that this can

deplete NAD^+ levels[39]. Severe NAD^+ depletion and PARP activation can impair ATP synthesis resulting in cell death[35]. Viable cells with reduced NAD^+ levels will have less substrate for sirtuin activity, impacting its pleiotropic and important functions in cell health[20]. Furthermore, NAM is a byproduct of PARP mediated NAD^+ consumption, and NAM itself can suppress sirtuin activity[40].

Sirtuins possess deacetylase activity and perform this function by breaking NAD^+ into nicotinamide and ADPR, while placing the acetyl group from their target on the ADPR moiety, forming O-acetyl-ADPR[41]. Some sirtuins can also modify targets by their ability to desuccinlyate, demalonyate, deglutarylate, decrontolyate, lipomidate, and remove fatty acids[20]. Interestingly, SIRT1 can deacetylate PARP-1, suppressing its activity[42]. However, this action is suppressed during PARP-mediated NAD^+ depletion, potentially increasing PARP activity and further lowering sirtuin activity[37].

The third class of NAD^+-consuming proteins are NADases. Cluster of differentiation 38 (CD38) is the major known NADase. CD38 is a transmembrane protein that utilizes NAD^+ to form cyclic ADP-ribose (cADPR). cADPR is a secondary messenger, regulating several biological functions through calcium signaling[43]. CD38 is constitutively active[43] and is a major NAD^+ consumer, with a lower K_m for NAD^+ than PARP-1[20]. Furthermore, CD38 expression/activity can be increased by several stimuli including inflammatory cytokines[44] and Ang II[45]. Inhibition of PARP[46] or CD38[47] increases NAD^+ levels and imparts protection in some models of disease by promoting sirtuin activity[20].

1.3 NAD^+ consumers and their role in resistance to oxidative stress and aging

1.3.1 Sirtuins, a critical protein family in preventing cardiovascular disease

As previously visited, sirtuins possess deacetylase activity and act on many targets regulating cellular health. Seven different sirtuins exists, with distinct cellular localization and deacetylation targets. SIRT1 is primarily found in the nucleus but can also be moved to the cytoplasm[48]. SIRT2, on the other hand, is located exclusively in the cytoplasm. SIRT3-5 are confined to the mitochondria and SIRT6/7 are predominately found in the nucleus.

Interest in sirtuins first grew from reports demonstrating their importance in enhancing yeast replicative lifespan[49]. Further studies demonstrated increased longevity when overexpressing sirtuins in nematodes[50] and fruitflies[51]. Although some contradicting studies emerged, these results have now been independently replicated[41]. Meanwhile, many molecular targets of sirtuins have been studied and continue to be elucidated. Briefly, sirtuins act on pathways involved in cell survival, DNA damage repair, senescence, anti-oxidant defense, inflammation and many more[52]. Sirtuin deacetylase activity can impact these pathways by regulating transcription, activity and localization of proteins involved in these pathways[53]. Accordingly, knock out (KO) and overexpression of sirtuins has profound effects in many models of disease, including vascular diseases.

In human abdominal aortic aneurysm (AAA) samples, SIRT1 expression and activity are reduced[54]. Furthermore, when aged mice are infused with Ang II, vascular smooth muscle cell (VSMC) SIRT1 KO decreases survival and increases AAA severity

and VSMC senescence[54]. Moreover, overexpression of SIRT1 in VSMCs has been reported to attenuate Ang II-induced hypertension, vascular remodeling and inflammation[55]. SIRT1 expression is also reduced in human atherosclerotic plaques. Inactivation of SIRT1 in VSMCs of high-fat-diet(HFD)-fed ApoE$^{-/-}$ mice leads to increased plaque area, DNA damage and apoptosis[56]. In mice fed a high-fat-high-sugar diet, VSMC SIRT1 KO inhibited overnight fasting-induced reductions in aortic stiffness[57]. Furthermore, SIRT1 overexpression/activation abrogated long term increases in aortic stiffness and ROS, while decreasing TNFα-induced aortic vascular cell adhesion molecule-1 (VCAM-1) expression. SIRT1 expression is also reduced by HFD and endothelial cell (EC) SIRT1 overexpression in ApoE$^{-/-}$ mice mitigates HFD-induced atherosclerotic lesions and reductions in endothelium-dependent vasodilation[58]. Accordingly, SIRT1 KO in high-cholesterol-fed ApoE$^{-/-}$ mice promotes atherosclerosis and associated inflammation[59]. SIRT6 KO also promotes atherosclerosis and impairs endothelium-dependent relaxation[60].

1.3.2 PARP-1 and its role in genomic integrity maintenance and cell death

As previously discussed, the PARP family of proteins consume NAD^+ to form ADP-ribose units on target proteins. PARP-1 is the most studied member of the PARP family and accounts for >90% of ADP-ribose synthesis[61]. However, 17 PARP protein family members exist, with distinct functions and subcellular localization. PARP-1 plays a role in both single[62] and double strand break repair[63,64]. Following DNA damage, PARP-1 is activated, consuming NAD^+ to create PAR chains at sites of DNA damage, on associated repair proteins, and even on itself[65]. This creates a docking site which is used

to recruit repair machinery and chromatin remodeling proteins to facilitate DNA repair[65]. PAR chains also promote phase separation at sites of damage, important for spatial organization of repair proteins by intracellular compartmentalization[66]. However, excessive PARP-1 activity and PARylation promotes cell death, possibly signaling DNA damage that is considered excessive/irreparable or by metabolic collapse.

Several modes of cell death involve PAR/NAD^+. Traditionally, Berger's cell suicide hypothesis proposed that NAD^+ consumption by PARP leads to ATP depletion, metabolic failure and necrotic death[67]. However, recent work has demonstrated that marked NAD^+ decline, induced by inhibiting NAMPT, does not necessarily directly impact ATP levels or glycolysis[68]. Instead, PARP activation-derived PAR can bind to and inhibit the glycolytic enzyme hexokinase (and perhaps other Krebs cycle enzymes) leading to ATP depletion. Others have also implicated hexokinase and demonstrated that PARP activation-induced reductions in ATP occur before NAD^+ decline[69]. Although not highlighted by the authors, eventual reductions in NAD^+ were associated with further drops in ATP, suggesting both mechanism may be at play.

Parthanatos is a form of regulated necrosis that is associated with PARP hyperactivity and NAD^+/ATP depletion[70]. Exclusive to Parthantos is nuclear translocation of apoptosis-inducing factor (AIF) from the mitochondria, inducing large scale chromatinolysis and cell death[70]. Although this pathway hasn't been fully elucidated, inhibition of the PAR clearance enzyme poly (ADP)-ribose glycohydrolase (PARG), leads to PARP activation-induced neuronal death without substantial NAD^+ depletion (mild ~15% NAD^+ decline)[71]. Furthermore, mutation of AIF's PAR binding site prevented translocation to the nucleus and cell death following PARP-1 activation[72].

Recent work has also demonstrated that PARG activity can promote AIF-mediated death, by liberating PAR, creating protein-free PAR chains, which can subsequently migrate to the mitochondria and interact with AIF[73]. Although other PAR clearance enzymes exist, PARG preferentially acts on protein-bound PAR and appears to create free-PAR small enough to traverse the nucleus[73]. Mitochondrial permeability transition (MPT) also appears to be important, as the MPT inhibitor cyclosporin A prevented AIF translocation and cell death[74]. NAD^+ repletion after oxidative stress also prevented MPT and cell death. Furthermore, depletion of NAD^+ with NADase induced AIF translocation and cell death, that was rescuable with pyruvate, even in PARP-$1^{-/-}$ neurons[75]. This suggests that NAD^+ depletion does directly cause metabolic changes that mediate AIF translocation. Pyruvate also prevented MPT and cell death. Further complicating matters, AIF interacts with hexokinase and could therefore play a role in ATP decline[76]. PARP activation has also recently been demonstrated to influence mitochondrial ROS production through actions on activating transcription factor 4 (ATF4)[77]. ATF4 silencing prevented improvements in cell survival by PARP inhibition after oxidative stress, suggesting this is an important contributor to cell death.

Necroptosis is another form of regulated necrosis and is mediated through receptor interacting protein kinases (RIPKs). Importantly, necroptosis appears to involve PARP. In TNF-related apoptosis inducing ligand (TRAIL)-induced necroptosis, PARP-1 appears downstream of RIPK1 and 3, and their inhibition reduced PARP activation[78]. Inhibition of PARP also reduced the number of TRAIL-induced necrotic cells.

PARP is generally considered to have little contribution to apoptosis[79]. In fact, cleavage and therefore inactivation of PARP by caspases is one of the first steps in

apoptosis[80]. Furthermore, apoptosis requires ATP which is not necessarily abundant during PARP activation[81]. In line with this, a number of studies have demonstrated no effect of PARP on extrinsic and intrinsic apoptosis[79]. However, PAR may still be important during apoptotic death as it promotes the release of the inflammatory stimulator high mobility group box 1 (HMGB1) from damaged cells[82]. PARylated HMGB1 binds to phosphatidylserine/RAGE (receptor for advanced glycation end products), impeding the clearance of apoptotic cells by the immune system[83]. A phenomenon that usually occurs before late-apoptotic cell membrane disruption, which triggers inflammation[84].

1.3.3 CD38, a NADase and major NAD^+ consumer

NADases are enzymes that hydrolyze NAD^+, forming nicotinamide and ADPR. Other commonly used names for these enzymes include NAD^+ glycohydrolases and NAD^+ nucleosidases[85]. Cluster of differentiation 38 (CD38) is the major known NADase, a constitutively active, ubiquitous transmembrane protein with ecto-enzyme activity[43]. CD38 primarily functions as a type 2 plasma membrane protein, with extracellular NADase activity[86]. However, it has also been identified with its catalytic domain facing intracellularly[87], as a soluble protein[88], and within the nuclear membrane[89]. Its homologue, cluster of differentiation 157, has also been identified but less thoroughly investigated. CD38 uses NAD^+ to form cADPR and nicotinamide and may also form NAADP from $NADP^+$[90]. As most CD38 activity exists extracellularly, it has been proposed that intracellular NAD^+ is transported extracellularly through connexin 43 hemichannels and cleaved to form cADPR, which is internalized by CD38 or through

nucleoside transporters[91]. cADPR is a second messenger that regulates intracellular calcium release[91]. This impacts several biological functions, and importantly, plays a role in Ang II-induced NADPH oxidase-derived oxidative stress[92,93]. CD38 is very inefficient, requiring hydrolysis of ~100 NAD^+ molecules to generate one cADPR[94]. Accordingly, CD38 KO can markedly increase NAD^+ levels, although with considerable variation between tissues and studies (2- to 27-fold[95], 0- to 6-fold[96], 0- to 2-fold[97], 3.5-fold[98]). This likely has to do with tissue specific expression of CD38 which also varies largely (5- to 24-fold)[95]. CD38 expression has been measured in several distinct rat arteries and can vary up to ~14-fold, with relatively low expression in the aorta[99] All together, CD38 is a major NAD^+ consumer and is considered to be an important regulator of NAD^+ levels.

1.4 NAD^+ decline during oxidative stress and aging

1.4.1 NAD^+ decline during acute oxidative stress

It has been well established that oxidative stress, in both an acute and chronic (e.g. age-related) manner, lowers the levels of intracellular NAD^+. The classical paradigm is that ROS damages DNA, triggering the activation of PARP-1, which consumes NAD^+ to initiate DNA repair[79]. ATP decline is a consequence of, or associated with, PARP activation-induced NAD^+ decline, as discussed in depth in section 1.2.2. This can result in necrotic cell death due to metabolic collapse, or contribute to programmed modes of cell death[38]. Furthermore, a PARP-mediated decline in NAD^+ lowers sirtuin activity, impacting cellular health[37]. This oxidative stress-induced decline in NAD^+ has also been hypothesized to contribute to chronic age-related NAD^+ decline[100].

1.4.2 NAD^+ decline during aging and disease

Animal and emerging human data have demonstrated that there is a progressive NAD^+ decline over the course of an organism's lifespan. The reason for this decline appears to be multifaceted, with evidence of increased NAD^+ consumption and decreased NAD^+ synthesis. Recent work in mice demonstrated that mRNA/protein expression and activity of the NADase CD38, *a major NAD^+ consumer*, increases in liver, adipose tissue, spleen and skeletal muscle with age[97]. Human adipose tissue was assayed and also exhibited increased CD38 mRNA with age. In the murine liver, where NMNAT mRNA was measured, a significant decline in NMNAT1 was observed along with nonsignificant drops in NMNAT2/3. CD38 upregulation in mice was associated with a fall in NAD^+, with no change in the protein/mRNA expression of NAMPT and a decline in PARP-1 protein expression. However, mice with CD38 KO did not have a decline in tissue NAD^+ content when aged to 32 months. This work implicates NAD^+ consumption by CD38 as the major cause of age-associated NAD^+ decline, while deemphasizing PARP and NAMPT's role. In contrast to this, another study reported age-related increases in PARP protein expression/activity in rat heart, liver, kidney and lung tissue, along with declining NAD^+[100]. A decrease in anti-oxidant capacity, oxidation and DNA damage was observed in these tissues with age. This suggests that age-related oxidative stress/DNA damage may decrease NAD^+ by increasing PARP activity. This study was repeated in the rat brain with identical results in the hippocampus, cortex, cerebellum and brainstem[101]. Here, NADase activity was also measured and significantly increased with age.

Importantly, translational work has demonstrated an age-associated decrease in brain NAD^+ in humans[102]. Furthermore, in human skin samples, age was negatively

correlated with NAD^+ levels and positively correlated with oxidation, DNA damage, and PARP activity in males[103]. In a recent study, liver samples obtained from patients <45 years old contained ~33% more NAD^+ than samples from patients >60 years old[104]. These samples also revealed a reduction in NAMPT protein and an increase in NMNAT protein expression. This pattern was also displayed in livers of 4- vs. 12- vs. 20-month-old mice. In the kidney of aged mice, NAMPT mRNA and NAD^+ decline has been observed, along with a reduction in NMNAT1/3 mRNA[105]. Overall, animal data has revealed age-related reductions in NAD^+ in the pancreases[106], spleen[97], liver[100], brain[107], heart[100], kidney[105], lung[100], skeletal muscle[106], muscle stem cells[108], white adipose tissue[106] and retina[109].

In addition, reductions in NAD^+ and related NAD^+-producing enzymes have been observed in disease. Important to this project, we have demonstrated that NAMPT expression is reduced in dilated vs. non-dilated ascending aortas[110]. Also of interest is a decline in endothelial progenitor cell (EPC) NAD^+/NAMPT seen in diabetic patients[111]. Here, blood EPC number correlated with NAD/NAMPT levels and was increased upon nicotinamide administration. Tissue NAD^+ decline has also been observed during HFD[106], ischemia[112], diabetic retinopathy[109], light-induced retinal degeneration[109], noise-induced hearing loss (n.s.)[113], muscular dystrophy[114] and environmental toxin exposure[115].

1.4.3 Supplemental NAD^+ precursors: Advantage nicotinamide riboside

A number of NAD^+ precursors have been synthesized and administered in models of disease to augment NAD^+ levels. However, the current evidence suggests that NR may

be one of the best NAD^+ precursor in several respects. Orally administered NR elevates liver NAD^+ similarly to orally delivered NAM, over the course of 12 hours (AUC: area under the curve)[28]. However, peak NAD^+ was ~2-fold greater with NR compared to NAM. More importantly, levels of ADP-ribose, indicative of NAD^+-consuming activity, were ~3-fold greater with NR, perhaps due to negative feedback of sirtuins by NAM. In line with this evidence, NAM was not as effective as NR in reversing muscle pathology induced by muscle-specific NAMPT KO[27]. Furthermore, evidence suggests NAM can induce lipotoxicity and glucose intolerance[116,117].

In contrast to NAM, oral NA elevates liver ADP-ribose to a similar extent as NR. However, ADP-ribose still remained ~25% greater with NR (n.s. n=3-4)[28]. Furthermore, NA only elevated liver NAD^+ AUC half as much as NR. Importantly, NA causes a mild to extreme flushing reaction which has reduced compliance in dyslipidemic patients taking NA, even with extended release formulations[118,119]. As well, the time course of NAD^+ elevation after gavage of NA/NAM/NR suggests that twice daily supplementation for any of the precursors would be required to elevate 24-hour NAD^+ metabolism[28]. This could be a difficult regimen to maintain given NA's side effects. In addition, NA has been demonstrated to reduce insulin sensitivity[120,121] and increase oxidative stress[122].

NMN has also been used to elevate NAD^+ levels and logically appears to be a good precursor, given that NR is first metabolized to NMN inside the cell before becoming NAD^+. However, NMN cannot be transported across the cell membrane and is first metabolized to NR extracellularly, before cellular uptake[29]. Some evidence also suggests that NR may be the preferred NAD^+ precursor during stress. Nerve transection elevated neuronal NRK2 >20-fold[123] and we observed an increase in aortic NRK1 after

NAMPT KO, suggesting a preference for NR-derived NAD^+ during NAD^+ deficiency[124]. However, this may be of little importance with oral NMN administration protocols, as NMN is converted to NR in the small intestine[25]. Still, whereas little is known about the absorption of NMN, some oral NR is absorbed intact and directly utilized to create NAD^+[27]. Unlike NMN, NR has undergone a comprehensive safety assessment, including administration of doses up to 5000 mg/kg/day[125]. NR has also been administered in a clinical trial and demonstrated to elevate human NAD^+ metabolism[28]. Lastly, although not statistically evaluated, oral administration[126] and IP injection[29] of NR appears to increase liver and muscle NAD^+ more than NMN.

In summary, NMN and NR both appear to be promising supplemental NAD^+ precursors. There is evidence that NR increases NAD^+ metabolism and attenuates pathology more than NA or NAM. Evidence also suggests that NR may elevate NAD^+ more than NMN in certain tissues.

1.5 Angiotensin II infusion as a model of hypertension, vascular oxidative stress and aging

1.5.1 Mechanism of Ang II-induced oxidative stress and associated vascular pathology

Decades of research has established the role of the renin-angiotensin system (RAS) in hypertension and vascular disease. Ang II is the main bioactive product of the RAS and has been widely used to induce experimental vascular pathology both *in vitro* and *in vivo*[127]. Ang II acts through angiotensin II type 1 (AT1R) and type 2 G protein-coupled receptors, which can generally be considered to have pathological and protective

downstream effects, respectively[127]. Through the AT1R, Ang II elevates blood pressure, which appears to involve oxidative stress in several tissues. This is associated with a blood pressure-independent hyperplasia of the ascending aorta, and aortic medial hypertrophy in the thoracic, suprarenal and infrarenal zones[128]. Furthermore, Ang II induces endothelial dysfunction[129], cardiac hypertrophy[130] and abdominal aortic aneurysms in some mouse strains[131].

Through the AT1R, Ang II upregulates NADPH oxidase (Nox) subunits at the epigenetic, transcriptional and post-transcriptional level, increasing superoxide generation[127,132]. Nox is a transmembrane catalytic protein, with seven isoforms (Nox1-5, Duox1-2) formed through interaction with up to 5 distinct/shared cytosolic regulatory subunits[133]. Nox1, 2, 3 and 5 have been found in ECs and VSMCs[133]. All vascular cell types can use Nox to create superoxide by transferring electrons from NADPH to molecular oxygen[134]. This superoxide directly contributes to oxidative stress, or is dismutated into hydrogen peroxide (H_2O_2)[133]. Oxidative stress is thought to act locally, as O_2^- is highly reactive, has a short half-life, and cannot passively cross cell membranes[134]. H_2O_2 on the other hand, has a slightly longer half-life and can cross the plasma membrane through aquaporin channels[135]. Both of these reactive oxygen species (ROS) directly react with cellular membranes, proteins and DNA, but can also give rise to other ROS. O_2^- can react with NO forming $ONOO^-$, and H_2O_2 can form OH^- in the presence of Fe^{2+} [127]. During Ang II infusion, H_2O_2 appears to be important in vascular hypertrophy[136] and endothelial dysfunction[137], as over/under expression of H_2O_2 clearing enzymes impacts these endpoints. Interestingly, a feed-forward loop exists where Nox-derived ROS upregulates AT1R expression[138]. Furthermore, NO can suppress AT1R expression

but as previously mentioned, reacts with O_2^-, forming $ONOO^-$[138].

Evidence exists to suggest a critical mediating role of Nox-derived oxidative stress in Ang II-induced hypertension. Ang II upregulates vascular Nox1 and its gene disruption ~halves Ang II-induced ROS production and hypertension[139], with conflicting reports on thoracic medial hypertrophy[140]. KO of Nox-2 reduces baseline blood pressure, but does not attenuate Ang II-induced blood pressure elevation, despite abrogating ROS and attenuating aortic thickening[141]. KO of the Nox1/2 regulatory subunit $P47^{phox}$ also prevented Ang II-induced vascular ROS production and halved hypertension[142]. Nox4 has been reported to exert protective and pathological functions. Meanwhile, less is known about Nox5 because it is not present in rodents[133]. Interestingly, evidence suggests neuronal ROS plays an important role in Ang II-induced hypertension. Nox disruption or anti-oxidant clearance of ROS in several brain regions lowers Ang II-induced ROS and hypertension[143,144]. Adding complexity, Ang II-induced superoxide production can also be blunted through vascular IL6 deficiency[145] and Cox-2 inhibition[146].

1.5.2 Cell death induced by Ang II

Cell death is one of the most significant consequences of Ang II-mediated cell damage. *In vitro* work has demonstrated that Ang II can induce apoptosis of ECs[147] and VSMCs[148]. As well, apoptosis of smooth muscle cells (cleaved-caspase-3) can be seen *in vivo* during long-term Ang II infusion, which is largely associated with areas of aortic hemorrhage[149]. It is not clear if cell death was a primary outcome of Ang II or secondary to hemorrhage-associated adverse microenvironment. In this study, EC apoptosis was not quantitatively reported and does not appear present in representative images at day 3, 10,

18 or 28[149]. In rats receiving 7 days of Ang II, aortic DNA laddering increased, indicative of apoptosis, and VSMC TUNEL was detected, although not quantified[150]. Investigators have also detected apoptosis in the heart[151] and kidney[152] after Ang II infusion.

Little is known about other cell death subroutines that may be stimulated by Ang II. This may be due to technical limitations in detection. A recently published abstract reported increased aortic smooth muscle cell receptor-interacting protein kinase 3 (RIP3) in mice receiving high fat diet and 28 days of Ang II infusion, suggesting that necroptosis may be present[153].

Uncontrolled and excessive death of ECs and VSMCs is detrimental to vascular homeostasis and health. Therefore, preventing their death during age and disease is an important endpoint in combating vascular pathology. Healthy ECs maintain the integrity of the vascular wall and secrete vasculoprotective compounds such as nitric oxide (NO)[154]. Loss of these functions during EC death has been proposed to promote disease[154,155]. For instance, loss of ECs increases transendothelial leakage[156], which can promote uptake of macromolecules such as LDL[157] and possibly inflammatory cells[158]. In fact, it has been suggested that endothelial leakiness is responsible for 90% of transendothelial LDL transport[159]. Furthermore, inflammatory cells infiltrating the media promote atherosclerosis[160], ECM degradation[161], and secrete ROS which can damage VSMCs[162]. Moreover, inflammatory signaling from dying ECs or VSMCs may enhance these processes[163]. Evidence also suggests EC death/injury promotes thrombosis by releasing platelet activating compounds and exposure of platelets to the endothelial cell basement membrane[154]. One interesting context for endothelial cell injury is malignant hypertension, which can lead to thrombosis at the capillary level (thrombotic

microangiopathy)[164]. VSMCs are responsible for maintaining vessel structure/integrity and tone. Death of VSMCs has been implicated in aneurysm formation[165], atherosclerosis and plaque rupture[166], pathologies in which elevated Ang II is considered a driving factor.

1.5.3 DNA damage, senescence and inflammation induced by Ang II

Oxidative stress damages DNA. Ang II infusion has been demonstrated to increase oxidation of guanine nucleobases and induce formation of the DNA damage response protein γ-H2AX in the kidney[167], heart[167] and VSMCs[167]. Biochemically, guanine is highly susceptible to oxidation forming 8-oxo-2'-deoxyguanosine (8-oxo-dG), one of the most frequent DNA lesions[168]. If un- or mis-repaired, 8-oxo-dG can cause G→T and A→C substitutions. Furthermore, recent work has demonstrated 8-oxo-dG lesions can silence transcription, even when occurring at non-transcribed/non-promoter DNA regions[169]. In addition, 8-oxo-dG lesions on promoter sequences promote NF-kB-driven gene expression[170]. Single and double strand DNA breaks also pose a risk to cellular function. When double strand breaks occur, histone H2AX is phosphorylated forming γ-H2AX to facilitate DNA repair[171]. Therefore, detection of γ-H2AX is frequently used to assess the extent of DNA double strand breakage. Mutations from unrepaired double strand breaks contribute to cell death, inflammation and senescence[172]. Importantly, these lesions have been detected in human VSMCs at sites of atherosclerosis[173] and aneurysm[174].

In vitro, it has been demonstrated that Ang II-induced damage to telomeric and non-telomeric DNA contributes to replicative senescence and stress-induced premature senescence, respectively[175]. Consistent with this, VSMC senescence has been detected

after infusion of Ang II[176,54]. Moreover, senescence or a predisposition to senescence is seen in VSMCs at sites of atherosclerosis[177] and aneurysm[178], both of which are associated with DNA damage. Senescence is a state of permanent cell cycle arrest. Senescent cells promote pathology by disrupting normal tissue structure and function, while secreting pro-inflammatory cytokines and matrix-degrading enzymes[179]. In replicative senescence, damage or natural shortening of telomeres exposes chromosomal DNA to replication mediated cleavage[9]. Hence a senescent phenotype is adopted to prevent proliferation of dysfunctional cells. In stress-induced premature senescence, telomere shortening-independent senescence occurs following prolonged oxidative stress and DNA damage[9]. Although several contributing signaling pathways to senescence exist, the $p16^{INK4A}$ pathway was investigated in this project. $p16^{INK4A}$ expression is increased during oxidative stress[180] and DNA damage[181]. $p16^{INK4A}$ signals through several intermediaries to suppress the expression of S-phase genes, preventing cell cycle progression and inducing senescence[179]. For this reason it is a commonly used biomarker of senescence and its expression can be increased by Ang II[176].

Ang II infusion also causes inflammation, which contributes to vascular pathology[182]. One important proinflammatory change is an increase in the expression of vascular cell adhesion molecule-1 (VCAM-1) in vascular cells[183]. Circulating inflammatory cells adhere to vessels through adhesion molecules, before transmigration into the media, where they promote disease[160]. VCAM-1 in particular has been implicated here[184,185]. Ang II stimulates cyto-nuclear transmigration of the transcription factor NF-kB, which promotes expression of VCAM-1. This has been demonstrated *in vitro*[186] and during infusion of Ang II[183]

1.5.4 Non-vascular pathology induced by Ang II

Ang II is a particularly noxious peptide that can induce pathology throughout many tissues outside of the vasculature. Ang II can induce muscle wasting by increasing ubiquitin proteasome system-dependent protein degradation through ROS[187], IGF-1 decline[188] and inflammation[189,190]. Muscle is the largest site of glucose disposable[191] and beyond reducing its size, Ang II induces insulin resistance and plays a role in pancreatic pathology[192]. In fact, after Ang II infusion, end organ damage is seen in tissues including the heart, kidney and brain, likely through ROS, inflammation and hemodynamic changes[193]. The kidney in particular is affected by Ang II and is important for the development of Ang II-induced hypertension[130, 194].

1.5.5 Physiological relevance of high dose Ang II infusion in mice

Ang II is formed from its precursor angiotensinogen. Therefore, the production rate of Ang II cannot exceed the production rate of angiotensinogen. Based on this, it has been proposed that an infusion of 1.44 mg/kg/day Ang II to a mouse reflects a 30-fold increase over the natural production rate of the Ang II precursor angiotensinogen[195]. Although a high Ang II dose, this may be physiologically relevant stimulus. In the murine thoracic aorta, Ang II increases ~25-fold from 2 to 12 months, with a further ~1.4-fold increase at 24 months (~35-fold vs. 2 months)[196]. Although, serum Ang II levels only increase from ~30 to ~35 to ~45 pg/ml over this time frame, local Ang II concentrations are independently regulated and can be 1000-fold higher than circulating levels[197,198]. As well, thoracic aortic AT1R expression more than doubles between time points (2 vs. 12

vs. 24 months) with similar declines in AT2R expression. Assuming 1:1 Ang II to AT1R signaling, these changes could reflect up to a >140-fold increase in aortic AT1R signaling from 2 to 24 months. In addition to this data, non-human primates exhibit a 5-fold increase in aortic intimal Ang II immunostaining with age (6 vs. 20 years)[199]. Similarly in humans, thoracic aortic intimal area fraction of Ang II immunostaining was found to increase by ~4-fold (possibly saturated based on representative image) with age (20 vs. 65 years)[200]. A 4-fold increase in intimal AT1R protein expression was also noted.

Acute increases in blood pressure and oxidative stress occur during hypertensive crisis[201], in which the RAS has been implicated[202,203]. Therefore, acute infusion of Ang II may represent a model of hypertensive crisis. More sustained infusion, with the associated oxidative stress and vascular remodeling, has features of vascular aging.

1.5.6 Ang II infusion and NAD^+ metabolism

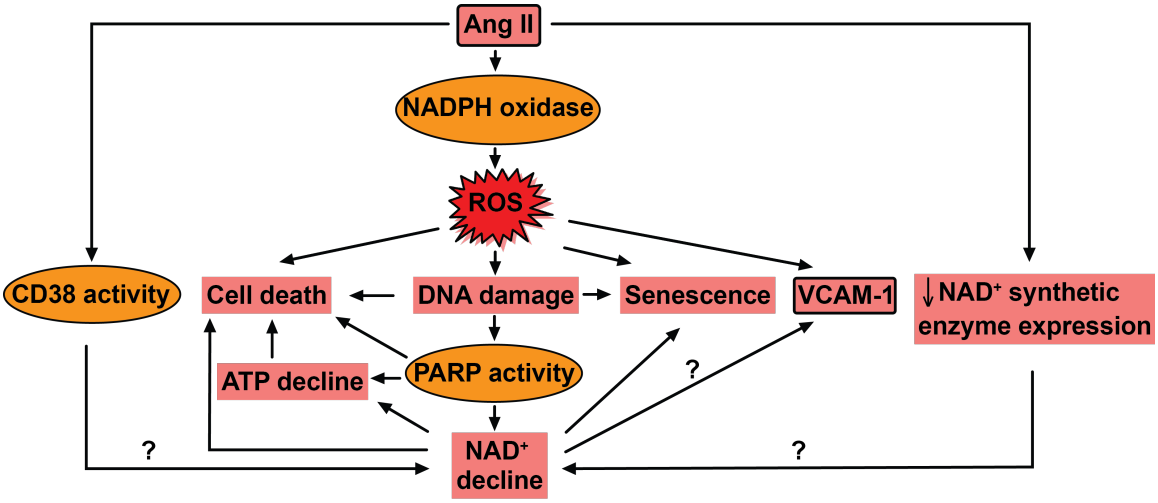
Intriguing evidence suggests a link between Ang II signaling and NAD^+ metabolism. AT1R KO, which improves murine lifespan by 26%, is associated with greater kidney NAMPT and SIRT3 mRNA[204]. Furthermore, 0.3 mg/kg/day Ang II for 14 days was found to reduce whole heart cell lysate NAD^+ by ~33%, which was prevented with PARP KO[205]. Ang II also increases activity of the NAD^+ consumer CD38, which might contribute to Ang II-induced NAD^+ decline[99]. Importantly, we have found that Ang II infusion reduces murine aortic NAD^+ and NAMPT content. Furthermore, loss of NAD^+ homeostasis through KO of NAMPT in VSMCs, promotes aortic pathology[110]. Given that Ang II and AT1R expression increase with age, these studies raise the possibility that Ang II contributes to age-associated NAD^+ decline. Similarly, more acute vascular

damage from Ang II might be linked with impaired NAD^+ metabolism.

Therefore, it is reasonable to consider whether improving NAD^+ supply through precursor supplementation might protect against Ang II-induced vascular damage. In this regard, it is noteworthy that a recent study has demonstrated oral NMN reduces vascular oxidative stress, aortic stiffness and improves endothelial function in aged mice[206]. In addition, cell culture work from our lab has found that NR supplementation partially inhibits H_2O_2-induced vascular cell death, DNA damage, and cell senescence[124]. Whether NR can protect against vascular pathology induced by Ang II is unknown, and a central question for this thesis.

Figure 1.3 Framework of Ang II-induced pathology and NAD^+ decline.

This figure illustrates the mechanism of Ang II-induced pathology and NAD^+ decline. Moreover, the contribution of NAD^+ decline to pathology is highlighted. Ang II can reduce synthesis and increase consumption of NAD^+, lowering its levels. ROS also play a role in Ang II-induced CD38 activity and NAD^+ synthetic machinery decline. Declining NAD^+ impacts sirtuin activity, exacerbating Ang II-induced pathology. Abbreviations used are: Ang II, angiotensin II; NAD^+, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule-1; CD38, cluster of differentiation 38.


Ang II
NADPH oxidase
ROS
CD38 activity
Cell death
DNA damage
Senescence
VCAM-1
↓NAD+ synthetic enzyme expression
ATP decline
PARP activity
?
?
?
NAD+ decline

1.6 Summary of rationale for thesis

NAD^+ homeostasis is vital for vascular cell health. Ang II induces oxidative stress and is considered a driver of vascular pathology and disease. Ang II signaling intensifies with age and has been demonstrated to suppress NAD^+ levels and those of its synthesizing enzymes. *In vitro*, NR can partially protect vascular cells from oxidative stress-induced NAD^+ decline, death, DNA damage and senescence.

The objective of my work was to determine whether oral administration of NR imparts resistance to Ang II-induced aortic cell damage, *in vivo*.

1.7 Hypothesis and specific aims

I hypothesize that nicotinamide riboside administration will protect the aorta from Ang II-induced cell death, DNA damage, senescence and, proinflammatory signaling.

To test this hypothesis I will address two aims:

1. To determine the effect of NR on acute Ang II-induced aortic EC and VSMC death and DNA damage
2. To determine the effect of NR on sustained Ang II-induced aortic EC and VSMC DNA damage, proinflammatory signaling and senescence.

2 MATERIALS AND METHODS

2.1 Animals

Male C57Bl/6 Retired Breeders were ordered from Charles River at 6-9 months of age. As these investigations were proof-of-concept, discovery-based research, no formal sample size calculation was made. The number of mice in the various treatment groups (5-9 for histologic endpoints, 4-14 for physiologic endpoints) was chosen based on an assessment of the literature pertaining to Ang II infusion and aortic pathology[128,207,208]. Mice were housed individually, had *ad libitum* access to food and water and were kept under 12 hour light-dark cycles. Mice were given at least one week to acclimate to the animal facilities before experiments were performed. Procedures performed were approved by Western University, the Animal Care Committee and were done in accordance with the Canadian Guide for Care and Use of Laboratory Animals. Only male mice were used for this study, as female mice are relatively resistant to Ang II-induced pathology[209].

2.2 NR administration

Based on average food intake and body weight, NR-containing food pellets were made by mixing NR-containing deionized water ($_{di}H_2O$) with powered Teklad Rodent Diet 8604 (naturally containing 64 mg/kg of nicotinic acid, a dose of ~eight mg/kg/day). NR capsules (N(r) - NIAGEN®, High Performance Nutrition) were opened and the contents were dissolved/vortexed in $_{di}H_2O$ before addition to powdered food. Each capsule contained 125mg of NR Chloride and was mixed with an appropriate amount of

$_{di}H_2O$/powdered food, to deliver ~400mg/kg/day of NR. This dose has been demonstrated to elevate NAD^+ levels in multiple tissues (eg. muscle, liver, brown adipose tissue) and studies, with associated benefits[126,108,210]. Vehicle-treated mice also received homemade food pellets, which were made with $_{di}H_2O$. Food was allowed to dry for 24-72 hours before administration and was remade every 1-2 weeks.

2.3 3-Day angiotensin II experiments

Nine- to 10-month old mice were given a NR- or vehicle-containing diet for 1 week. While continuing their designated diet, osmotic pumps (Alzet model 1003D, Cat#: 0000289) were implanted subcutaneously on the flank, to continuously infuse 1.44 mg/kg/day of Ang II (Sigma-Aldrich, Cat#: A9525) or saline for 3 days. Osmotic pumps were filled 1-2 hours before implantation, which was performed under isoflurane anesthesia. Tail-cuff-derived blood pressure was recorded before diet initiation and 2 days after osmotic pump implantation (n=7-11 mice). On day 3, mice were euthanized and studied for DNA strand breakage (comet assay) (n=106-172 cells from the aortic wall), cell death (n=5-8 mice) or liver NAD^+ content (n=4-8 mice), as described in the appropriate sections below.

2.4 28-Day angiotensin II experiments

C57Bl/6 mice were followed for 6 weeks to determine baseline blood pressure, weight and food intake. Mice were then randomly assigned to receive vehicle-containing

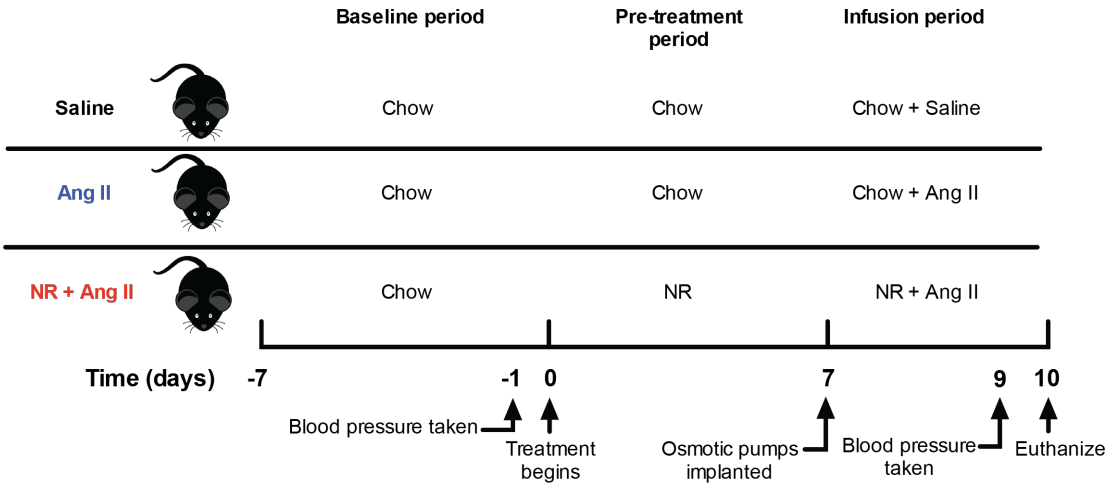
diet or NR-containing diet for 3 weeks. After 3 weeks, osmotic pumps (Alzet model 1004, Cat#: 0009922) were implanted subcutaneously on the flank to continuously infuse mice with Ang II (1.44 mg/kg/day) or saline for 4 weeks, while continuing their designated dietary treatment. Blood pressure (n=6 mice) and body weight (n=7-14 mice) measurements were taken weekly during the study period. After 4 weeks of Ang II, mice were anesthetized with isoflurane and euthanized by injection of 1M KCl into the inferior vena cava. Animals were perfused with PBS by injection into the left ventricle, after which a portion of the liver was excised and frozen in liquid nitrogen for NAD^+ assay. Whole mouse fixation was performed via cardiac infusion of 4% formaldehyde. Tissues were processed for histology, as described below, then investigated for medial thickening (n=6-9 mice), DNA oxidation (n=6-9 mice), VCAM-1 (n=6 mice) and $p16^{INK4A}$ (n=5-6 mice). Ang II/saline delivery was verified by measuring remaining osmotic pump volume to ensure pump contents had been dispensed.

Figure 2.1 Experimental timeline for 3-day and 28-day Ang II experiments

A. C57Bl/6 (9-10 month-old retired breeders, Charles River, n=4-11) mice were pre-treated with NR- (400 mg/kg/day) or vehicle-containing diet for 1 week. While continuing their designated treatment, an osmotic pump was implanted subcutaneously, infusing saline or Ang II (1.44 mg/kg/day) for 3 days. After 3 days, mice were sacrificed and tissues/cells were collected for histology, comet assay, or NAD^+ assay. **B.** C57Bl/6 (7-10 month old retired breeders, Charles River, n=5-14) mice were pre-treated with NR-diet (400 mg/kg/day) or vehicle-diet for 3 weeks. While continuing their designated treatment, Ang II (1.44 mg/kg/day) or saline was delivered for 4 weeks. Mice were then euthanized and tissues were harvested for histological analysis. Blood pressure measurements were taken twice a week, on the same days and at the same time, for the duration of the study.

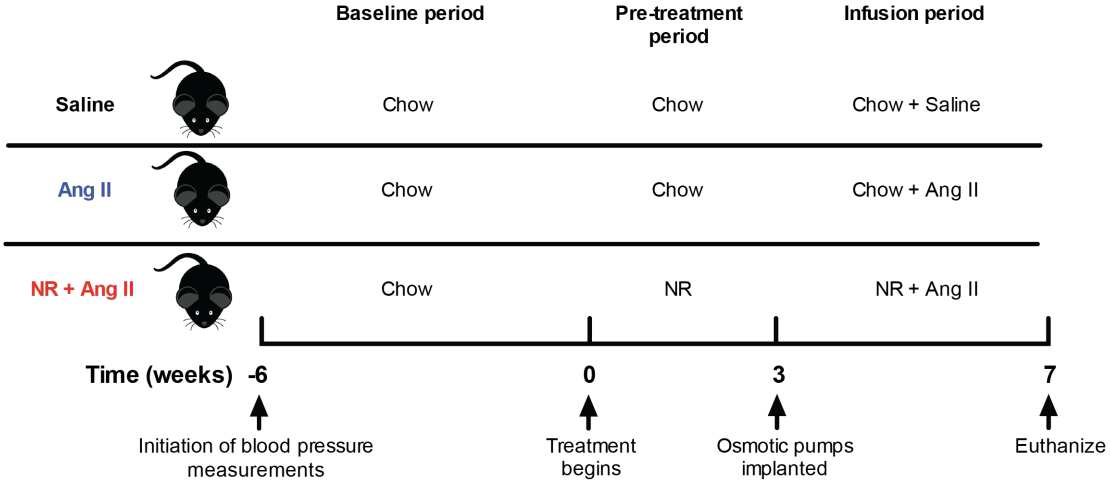
A

Three-day Ang II protocol



B

Twenty eight-day Ang II protocol



2.5 *In vivo* cell death assay

Following dietary treatment and 3 days of Ang II, mice were anesthetized via isoflurane inhalation and injected intravenously with Ethidium Homodimer-III (EthD-III, 300μM in PBS, Biotium, Cat#:40050) through the dorsal penile vein. After 10 minutes, mice were killed via isoflurane overdose and perfused with PBS through the myocardium for ~5 minutes. The liver was collected for NAD^+ assay and immediately frozen in liquid nitrogen. Mice were then infused with 4% formaldehyde through the left ventricle, for 10 minutes to fix tissues. The ascending, thoracic and suprarenal aorta were excised and frozen in OCT. Heart, liver and kidney tissue was also collected and frozen in OCT.

2.6 Acute aortic cell isolation and comet assay

Following dietary treatment and 3 days of Ang II, mice were euthanized via isoflurane overdose, the chest cavity was opened, and the aorta was flushed with PBS through the left ventricle. The aorta was excised, ligated distally near the thoracoabdominal transition zone and placed in a petri dish containing culture media (M199). To isolate ECs, collagenase II (480 units/ml in M199, Worthington) was injected into the lumen through the proximal opening and the aorta was incubated at 37°C for 35 minutes. The ligation was removed and the lumen was flushed with M199 to collect ECs. The aorta was kept on ice while ECs were counted, filtered through a cell 70-μm cell strainer, and processed for comet assay. Next the aorta was cleaned of adventitia, placed in a collagenase I (260 units/ml in M199, Worthington) + dispase (1.8 units/ml in M199, Worthington) solution, cut finely using scissors and incubated at 37°C for 1.5 hours.

Liberated VSMCs were counted, filtered through a 100-μm pore-size cell strainer and processed for comet assay. Cells were protected from light and comet assay was performed according to the manufacturer's instructions (Trevigen, Cat#:4250-050-K), with electrophoresis performed at 4°C. Cells were stained with Sybr-Gold to visualize DNA and fluorescent images were captured (Olympus BX51 microscope). Open Comet plugin for ImageJ was used for analysis and supplemented with manual analysis when necessary.

2.7 Blood pressure measurements

Blood pressure measurements were taken using tail-cuff non-invasive volume-pressure recording with CODA 6 software (Kent Scientific). Measurements were taken at the same time of day within each cohort of animals. Animals were brought to the blood pressure room one hour before measurements to reduce stress from transport. Mice were loaded in holders and placed on a heating block, which was set on low. Recording cuffs were placed on the tail and mice were given 15 minutes to acclimate. Five acclimation and 30 blood pressure measurements were recorded, the latter of which were averaged to give the daily recorded blood pressure.

2.8 NAD^+/NADH assay

Liver NAD^+ levels were determined using a colorimetric kit (Biovision, Cat#: K337). The assay measures NADH and relies upon an enzymatic cycling reaction, which

converts extracted NAD^+ into NADH. By loading samples with decomposed and intact NAD^+, NAD^+ concentration can be measured indirectly, as the difference in measured NADH concentration. To extract tissue NAD^+/NADH, 20 mg of tissue was homogenized (QIAshredder) in 800 uL of lysis buffer. Samples were then spun at 4°C at 16,000g for 10 minutes. Supernatants were transferred to a new tube from which 500 uL was loaded in 10-kDa Spin Columns and spun at 4°C at 12,000g for 30 minutes to deproteinize samples. Remaining supernatant was used for BCA protein assay **(**Pierce™ BCA protein assay kit, Thermo Fischer Scientific**)**. In a 96 well plate, 15-40 µL of filtrate and decomposed filtrate (heated at 60°C for 30 minutes to decompose NAD^+) were loaded in duplicate. Wells were brought up to 50 µL using lysis buffer and a NADH standard curve was loaded (0-100 pmol/well). 100 uL of cycling mix was added to each well and the plate was mixed and incubated at room temperature for 5 minutes. Next, the reaction was started by adding 10 uL of NADH developer to each well and was left to cycle at room temperature for 1 hour. After 1 hour, absorbance at 450 nm was read on a colorimetric plate reader (Thermo Electron Corporation, Multiskan Ascent software).

2.9 Processing of formaldehyde-fixed tissue and delineation of aortic regions

After infusing mice with 4% formaldehyde, tissues were excised, left in 4% formaldehyde overnight and subsequently immersed in 70% ethanol. The aorta was cleaned, cut into ascending/thoracic/suprarenal regions, embedded in paraffin and five-µm cross-sections were cut. The ascending aorta was defined as the region located one to three mm distal of the termination of the aortic valve leaflet stubs. The thoracic aorta

comprised the region five to seven mm posterior of the left subclavian artery. The suprarenal region was located two mm above of the superior mesenteric artery. These precise sites were also used when collecting frozen sections of the aorta.

2.10 Cell death (EthD-III) visualization and quantitation

After freezing aortic tissue in OCT, 10-μm cross-sections were made from the ascending, thoracic and suprarenal aortic regions. Sections were mounted with DAPI-containing media (DAPI Fluoromount-G®, Southern Biotech), sealed with nail polish and allowed to dry for 20 minutes. Fluorescent images of EthD-III ($\lambda_{Ex}/\lambda_{Em}$: 530/620 nm) and DAPI ($\lambda_{Ex}/\lambda_{Em}$: 350/455 nm) were taken immediately and stacked using ImageJ. File IDs were blinded using NameChanger (MRR Software) before quantification was performed. DAPI signal was used to identify cells, which were considered positive when exhibiting EthD-III signal intensity above levels seen in elastin background.

2.11 Cleaved caspase-3 immunostaining of frozen sections

Immunostaining for cleaved caspase-3 was performed on frozen thoracic cross-sections from mice infused with Ang II for 3 days. A frozen section of liver from an Ang II-infused mouse was also stained as a positive control. Sections were exposed to H_2O_2 for 30 minutes before 1 hour block with donkey serum (Sigma) and subsequent overnight incubation with rabbit polyclonal anti-cleaved-caspase-3 (1:50, Cell Signaling

Technologies, Cat#: 9661S). To detect bound primary against cleaved-caspase-3, HRP-conjugated donkey anti-rabbit secondary antibody (1:200, Vector Labs) was applied for 2 hours and visualized with DAB (Vector Labs). Haematoxylin counterstaining was performed to label nuclei. Sections were thoroughly investigated and light micrographs were obtained (Olympus BX51 microscope).

2.12 H&E staining & morphology quantitation

Five-micron formaldehyde-fixed paraffin-embedded sections from the ascending, thoracic and suprarenal aorta were stained with H&E **(**Leica Autostainer XL**)**. Light micrographs were captured using a 20x objective (Olympus BX51 microscope) and stitched together using Grid/Collection stitching[211] in FIJI, to create full circumference cross-sectional images of the aorta. These images were used to quantify histological data. The medial area was quantified by measuring the area within the inner and outer most elastin layer, using ImageJ. Areas of branching or hemorrhage were avoided and the lumen perimeter of quantified zones was used to standardized medial area measurements.

2.13 Immunostaining of formaldehyde-fixed paraffin-embedded sections

Immunostaining was performed on 5-micron paraffin-embedded thoracic cross-sections from mice infused with Ang II for 28 days. Briefly, slides were deparaffinised and antigen retrieval (Retriever 2100, Prestige Medical) was performed with 100 mM sodium citrate buffer. Sections were blocked with H_2O_2 for 30 minutes before 1 hour

block with sheep or donkey serum (Sigma) as appropriate and subsequent overnight incubation with the primary antibody for the investigated molecular target. Primary antibodies included: mouse monoclonal anti-8-oxo-dG (1:300, Northwest Life Sciences, NWA-MOG020), rabbit polyclonal anti-VCAM-1 antibody (1:30, Santa Cruz, Cat#:sc-1504-R) and mouse monoclonal anti-$p16^{INK4A}$ (1:500 Santa Cruz, Cat#:sc-1661). To detect bound primary antibody, HRP-conjugated sheep anti-mouse or HRP-conjugated donkey anti-rabbit secondary antibody (1:200, Vector Labs) was applied for 2 hours and visualized with DAB (Vector labs). 8-oxo-dG and $p16^{INK4A}$ stained sections were counterstained with haematoxylin. Light micrographs were captured, files IDs were blinded using NameChanger (MRR Software) and quantitation was performed. Positive cells were identified by brown DAB signal within the nucleus. One section was quantified per animal. For $p16^{INK4A}$, two slides containing aortic sections were stained from each animal and positivity was determined by averaging the percentage of positive cells from one section on each slide. For VCAM-1, a threshold was chosen using ImageJ, which captured DAB signal lining the endothelium without capturing the adjacent elastin. This threshold was applied to all images and used to measure the area of DAB signal. This area was then standardized to the perimeter of the aorta.

2.14 Statistical analyses

Statistical differences ($P < 0.05$) were determined using GraphPad Prism software version 6.0. Unless otherwise stated below, One-Way ANOVA was used to assess the effects of one independent variable on a dependent variable. This test was followed by

Dunnett's Multiple Comparison Test, comparing Saline and NR+Ang II groups, to Ang II. For non-uniform data, Kruskal-Wallis Non-Parametric One-Way ANOVA was used to assess the effects of one independent variable on a dependent variable (Fig. 3.1A, 3.2C). This test was followed by Dunn's Multiple Comparisons Test, comparing Saline and NR+Ang II groups, to Ang II.

3 RESULTS

3.1 Dietary NR supplementation augments liver NAD^+ levels during Ang II infusion

A decline in cellular NAD^+ levels has been observed during conditions of oxidative stress[212,213]. Ang II can be damaging to the vasculature and may lower NAD^+ levels. To determine if NR supplementation impacts Ang II-induced vascular damage, I conducted experiments whereby middle-aged C57Bl/6 mice were given a chow diet supplemented with NR (400mg/kg/day) or vehicle for 1 week. While continuing their respective diet, mice then received a continuous infusion of Ang II (1.44 mg/kg/day) for three days. A group of vehicle-treated mice was also infused with saline for three days, as control. I first evaluated whether this Ang II infusion protocol affected NAD^+ metabolism. For this, mice were sacrificed after three days of Ang II and liver NAD^+ content was assayed. I found that Ang II infusion decreased liver NAD^+ content to 55.2% of that present in saline-infused mice ($p=0.1068$, Fig. 3.1A). In contrast, Ang II-infused mice that received NR did not have a decrease in NAD^+ content. Liver NAD^+ content in these mice was significantly higher than in Ang II-infused mice on control diet ($p=0.0321$).

3.2 Dietary NR supplementation does not abrogate Ang II-induced hypertension

Ang II infusion elevates blood pressure[214]. Therefore, I next tested the effect of NR on blood pressure during Ang II infusion. In mice implanted with a 3-day Ang II

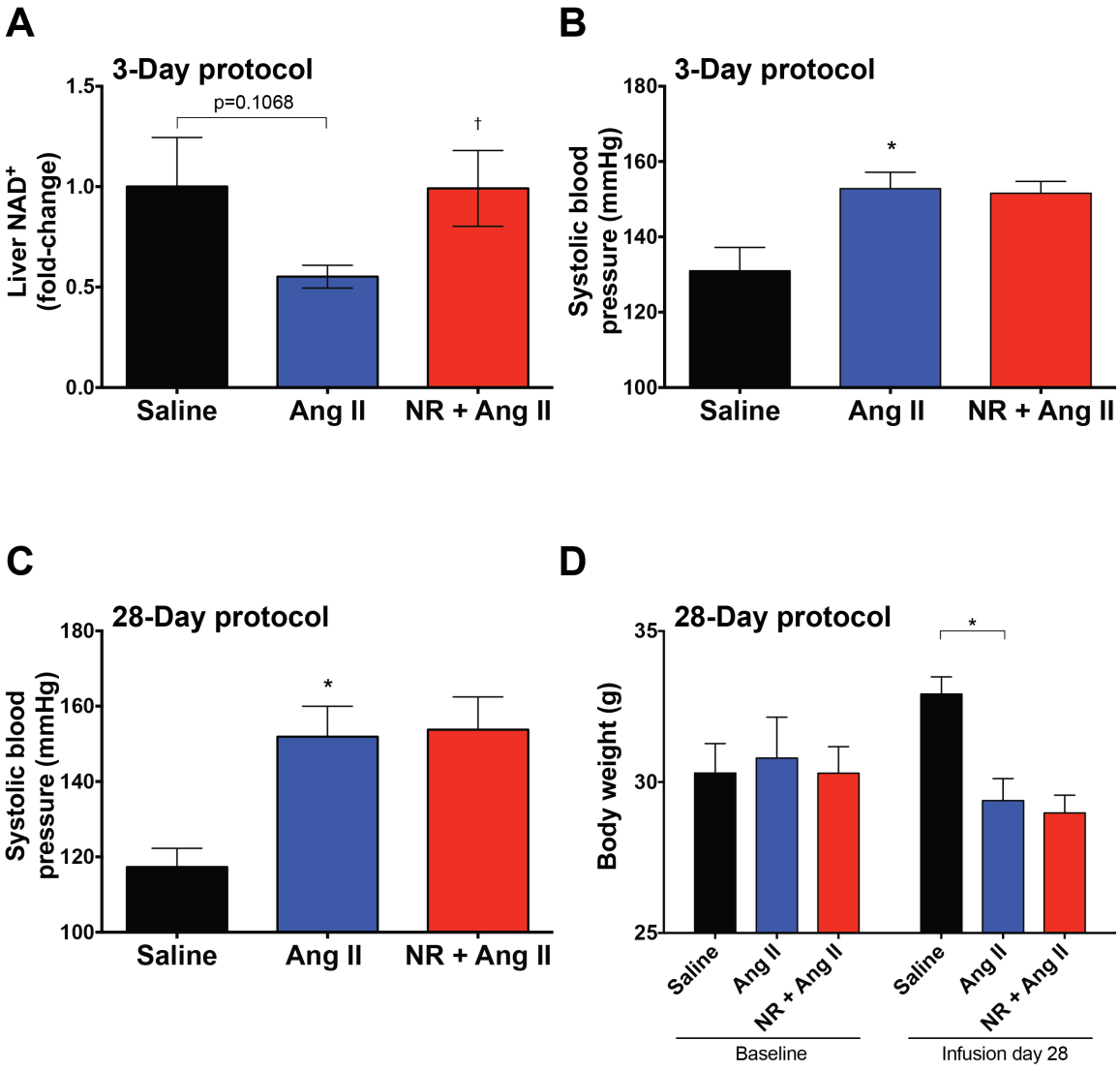
infusion pump, systolic blood pressure on day 2 had increased by 16.6% (~20 mmHg) ($p<0.01$) (Fig. 3.1B). Notably, there was a similar increase in BP in mice on the NR-supplemented diet (Fig. 3.1B).

I also measured blood pressure in mice implanted with 28-day Ang II infusion pumps. Blood pressure on day 27 of Ang II infusion in mice receiving vehicle-diet increased by ~30 mmHg ($p<0.01$) (Fig 3.1C). Again however, an NR-diet had no impact on systolic blood pressure increase in response to Ang II.

3.3 Dietary NR supplementation does not affect Ang II-induced bodyweight decline

Ang II infusion can also lead to body weight loss. This is reportedly due to anorexia[215] and muscle wasting[216], which can be mitigated by AT1R blockers[217]. I therefore tested if NR administration could impact bodyweight. At baseline, body weight was not significantly different between treatment groups (~30g). After four weeks of Ang II infusion, body weight was ~four grams lower in Ang II-infused mice compared to saline-infused mice ($p<0.05$, Fig. 3.1D). However, NR-diet did not impact this body weight disturbance ($p=0.9330$).

Figure 3.1 NR can maintain NAD^+ levels during Ang II infusion, but does not affect hypertension or bodyweight disturbance A-B. Liver NAD^+ levels (A, n=4-8, †p=0.0321 vs. Ang II) and tail-cuff systolic blood pressure measurements (B, n=7-11, *p=0.006 vs. Saline) from C57Bl/6 mice receiving vehicle- or NR-containing diet for 10 days, with Ang II- or saline-infusion during the last 3 days. Blood pressure was measured on day 2 of infusion. Values are the mean ± S.E.M. **C-D.** Tail-cuff systolic blood pressure (C, n=6, *p=0.009 vs. Saline**)** and body weight measurements **(**D, n=7-14, *p=0.0415 vs. Ang II**)** from C57Bl/6 mice receiving vehicle- or NR-containing diet for 3 weeks, followed by infusion of Ang II or saline for 28 days, while staying on the designated diets. Blood pressure was taken on day 27 of infusion. Body weight was taken at baseline (before diet initiation) and on day 27 of infusion. Values are the mean ± S.E.M.


A
3-Day protocol
p=0.1068
†
Liver NAD+ (fold-change)
1.5
1.0
0.5
0.0
Saline
Ang II
NR + Ang II
B
3-Day protocol
*
Systolic blood pressure (mmHg)
180
160
140
120
100
Saline
Ang II
NR + Ang II
C
28-Day protocol
*
Systolic blood pressure (mmHg)
180
160
140
120
100
Saline
Ang II
NR + Ang II
D
28-Day protocol
*
Body weight (g)
35
30
25
Saline
Ang II
NR + Ang II
Saline
Ang II
NR + Ang II
Baseline
Infusion day 28

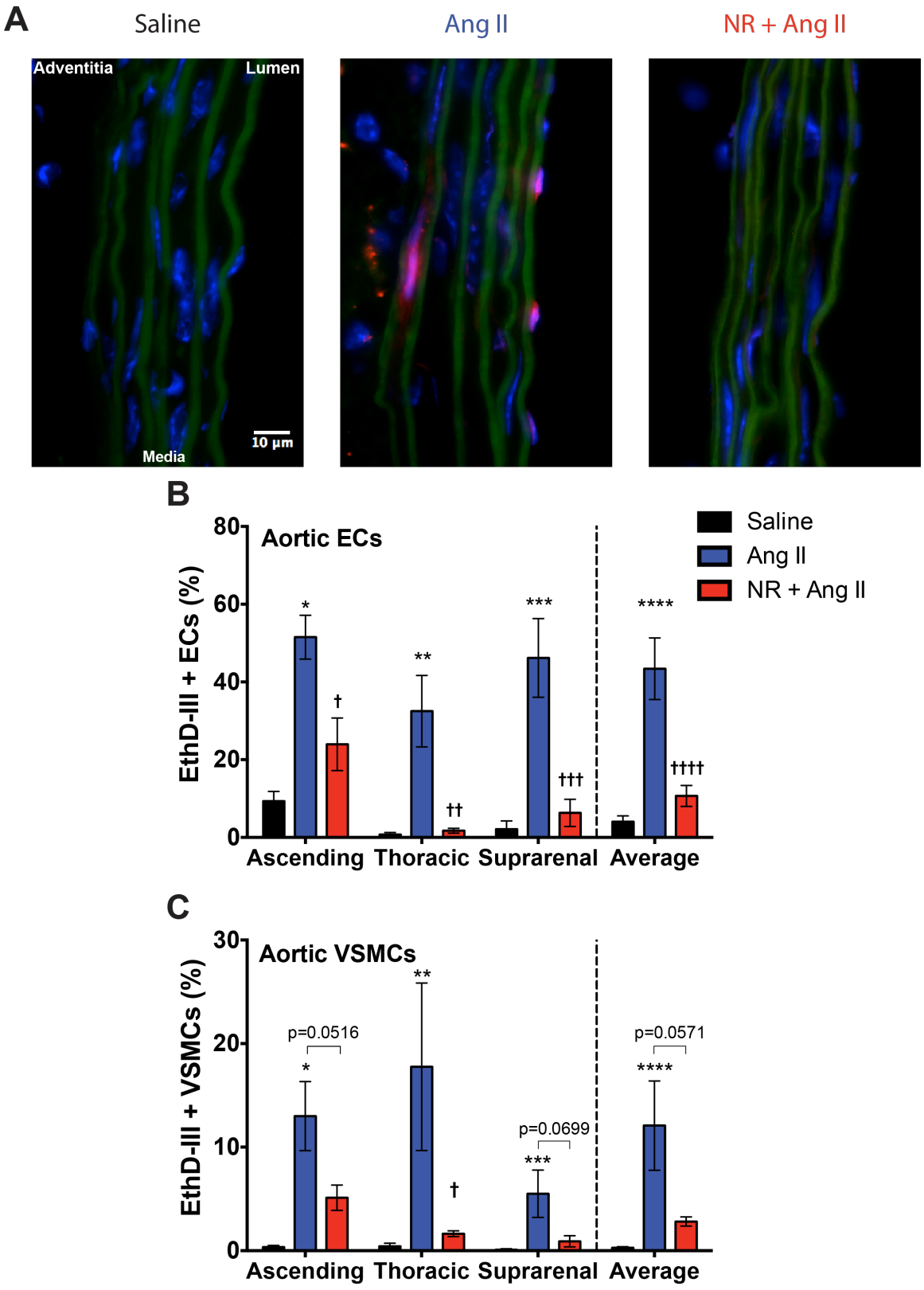
3.4 Dietary NR protects aortic vascular cells from acute Ang II-induced death, *in vivo*

Ang II induces apoptosis of vascular cells *in vitro*[147,218] and during sustained infusion in mice[149]. However, the true extent of Ang II-induced cell death, which may occur through many non-apoptotic routines, is unknown. Furthermore, it is not known if an acute infusion of Ang II can induce cell death or if supplementation with NR has any protective effect. Therefore, we used a vital dye to investigate Ang II's effect on vascular cell viability, and whether NR has any impact. To do this, mice were treated with NR- or vehicle-diet for 1 week, before also receiving a continuous infusion of Ang II or saline for 3 days. Ten minutes before sacrifice, mice were injected intravenously with the vital dye, Ethidium Homodimer-III (EthD-III). EthD-III is a cell membrane impermeable dye, which fluoresces red upon binding with DNA. Because EthD-III is cell membrane impermeable, it can only enter dead/damaged cells, which have a compromised cell membrane[219]. After sacrifice, the aorta was harvested, frozen in OCT and cryosections were obtained. Cross-sections of the ascending, thoracic and suprarenal aorta were mounted with DAPI-containing media and EC/VSMC EthD-III positivity was assessed. This revealed that Ang II induced marked EC death in the ascending, thoracic and suprarenal aorta. This death response was attenuated by NR supplementation (Fig. 3.2A). On average, EthD-III-positive ECs increased from 4.1% in saline to 43.4% in mice subjected to Ang II infusion ($p=0.0005$, Fig. 3.2B). With NR treatment, EC EthD-III positivity decreased to 10.7% ($p=0.0012$).

The ascending, thoracic and suprarenal aorta also exhibited a significant increase in VSMC EthD-III positivity upon 3 days of Ang II infusion (Fig. 3.2C). In the thoracic

aorta, NR administration significantly reduced the number of EthD-III-positive cells ($p=0.0275$). On average, EthD-III-positive VSMCs increased from 0.3% in saline-infused mice to 12.1% in Ang II-infused mice ($p=0.0321$). With NR supplementation, only 2.8% of VSMCs exhibited EthD-III positivity ($p=0.0571$).

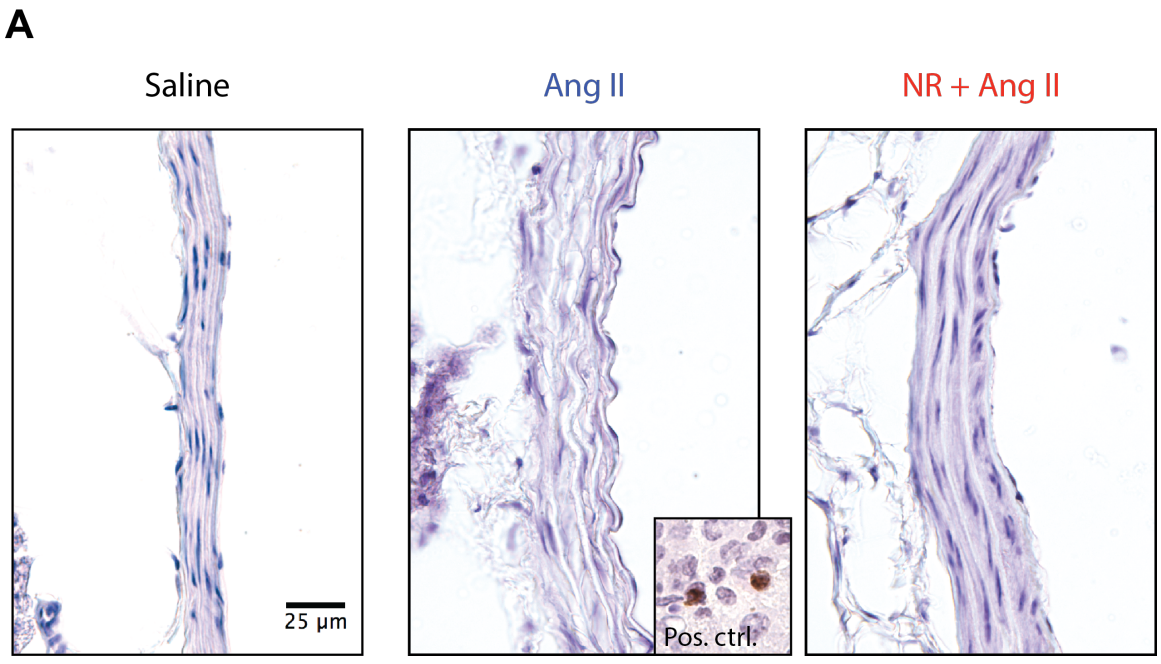
Figure 3.2 NR protects vascular cells from acute Ang II-induced death and membrane permeability A. Fluorescent images depicting EthD-III (red), elastin background (green) and DAPI (blue) signal, from the thoracic aorta of mice treated with NR- or vehicle-diet for 1 week, before also receiving a continuous infusion of Ang II or saline for 3 days. **B-C.** Quantitation of the percentage of EthD-III-labeled ECs (B, n=5-8, *p=0.0003, **p=0.0075, ***p=0.00128, ****p=0.0005 vs. Saline. †p=0.0047, ††p=0.0046, †††p=0.0018, ††††p=0.0012 vs. Ang II) and VSMCs (C, n=5-8, *p=0.0053, **p=0.0004, ***p=0.0116, ****p=0.0321 vs. Saline. †p=0.0275 vs. Ang II) Bars represent mean ± S.E.M.


A
Saline
Ang II
NR + Ang II
Adventitia
Lumen
Media
10 µm
B
Aortic ECs
EthD-III + ECs (%)
Saline
Ang II
NR + Ang II
Ascending
Thoracic
Suprarenal
Average
C
Aortic VSMCs
EthD-III + VSMCs (%)
p=0.0516
p=0.0699
p=0.0571

3.5 Ang II-induced death at day 3 is caspase-3-independent

Long-term Ang II infusion can induce apoptosis in vascular cells of the aorta[149]. We stained for the apoptotic mediator cleaved caspase-3, to determine whether cell death at day 3 of Ang II occurs through a caspase-3-dependent apoptosis program. In the ascending, thoracic and suprarenal aorta, ECs and VSMCs in all treatment groups did not stain positively for cleaved caspase-3 (Fig. 3.3). A liver section from an Ang II-infused mouse was used as a positive control, which stained positively for cleaved caspase-3 (Fig. 3.3, middle panel).

Figure 3.3 Acute Ang II-induced EC and VSMC death is caspase-3-independent A. Cleaved caspase-3 immunostaining in the thoracic aorta of mice fed a diet with NR or vehicle for 1 week, before also receiving a continuous infusion of Ang II or saline for 3 days. Positive control represents a sample of liver taken from an Ang II-infused mouse. Staining was performed in the ascending, thoracic and suprarenal aorta and appeared negative in each.

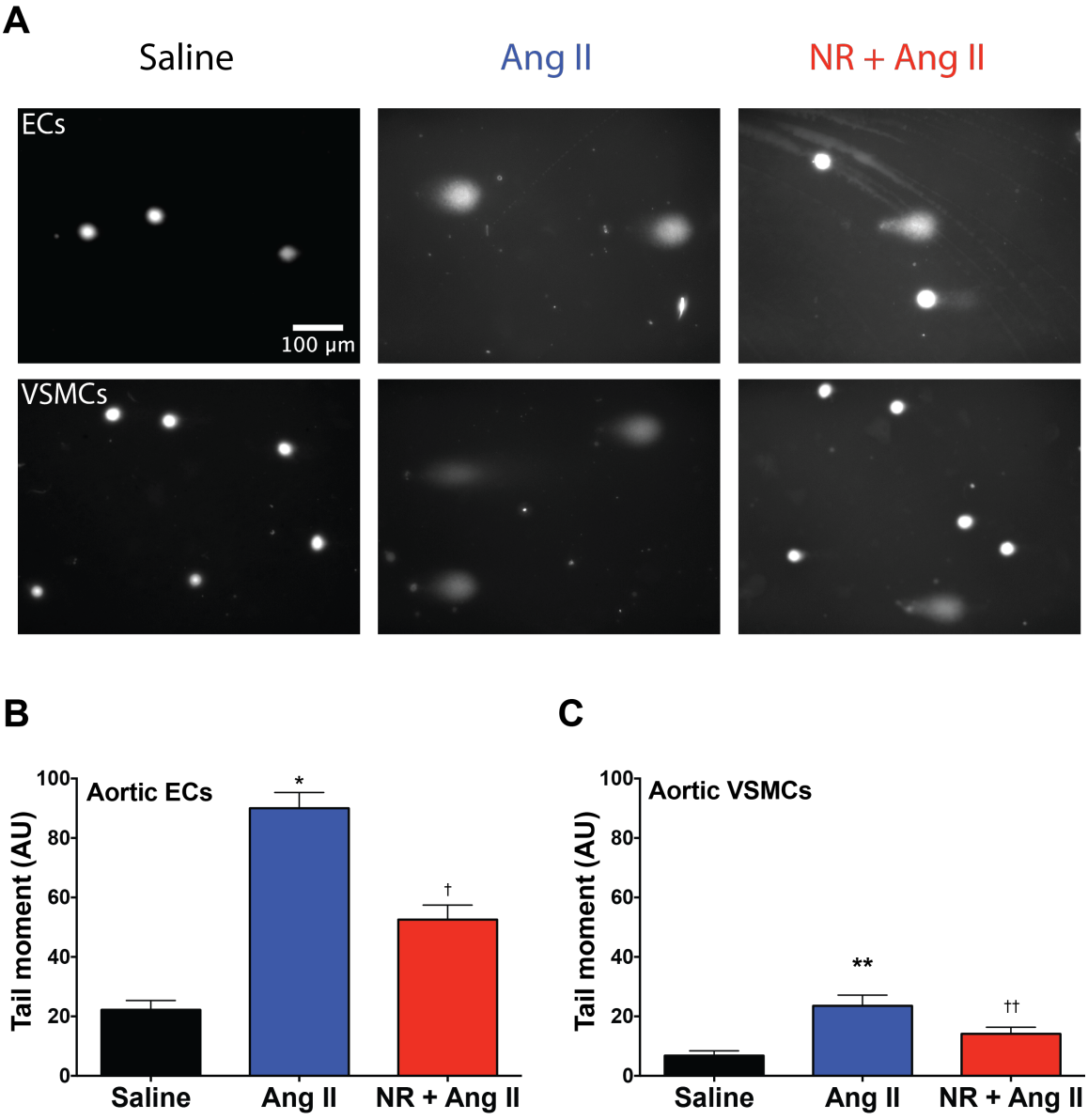

A
Saline
Ang II
NR + Ang II
25 µm
Pos. ctrl.

3.6 NR protects aortic vascular cells from acute Ang II-induced DNA damage

Ang II can induce DNA strand breakage[167]. *In vivo*, this has been assessed only indirectly by measuring levels of DNA damage response proteins[176]. As yet, there is no direct evidence that Ang II can cause DNA strand breakage in ECs or SMCs within the vessel wall. To assess this, I developed a strategy that builds on the "comet assay". The comet assay uses gel electrophoresis to pull cleaved DNA - which has lost its supercoiling, exposing its negative charge - from the nucleus. This forms a comet shaped structure, with intact DNA in the "head" and damaged DNA in the "tail"[220]. Previous *in vitro* work in our lab has demonstrated that NR can protect vascular cells from H_2O_2-induced DNA damage. To investigate NR's effect on Ang II-induced DNA damage, *in vivo*, mice received a NR- or vehicle-containing diet for 1 week, before also receiving a continuous infusion of Ang II or saline for 3 days. After 3 days, mice were euthanized and ECs were immediately collected by exposing the aortic lumen to collagenase II for 35 minutes at 37°C. To collect VSMCs, the aorta was cleaned of adventitia and digested in collagenase I and dispase. Comet assay was then performed on the acutely dispersed cells. This revealed that, remarkably, short term Ang II infusion induced DNA damage in both ECs and VSMCs (Fig. 3.4A). Importantly, in mice receiving NR in the diet, both cells types displayed reduced DNA cleavage. Compared to saline infusion, ECs from Ang II-infused mice exhibited a 4.1-fold increase in tail moment ($p<0.001$). However, cells from NR-supplemented mice exhibited a 41.6% reduction in tail moment following Ang II infusion ($p<0.0001$). Similarly, Ang II infusion resulted in VSMCs exhibiting a 3.5-fold increase in tail moment ($p<0.0001$). Like for ECs, this DNA damage was reduced by

40.1% in mice receiving the NR-diet ($p=0.0158$).

Figure 3.4 NR protects aortic vascular cells from Ang II-induced acute DNA damage A. Sybr-Gold staining, depicting comet tails of ECs and VSMCs isolated from mice that had received a diet containing NR or vehicle for 1 week before also receiving a continuous infusion of Ang II or saline for 3 days. **B-C.** Quantitation of tail moments from ECs (B, n=142-172 cells from 2 mice per condition, *$p<0.0001$ vs. Saline, †$p<0.0001$ vs. Ang II) and VSMCs (C, n=106-180 cells from 1 saline mouse and 2 mice from Ang II infused groups, **$p<0.0001$ vs. Saline, ††$p=0.0158$ vs. Ang II) using open comet software. Bars represent mean ± S.E.M.

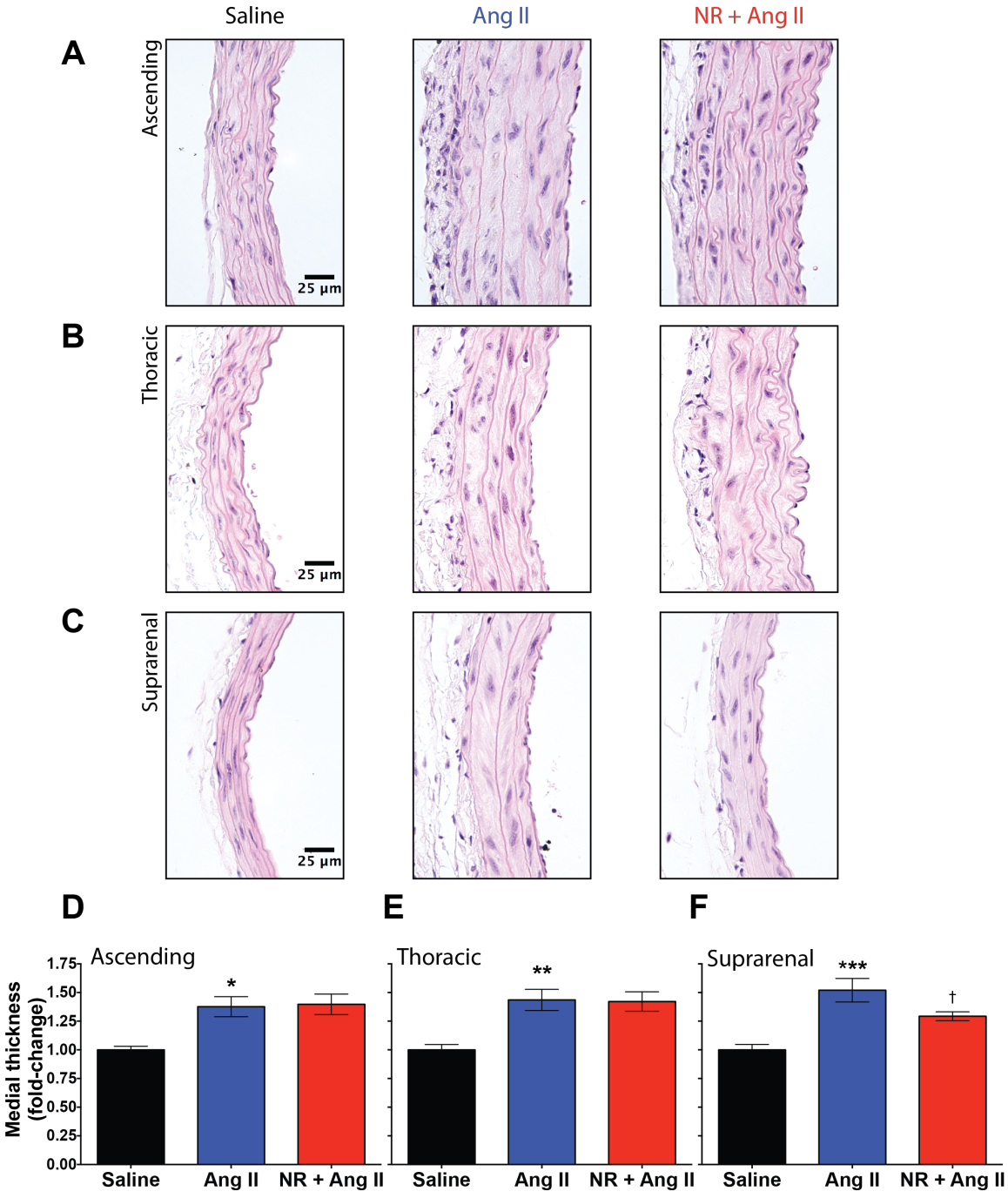

A
Saline
Ang II
NR + Ang II
ECs
100 μm
VSMCs
B
Aortic ECs
Tail moment (AU)
100
80
60
40
20
0
*
†
Saline
Ang II
NR + Ang II
C
Aortic VSMCs
Tail moment (AU)
100
80
60
40
20
0
**
††
Saline
Ang II
NR + Ang II

3.7 NR confers site-specific reduction in Ang II-induced medial thickening

Chronic Ang II infusion results in thickening of the aortic media. Having shown that early cell death can be prevented by NR, I next investigated whether medial area after 28 days of Ang II infusion was affected by NR pre- and co-treatment. After Ang II infusion, medial area in the ascending, thoracic and suprarenal aorta was significantly increased (Fig. 3.5A to C). Interestingly however, NR treatment did not impact medial area in the ascending or thoracic aorta. On the other hand, NR did confer mild protection in the suprarenal region. Compared to saline-infused mice, ascending aortic medial area in mice receiving either Ang II or Ang II plus NR pre- and co-treatment, was increased by 37.6% and 39.7% respectively ($p<0.05$) (Fig. 3.5D). Similarly, in the thoracic aorta, medial area was 43.6% and 42.1% greater in mice receiving Ang II and Ang II plus NR treatment, respectively ($p<0.01$, Fig. 3.5E). However, in the suprarenal aorta, Ang II infusion resulted in a 52% increase in medial area, but in mice administered NR, this increased by only 29.3% ($p=0.0335$).

Figure 3.5 NR confers site-specific reduction in Ang II-induced medial thickening

A-C. H&E staining of ascending (A) thoracic (B) and suprarenal (C) aortic sections from mice on a diet supplemented with vehicle or NR and subjected to 4 weeks of Ang II infusion (n=6-9). **D-F.** Quantification of medial area, standardized to lumen perimeter from the ascending (D, *p=0.0051 vs. Saline) thoracic (E, **p=0.0022 vs. Saline) and suprarenal aorta (F, ***p=0.0003 vs. Saline. †p=0.0335 vs. Ang II). Bars represent mean ± S.E.M.

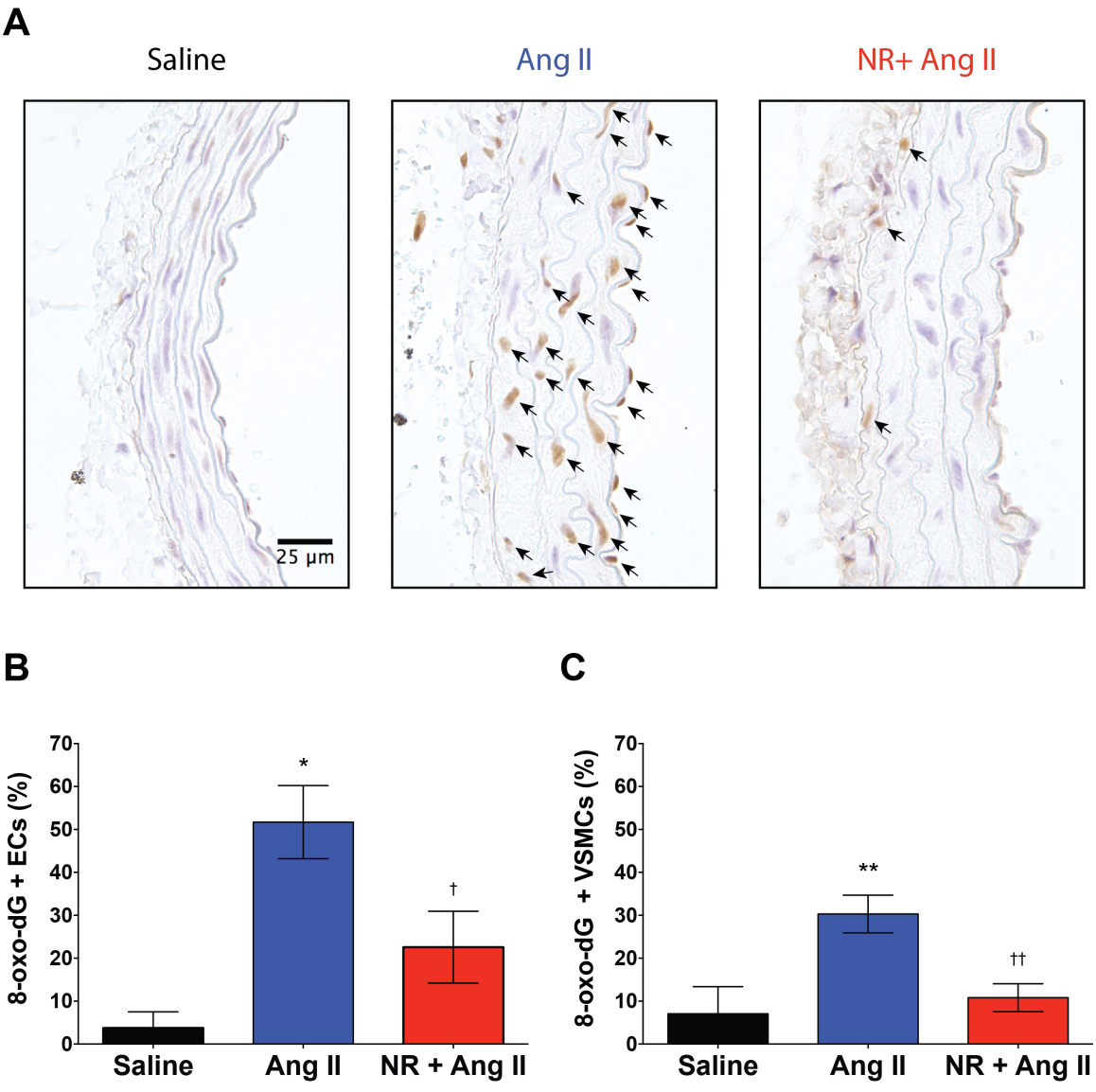

Saline
Ang II
NR + Ang II
A
Ascending
25 µm
B
Thoracic
25 µm
C
Suprarenal
25 µm
D
E
F
Ascending
Thoracic
Suprarenal
Medial thickness (fold-change)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
**
***
†
Saline
Ang II
NR + Ang II

3.8 NR attenuates Ang II-induced DNA damage in vascular cells at day 28

I next asked whether NR pre- plus co-treatment would protect vascular cells from genomic damage accumulated after prolonged infusion of Ang II. For this, I immunostained the thoracic aorta for the DNA damage biomarker, 8-oxoguanine (8-oxo-dG), following 28 days of Ang II infusion. 8-oxogaunine is a DNA damage product, formed when guanine nucleobases undergo oxidation[168]. Ang II significantly increased the percentage of 8-oxo-dG-positive ECs (Fig. 3.6A, B) and VSMCs (Fig. 3.6A, C) in the thoracic aorta (3.8% vs. 51.7% in ECs p=0.0042, 7.1% vs. 30.3% in VSMCs p=0.0012). However, Ang II-infused mice supplemented with NR exhibited a significant reduction in the percentage of 8-oxo-dG-positive cells (51.7% vs. 22.6% in ECs p=0.0042, 30.3% vs. 10.8% in VSMCs p=0.0227).

Figure 3.6 NR attenuates Ang II-induced DNA oxidative damage in vascular cells

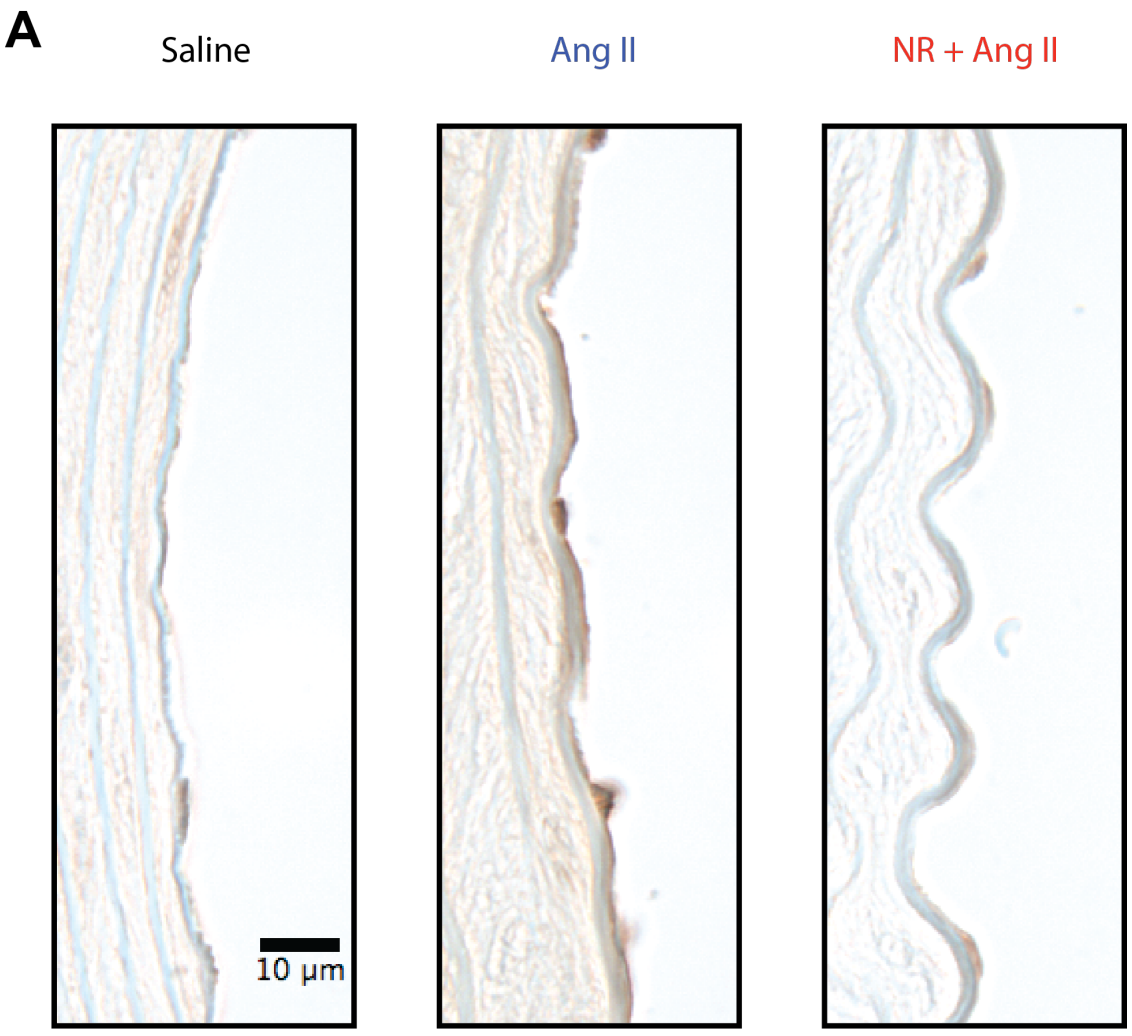
A. Light micrographs depicting 8-oxo-dG immunostaining in the thoracic aorta of mice infused for 4 weeks with saline, Ang II, or Ang II with pre-treatment (3 weeks) and co-treatment (4 weeks) with NR (n=6-9). Arrows indicate 8-oxo-dG-positive cells. **B-C.** Percent of 8-oxo-dG-positive ECs (B, *p=0.0042 vs. Saline, †p=0.0069 vs. Ang II) and VSMCs **(**C, **p=0.0012 vs. Saline, ††p=0.0227 vs. Ang II) in thoracic aortic sections. Bars represent mean ± S.E.M.


A
Saline
Ang II
NR+ Ang II
25 µm
B
8-oxo-dG + ECs (%)
70
60
50
40
30
20
10
0
*
†
Saline
Ang II
NR + Ang II
C
8-oxo-dG + VSMCs (%)
70
60
50
40
30
20
10
0
**
††
Saline
Ang II
NR + Ang II

3.9 NR prevents Ang II-induced endothelial VCAM-1 expression

VCAM-1 has been implicated in several inflammatory diseases, including atherosclerosis[221]. Expression of VCAM-1 increases during Ang II infusion[222]. To determine if NR impacts this, immunostaining for VCAM-1 was performed in the thoracic aorta, following 28 days of Ang II infusion (Fig. 3.7A). Endothelial cell VCAM-1 expression was 2-fold higher in Ang II-infused mice compared to saline-infused mice (Fig. 3.7B) ($p=0.0092$). Treatment with NR completely abrogated this increase ($p=0.0153$).

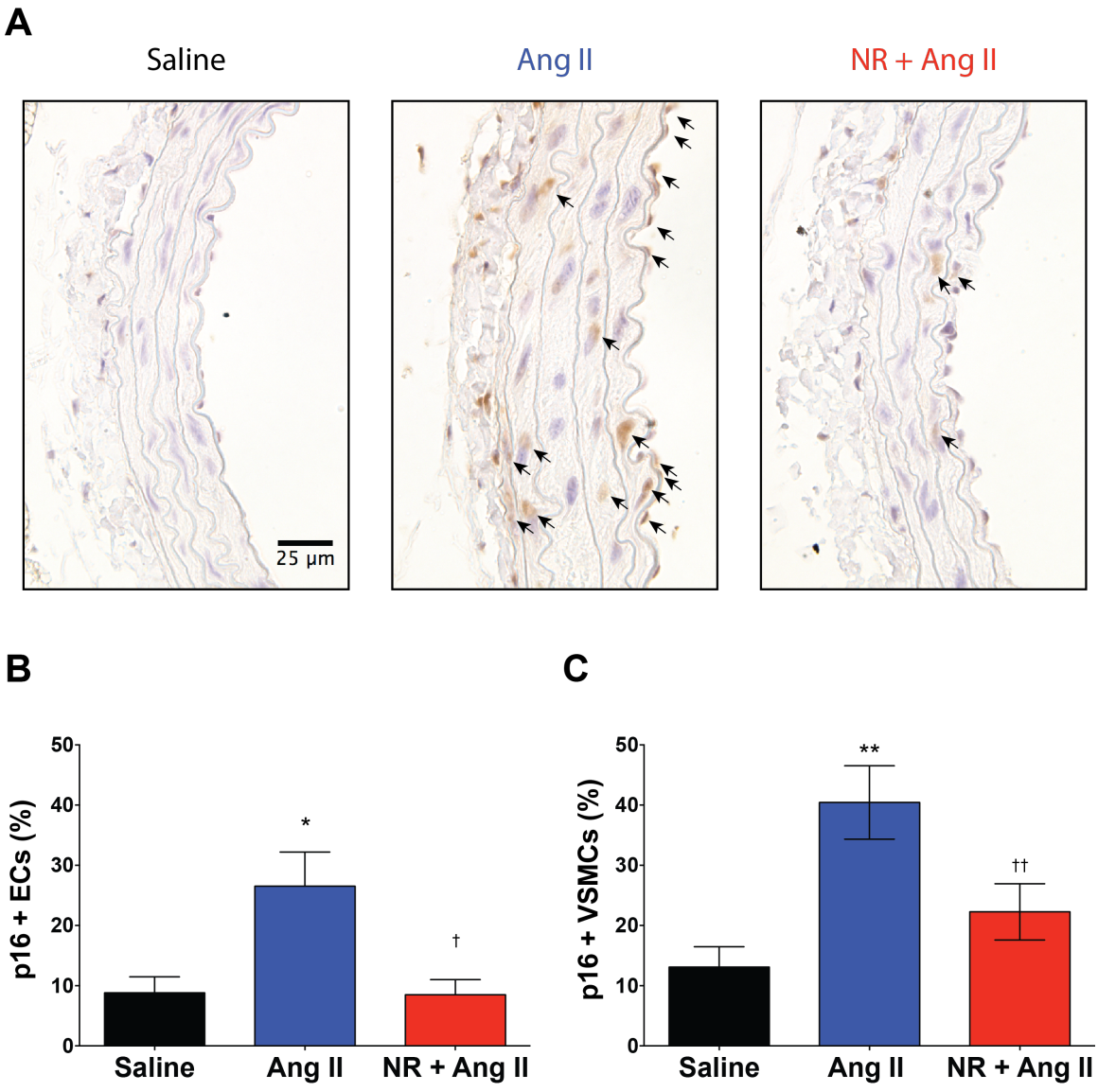
Figure 3.7 NR prevents Ang II-induced endothelial VCAM-1 expression A. VCAM-1 immunostaining in the thoracic aorta of mice infused for 4 weeks with saline, Ang II, or Ang II with pre-treatment (3 weeks) and co-treatment (4 weeks) with NR. **B.** Quantification of VCAM-1-positive area lining the endothelium, standardized to intimal perimeter, and expressed relative to the signal in saline-infused mice. Bars represent mean ± S.E.M. (n = 6 mice, *p=0.0092 vs. Saline, †p=0.0153 vs. Ang II).


A
Saline
Ang II
NR + Ang II
10 µm
B
Endothelial VCAM-1 expression (fold-increase)
2.5
2.0
1.5
1.0
0.5
0.0
*
†
Saline
Ang II
NR + Ang II

3.10 NR ameliorates Ang II-induced $p16^{INK4A}$ expression

Prolonged oxidative stress and DNA damage can result in cellular senescence[223]. *In vitro* work in our lab has demonstrated NR can protect ECs and VSMCs from H_2O_2-induced senescence[124]. Therefore, I next investigated whether NR impacted Ang II-induced senescence *in vivo*, by performing immunostaining for the senescence biomarker, $p16^{INK4A}$. $p16^{INK4A}$ is a tumor suppressor that induces cell cycle arrest and is therefore commonly used to identify senescent cells[223]. As depicted in Fig. 3.8, the percentage of $p16^{INK4A}$-positive ECs and VSMCs was significantly higher in Ang II-infused mice compared to that observed in saline-infused counterparts (26.6% vs. 8.8% in ECs p=0.0109, 40.4% vs. 13.1%. in VSMCs p=0.0022) (Fig. 3.8B). Treatment with NR reduced $p16^{INK4A}$ positivity in both ECs (8.5% vs. 26.6% p=0.0097) and VSMCs (22.3% vs. 40.4% p=0.0398).

Figure 3.8 NR ameliorates Ang II-induced p16^{INK4A} expression A. p16^{INK4A} immunostaining in the thoracic aorta of mice infused for 4 weeks with saline, Ang II, or Ang II with pre-treatment (3 weeks) and co-treatment (4 weeks) with NR (n=5-6). Arrows indicate p16^{INK4A}-positive cells **B-C.** Quantification of p16^{INK4A}-positive ECs (B, *p=0.0109 vs. Saline, †p=0.0097 vs. Ang II) and VSMCs (C, **p=0.0022 vs. Saline, ††p=0.0398 vs. Ang II) in whole aortic cross-sections. Bars represent mean ± S.E.M.


A
Saline
Ang II
NR + Ang II
25 μm
B
p16 + ECs (%)
50
40
30
20
10
0
*
†
Saline
Ang II
NR + Ang II
C
p16 + VSMCs (%)
50
40
30
20
10
0
**
††
Saline
Ang II
NR + Ang II

4 DISCUSSION

The major findings of this thesis are:

1. In middle-aged mice infused with Ang II, oral NR administration elevates liver NAD^+ content.
2. Administration of NR does not impact the immediate or sustained blood pressure rise induced by Ang II in middle-aged mice, nor prevent body weight loss induced by sustained Ang II infusion.
3. Ang II infusion induces aortic EC and VSMC death. This early death response is prevented by NR administration.
4. Administration of NR inhibits oxidation of DNA, VCAM-1 expression, and senescence in ECs and VSMCs, induced by chronic Ang II infusion.

4.1 Oral NR supplementation augments liver NAD^+ in mice infused with Ang II

During oxidative stress and aging, NAD^+ levels decline. This has the potential to impair the activity of proteins important to cellular health and function[100]. To counteract this, several precursors to NAD^+ have been synthesized or purified and studied to ascertain if their delivery productively boosts NAD^+ synthesis[41]. In 2012, Canto et al. demonstrated that oral NR administration increases NAD^+ levels in several tissues in mice[126]. I employed a similar feeding protocol and show that Ang II-infused mice that were fed a diet with NR had higher liver NAD^+ content than those subjected to Ang II and fed a vehicle-diet. This is the first study to report a NR-mediated augmentation of liver NAD^+ in mice infused with Ang II. This finding provides assurance that the NR-

containing diet was properly prepared and administered, and mice receiving NR utilized it to generate NAD^+. Although vascular cell NAD^+ measurements would be desirable, the aorta contains relatively few cells, making measuring NAD^+ and detecting differences in NAD^+ difficult. For instance, despite demonstrating impressive vascular benefits, a study administering an alternative precursor (NMN) to aged mice did not detect an increase in aortic NAD^+[206]. The authors speculate this was due to rapid metabolism of generated aortic NAD^+. Another study sought to measure NAD^+/NADH ratios in the heart after NR treatment but did not report these values because they were deemed "unreliable"[224]. In a third study, NMN administration led to a non-significant trend to increase NAD^+ in liver, skeletal muscle, cortex and brown adipose tissue[225]. The authors noted the difficulty of measuring small fluctuations in NAD^+ and used labeled NMN to confirm its contribution to the tissue NAD^+ pool.

In keeping with my finding with respect to Ang II and NAD^+, experiments *in vitro*, have revealed a rescuable decline in VSMC NAD^+ after 3 days of Ang II[176]. Similarly, *in vitro* work in our lab has demonstrated NR delivery increases NAD^+ content and buffers NAD^+ decline in ECs and VSMCs exposed to H_2O_2[124].

4.2 NR does not impact Ang II-induced hypertension

Certain hypertensive medications can lower Ang II-induced hypertension in mice, particularly those that block RAS signaling[226,227]. I demonstrated that NR has no impact on acute and chronic Ang II-induced hypertension in middle-aged C57Bl/6 mice. Consistent with this, administering the NAD^+-boosting compound, PNU-282987, did not impact tail-cuff-derived blood pressure after 14 days of Ang II (0.576 mg/kg/day)[176].

Furthermore, CD38 KO, *an intervention that can markedly increase NAD^+ levels*, did not abrogate the rise in tail-cuff-derived blood pressure induced by 2.16 mg/kg/day of Ang II for 14 days[228].

On the other hand, human data has raised the possibility that NAD^+ precursors may provide mild blood pressure lowering in subsets of patients. In humans, the NAD^+ precursor niacin has been used to treat dyslipidemia for several decades. In dyslipidemic patients administered 1-2 g/day of niacin, systolic/diastolic blood pressure (SBP/DBP) was reduced by ~2/3 mmHg after 24 weeks[229]. In another trial, there was no reduction in BP with 3 g/day of niacin in MI survivors[230], but a post-hoc analysis suggested a 3/2 mmHg decline in SBP/DBP in patients considered to have metabolic syndrome[231]. In contrast, of five studies administering niacin combined with other drugs to patients with cardiovascular disease, only one found a decrease in blood pressure[232]. Collectively, these studies suggest that NR's anti-hypertensive potential should not be ruled out entirely, particularly in the context of metabolic syndrome/dyslipidemia. However, our results demonstrate that in the context of aggressive Ang II delivery, BP is not affected by NR. This is noteworthy because despite this ongoing hypertensive milieu, NR was found to protect vascular cells from pathology, discussed below.

4.3 NR does not impact Ang II-induced body weight decline

Skeletal muscle wasting occurs during chronic heart failure (CHF) and is an independent risk factor for mortality in these patients[233,234]. Ang II is elevated in CHF[235] and its infusion in mice results in skeletal muscle atrophy/cachexia[236]. In our study, Ang II infusion resulted in body weight loss. However, this was not impacted by NR

treatment. In agreement with this, mdx-muscular-dystrophy mice treated with NR did not increase bodyweight, lean mass, or lower limb muscular wet weight, despite an increase in muscle NAD^{+}[114]. Following hepatectomy[237] or intracerebral hemorrhage[238], NR/NMN did promote bodyweight gain, although this may be secondary to other functional improvements.

It is possible in my study that NR did not elevate muscle NAD^{+} levels, that declined in response to Ang II infusion. NAD^{+} does appear to be important for the maintenance of bodyweight and lean mass, which are reduced in mice with muscle-specific NAMPT KO[27]. These mice exhibit severe skeletal muscle NAD^{+} depletion (~0.75 vs. ~0.18 nmol/mg) and when treated with NR, only a non-significant trend to increase muscle NAD^{+} (~0.25 nmol/mg) or muscle mitochondrial NAD^{+} was observed. Using an isotope-labeled NR tracer, the authors found that NR was directly utilized to synthesize NAD^{+} in the liver, but in muscle, NR was largely converted to NAM, with a minor indirect contribution to the muscle NAD^{+} pool. This suggests that NR may be less effective at elevating NAD^{+} in skeletal muscle, particularly when NAMPT expression is reduced. Several studies have demonstrated increased muscle NAD^{+} following NR/NAD^{+} precursor administration[126,114,29]. But the aforementioned data raises the possibility that NR did not elevate muscle NAD^{+} levels during the systemic oxidative stress of Ang II infusion.

4.4 NR abrogates acute Ang II-induced cell death within the aortic wall

Oxidative stress increases with age and has been implicated in several age-related diseases, including vascular disease[239]. Reductions in NAD^+ have been observed during oxidative stress and linked to cell death[212]. Our lab has demonstrated that overexpression of the NAD^+ generating enzyme, NAMPT, improves survival of vascular cells during oxidative stress[240,241]. Furthermore, in *vitro* work has demonstrated that administration of NR can protect cells from H_2O_2-induced death[124]. In the current study, NR was orally administered to mice infused with Ang II. I found that this protected ECs and VSMCs from acute Ang II-induced death.

This was the first study using a vital dye to investigate vascular cell death in an Ang II infusion model. In doing so, I am the first to report that an acute infusion of Ang II induces EC and VSMC death. This data, coupled with a lack of staining for the apoptotic mediator cleaved caspase-3, provides the first evidence for a non-apoptotic death response to Ang II infusion. This is particularly noteworthy as Ang II infusion has been used for decades to induce experimental pathology and this finding expands our understanding of Ang II's pathological effects.

To label dead cells, I used EthD-III, as it has been used to label dead endothelial cells *in vivo,* and importantly is a sensitive marker of death[242] - reduced to near non-detectable levels by chemical/genetic suppression of cell death mediators[242]. EthD-III is a cell membrane impermeable dye, therefore cells that uptake EthD-III have lost their plasma membrane integrity, fulfilling one of the *in vitro* criterion needed to consider cells dead, as established by the Nomenclature Committee on Cell Death[243]. EthD-III allows detection of cell death regardless of the mode of death. Furthermore, currently used

molecular markers of cell death can be transient, may not necessarily reflect the true incidence of cell death and only inform on specific death routines.

Vital dyes based on membrane permeability have been used for decades to assess cell viability[244,245], although generally not *in vivo*. This is a strength of my study. It should nonetheless be considered that an increase in membrane permeability may reflect repairable damage to the cell membrane[246]. Therefore, I cannot exclude the possibility that some of the EthD-III-positive cells were stressed but may have recovered.

Improvements in cell survival have been demonstrated with delivery of NAD^+ and certain NAD^+ precursors in models of acute kidney injury[105], liver injury[247], intracerebral hemorrhage[238], spinal cord I/R injury[248], Alzheimer's disease[249] and Parkinson's disease[250]. In my study, Ang II-induced cell death was not associated with cleaved caspase-3, at day 3. Another study, infusing the same dose of Ang II to 12 week old mice, also failed to find cleaved caspase-3 in the ascending aorta at day 3[149]. However, caspase-3 was detected at sites of hemorrhage during long-term infusion. Therefore, it appears that acute Ang II-induced death is caspase-3-independent. Potential casapse-3-independent forms of cell death that may be abrogated by NR include necrosis, parthanatos, necroptosis, autophagic death or caspase-3-independent apoptosis[251]. Interestingly, intracellular NAD^+ has been found to promote TNF-α-induced necroptosis[252], although NR/NMN have also reliably decreased TNF-α in several studies[238,104,253,254,255]. I speculate that increasing NAD^+ levels may have protected cells by lowering oxidative stress, reducing DNA damage, increasing DNA repair, or preventing ATP depletion[38]. Mechanisms which may have been mediated by NAD^+-dependent sirtuins.

4.5 NR abrogates acute Ang II-induced DNA damage

DNA damage contributes to vascular disease[9]. Although Ang II-induced DNA damage has been studied, I am the first to directly identify aortic DNA strand breakage, *in vivo*. Using a comet assay approach, I show for the first time that Ang II causes single and/or double strand breaks in the aorta, and that this occurs rapidly, at day 3 of infusion. Importantly, this rapid destruction of DNA strands was abrogated by NR treatment.

Several potential mechanisms may explain NR's protective effect. Sirtuin proteins require NAD^+ for their activity and regulate DNA repair pathways triggered in response to single – nucleotide excision repair and base excision repair – and double strand breaks – non-homologous end joining and homologous recombination[256]. SIRT1 and 6 are particularly important. These enzymes deacetylate proteins involved in DNA repair, promoting their interaction, assembly, activity and stability[256]. Sirtuins can localize these proteins to sites of damage[257] or promote their expression by localizing transcriptional regulators[258]. Sirtuins can also promote remodeling of the chromatin necessary for repair[259] or delay apoptotic signaling increasing the window for repair[260]. In addition, sirtuins regulate NOX-derived ROS generation[261] and anti-oxidant expression[52], which may have directly lowered oxidative stress and secondarily DNA damage. Importantly, aged mice (26-28 months) treated with NMN displayed reductions in vascular superoxide production and *ex vivo* incubation of aortas with NMN upregulated superoxide dismutase 2[206].

Another NAD^+ consumer, PARP-1, could also play a role. In response to DNA damage, PARP1 utilizes NAD^+ to create poly(ADP)-ribose chains at damaged sites, forming a docking site for DNA repair proteins[61]. By augmenting NAD^+ levels, NR may

have promoted DNA repair by transiently enhancing PARP-1-mediated repair. Sirtuins can also promote PARP activity[262] and even without cleavage, NAD^+ can promote PARP-1 activity by binding to its negative regulator, DBC-1[263].

Reports from other investigators support my findings. In muscle stem cells harvested from young (1 month) and old mice (22-24 months) administered NR, fewer cells stained positivity for the DNA damage biomarker, γ-H2AX[108]. Furthermore, comet assay performed on these cells revealed lower levels of DNA damage with NR treatment. NR administration also lowered the number of γ-H2AX positive hepatocytes in a hepatocyte carcinoma model driven by NAD^+ synthesis deficits and DNA damage[264]. Moreover, liver cells of mice exposed to γ-radiation have lower levels of 8-oxo-dG and fewer AP sites when mice are treated with nicotinamide[265]. *In vitro,* NAD^+ and its precursors have protected PBMCs[266], PC12 cells[250], and cortical neurons[249] from DNA damage.

Many of the aforementioned studies and studies involving Ang II-infusion, have detected breakage of DNA indirectly, by measuring levels of γ-H2AX[176]**.** Early in the DNA damage response, histone H2AX is phosphorylated, forming γ-H2AX and recruiting repair proteins to sites of damage[267]. However, H2AX has been shown to be phosphorylated during cell cycle progression[268], which may confound its use as a DNA damage biomarker. Therefore, an increase in γ-H2AX may be due to mitotic arrest or increased proliferation, which can be induced by Ang II[269,270]. γ-H2AX can also be seen in senescent cells, which has been argued to reflect persistent DNA damage foci[271] and/or sites of chromatin remodeling that are not associated with DNA damage[272]. Direct

assessment of DNA damage with the comet assay overcomes these limitations caused by indirect measures.

In summary, my work demonstrates, for the first time, that vascular cells within the aorta are susceptible to early destruction of DNA – with DNA breaks in both ECs and VSMCs. Oral administration of NR can abrogate this.

4.6 NR abrogates sustained Ang II-induced DNA oxidative damage

I found that after 28 days of Ang II infusion, oxidation of guanine nucleobases in vascular cells of the thoracic aorta was also lower in mice fed NR. Interestingly, DNA damage at day three, expressed as tail moment, and at day 28, expressed as the percentage of 8-oxo-dG-positive cells, was greater in ECs compared to VSMCs. This indicates that aortic ECs are more susceptible to Ang II-induced DNA damage than VSMCs. This differential pattern was also seen for the cell death response in the aorta after three days of Ang II.

4.7 NR confers region-specific protection from Ang II-induced aortic thickening

Infusion of Ang II for 28 days thickens the aorta, independent of blood pressure and via actions on the AT1 receptor[128]. In the ascending aorta, thickening occurs due to hyperplasia, while cell hypertrophy is responsible in other aortic regions[128]. In agreement with other studies, I observed thickening in the ascending, thoracic and suprarenal regions of the aorta[128]. NR did not impact thickening in the ascending and thoracic region. However, thickening was blunted at the suprarenal region. This region-specific

difference in NR's effect is interesting. It may be due to the embryological origins of VSMCs, which differ between the ascending, thoracic and abdominal aorta[273]. These VSMCs originate from the neural crest, somite, and splanchnic mesoderm, respectively. Interestingly, VSMCs isolated from these sites have exhibited differential growth and transcriptional responses, raising the possibility that they may respond differently to NAD^+ precursors[273,274]. In addition, CD38 expression varies markedly between vascular regions[99]. Therefore, it is possible that the aorta exhibits regional variations in the expression of enzymes involved in NAD^+ homeostasis, and thus responses to precursors.

4.8 NR prevents sustained Ang II-induced VCAM-1 expression

The role of inflammation in cardiovascular diseases, and particularly atherosclerosis, has been widely studied. The cell adhesion molecule VCAM-1 allows for the adhesion of monocytes to the vessel lumen[160]. Monocytes can subsequently enter the vessel wall (transmigration), differentiate into macrophages and phagocytose lipid, becoming foams cell and forming an atherosclerotic plaque[160]. Therefore, reducing the expression of VCAM-1 may be an important step towards preventing atherosclerotic plaque formation. In agreement with other studies, I found increased VCAM-1 expression after Ang II infusion, indicative of chronic EC dysfunction and a proinflammatory vascular state[183]. Importantly, this was suppressed by NR administration.

Transcription of VCAM-1 is mediated by NF-κB and is responsible for its upregulation by Ang II[186]. SIRT1 suppresses NF-κB activity by deacetylating its p65 subunit, a process which is prevented during NAD^+ depletion[275]. Therefore, NR may

have augmented NAD^+ levels, fueling SIRT1 mediated NF-κB suppression. Beyond directly boosting NAD^+, reductions in oxidative stress and DNA damage could play a role, because PARP-1[276] and 8-oxo-dG[170] are linked to NF-κB-driven transcription. Importantly, I found that NR lowered the number of $p16^{INK4A}$-positive ECs, suggesting fewer senescent cells. This may also be responsible as subsets of senescent ECs exhibit prolonged VCAM-1 expression[277].

In agreement with our results, NR abrogated a HFD-induced increase in liver VCAM-1 transcript abundance. However, in contrast, NMN did not lower VCAM-1 expression in a model of intracerebral hemorrhage. NMN was injected 30 minutes after hemorrhage and inflammation was assessed after 3 days, while we assessed VCAM-1 after long-term NR treatment and Ang II infusion. Differences in models, cell types, NAD^+ precursors and route of administration may be responsible. NAD^+ precursors have also mitigated inflammation in aging[108], muscle damage[108] and metabolic disease models[104,106,253,255].

4.9 NR attenuates chronic Ang II-induced $p16^{INK4A}$ expression

During aging, heavily damaged cells can adopt a senescent phenotype. This is characterized by permanent cell cycle arrest and the secretion of pro-inflammatory cytokines. Clearance of these cells has improved health[278,279] and lifespan[280] in animal models, including vascular endpoints such as carotid artery vascular reactivity[281]. $p16^{INK4A}$ is a commonly used biomarker of senescence, and prevents cell cycle progression by suppressing the expression of S-phase genes[179]. I found that NR abrogated EC and VSMC $p16^{INK4A}$ expression induced by Ang II infusion.

The observed reduction in senescence may be due to NR-mediated preservation of genomic integrity. This is because prolonged or severe DNA damage may trigger senescence as a means to prevent the propagation of mutated DNA, which could give rise to dysfunctional proteins and cells[9]. In addition, NAD^+-driven sirtuins can prevent senescence by negatively regulating senescence-inducing proteins including p53, forkhead box protein O1, Notch intracellular domain and plasminogen activator inhibitior-1[282].

Similar to my results, Ang II-induced VSMC senescence was found to be reduced in mice administered the NAD^+-boosting compound PNU-282987[176]. NR has also ameliorated senescence in muscle stem cells from mdx-muscular-dystrophy mice and protects neural and melanocyte stem cells from senescence[108]. Furthermore, we[124] and others[283] have found cultured cells supplemented with NAD^+ precursors are protected from H_2O_2-induced senescence.

One limitation of my assessment of senescence is the sole use of $p16^{INK4A}$. p16 upregulation is known to induce cell cycle arrest, but mutations in downstream proteins responsible for executing senescence can prevent this[179]. Moreover, transient p16 upregulation has been observed during wound healing and tissue repair[284]. This may reflect the clearance of senescent cells, or a transient increase in p16 that is not associated with senescence. Using two biomarkers, such as p21 and senescence associated β-galactosidase (β-Gal), may strengthen findings. Nevertheless, Ang II has been well established to induce vascular cell senescence in the aorta, including in our previous study[110] and that of others[54]. Thus it can be more confidently concluded that cells positive

for $p16^{INK4A}$ were senescent. As well, we have previously demonstrated that NR lowers β-Gal positivity in ECs and VSMCs exposed to H_2O_2[124].

My results indicate that an abrupt increase in Ang II, followed by sustained delivery in middle-aged mice, causes vascular cell senescence. This is a pathological state that can be prevented by NR supplementation.

4.10 Limitations

There are limitations to my study. This study was only performed in male mice. Whether female mice are also susceptible to acute Ang II-induced cell death and DNA damage, and whether these can be abrogated by NR warrants specific study. In addition, NAD^+ levels within the aorta were not measured so I cannot be certain that NR augmented NAD^+ levels in ECs and VSMCs. Regarding my assessment of cell death, EthD-III-positive cells could include cells with transient but repairable cell membrane damage. In addition, the sole use of $p16^{INK4A}$ to assess senescence is a limitation. This could be overcome by using additional markers for senescence, as noted in section 4.9.

4.11 Future directions

Ang II infusion has been used experimentally for decades to induce vascular pathology. A novel and important finding of this thesis is the extent of EC death/injury following acute Ang II infusion. Future work should thoroughly investigate the mode of Ang II-induced cell death, by blocking specific death subroutines through genetic or

chemical means. Furthermore, the downstream impact of these dead cells on vascular homeostasis and their incidence during natural aging would be of interest.

The use of NR in other contexts of vascular injury, such as atherosclerosis and plaque rupture would also be interesting. In addition, comprehensive evaluation of NAD^+ consuming/synthesizing enzyme expression throughout the aorta and their change during age and Ang II should be studied. Moreover, studies of oral NR digestion/absorption are needed to inform drug administration protocols. Whether protection from Ang II-induced damage can be conferred without NR pre-treatment should also be investigated.

Translationally, administration of NR in vascular diseases associated with ROS/DNA damage/inflammation/senescence should be considered. Directly assessing DNA effects would be challenging, but may be possible in patients undergoing surgical procedures. For instance, comet assay has been performed on VSMCs taken from atherosclerotic plaques from patients undergoing endarterectomy[173]. Less invasively, others have acutely infused Ang II to humans and used plasma oxidation markers to assess efficacy of a therapeutic intervention[285]. Hypertensive emergencies are associated with endothelial cell damage[201], ROS[201] and inflamation[286]. During these events, patients visit the emergency room and receive medication to lower blood pressure. An intriguing trial would involve acute administration of NR, and collection of blood to assess oxidation, endothelial cell damage (vWF) and inflammation (soluble VCAM-1, C-reactive protein).

Pre-clinical studies of NAD^+ precursors have demonstrated potential therapeutic benefits. The results of this thesis demonstrate that vascular diseases and vascular

endpoints should be considered when designing clinical trials to evaluate the benefits of supplementation with NR and other NAD^+ precursors.

4.12 Conclusion

In conclusion, I have found that NR protected the aorta of middle-aged mice from oxidative stress-associated pathology induced by Ang II infusion. These results support my overriding hypothesis. Specifically, NR abrogated acute early cell death and DNA damage induced by infusion of Ang II. As well, improvements in genomic integrity were evident following sustained Ang II infusion. This was associated with reduced proinflammatory signaling and reduced senescence in ECs and VSMCs. Collectively, these results suggest that NR has the capacity to protect the vasculature from acute and chronic vascular insults.

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6 APPENDICES

Appendix A: Animal Use Protocol

AUP Number: 2010-244
PI Name: Pickering, Geoffrey
AUP Title: Smooth Muscle Cells and Vascular Disease

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2010-244 has been approved.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Thompson, Sharla H
on behalf of the Animal Use Subcommittee

CURRICULUM VITAE

SINA GHOREISHI

UNIVERSITY EDUCATION

Candidate for Master of Science - Medical Biophysics **Anticipated completion: August 2017**
Western University, London, ON

Bachelor of Medical Science, Honours Double Major in Medical Science and Medical Cell Biology **2014**
Western University, *London, ON*

HONOURS, SCHOLARSHIPS AND AWARDS

Western Graduate Research Scholarship **2014-2016**
CIHR Canada Graduate Scholarship – Masters **2015-2016**
Western Gold Medal for the Major in Cell Biology **2014**
Dean's Honour List **2011-2014**
Queen Elizabeth II Aiming for the Top Scholarship **2010**
Western Scholarship of Excellence **2010**
A. E. Gillis Memorial Bursary **2010**
Earth and Space Science Award **2010**

LAB EXPERIENCE

Masters in Medical Biophysics Graduate Student
Pickering Vascular Lab, Robarts Research Institute, London, ON **2014-present**

- Used published literature to design and perform novel experiments investigating the effect of the nutraceutical, nicotinamide riboside, on vascular health in mice. Manuscript for publication in preparation.
- Developed laboratory skills for mice handling/surgery, blood pressure measurement, cryosectioning, histological staining/analysis, bright-field/fluorescent/confocal microscopy, NAD^+ assay, comet assay & more

Lab Manager Assistant
Siebens-Drake Medical Research Institute, London, ON **2013**

- Assisted in immunology lab research activities, including flow cytometry, superantigen production, BCA assay and ELISA
- Maintained lab and performed administrative duties

CERTIFICATIONS AND TRAINING

Comprehensive WHMIS Training, *Western University* **2016**
Basic Animal Care and Use, *Western University* **2014**
Standard First Aid with CPR C + AED, *St. John Ambulance* **2014**
Biosafety Training, *Western University* **2013**
Radiation Safety Training, *Western University* **2013**
General Laboratory Safety and Hazardous Waste Management Training, *Western University* **2013**
Occupational Health and Safety Orientation, *Western University* **2013**
Accessibility in Service, *Western University* **2013**

Safe Campus Community, *Western University* **2013**

POSTERS AND PRESENTATIONS

Molecular Medicine Data Club **2017**
Robarts Research Institute, London, ON
- Presentation: Nicotinamide riboside protects the aorta from angiotensin II-induced pathology

Robarts Research Retreat **2016**
Robarts Research Institute, London, ON
- Poster: Nicotinamide riboside attenuates angiotensin II-induced NAD^+ decline and DNA damage

Medical Biophysics Seminar Series **2016**
St Joseph's Hospital, London, ON
- Presentation: Effect of NAD^+ boosting on vascular stress

Molecular Medicine Data Club **2015**
Robarts Research Institute, London, ON
- Presentation: Effect of a NAD^+ boosting therapy on vascular stress, in vivo

Robarts Research Retreat **2015**
Somerville House, London, ON
- Poster: Effect of nicotinamide riboside on angiotensin II–induced vascular stress

Medical Biophysics Seminar Series **2015**
St Joseph's Hospital, London, ON
- Presentation: Effect of nicotinamide riboside on angiotensin II–induced vascular stress

London Health Research Day **2015**
London Convention Centre, London, ON
- Presentation: Effect of nicotinamide riboside on angiotensin II–induced vascular stress

CURRENT VOLUNTEERISM

Outreach Volunteer **2014-present**
Let's Talk Science, London, ON
- Responsible for giving hands on demonstrations of science based activities to children in the community

Physiotherapy Buddy **2015-present**
Participation House, London, ON
- Assist disabled individual with physiotherapy routine and provide friendship