Peroxynitrite, Pumps and Perivascular Adipose Tissue: Studies Across the Physiological Spectrum.

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

Peroxynitrite, Pumps and Perivascular Adipose Tissue: Studies Across the Physiological Spectrum.

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and hereby certify that, in their opinion, it is worthy of acceptance.

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To Darcy:

I can't wait to start our new life together in Atlanta. Thank you for the love, friendship and understanding you have given me. I am very lucky to have you in my life and I love you very much.

To my family:

I would like to thank my Mother for all of her love and support, my Father for teaching me the value of hard work and for always providing solid advice no matter the situation; my brothers Jeff and David for keeping me laughing through the good and bad times. You guys are the best.

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PEROXYNITRITE, PUMPS AND PERIVASCULAR ADIPOSE TISSUE: STUDIES ACROSS THE PHYSIOLOGICAL SPECTRUM

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ABSTRACT

Peroxynitrite ($ONOO^-$) is a reactive nitrogen species produced when nitric oxide (NO) and superoxide (O_2^-) react. In vivo studies suggest that reactive oxygen species and perhaps peroxynitrite can influence Na,K-ATPase (Na pump) function. However, the direct effects of peroxynitrite on Na,K-ATPase function remain unknown. We show that a single bolus addition of peroxynitrite inhibit purified renal Na, K – ATPase activity with an IC_{50} of 107 ± 9 μM. Peroxynitrite treatment produced 3-nitrotyrosine residues on the α, β and FXYD subunits of the Na pump and also modified cysteine residues. Taken together these results show that peroxynitrite is a potent inhibitor of Na,K-ATPase activity and that peroxynitrite can induce specific amino acid modifications to the pump. We also investigated if the Na pump was a "target" of peroxynitrite in vivo under cellular conditions. Preliminary evidence suggests that LLC-PK1 cells do contain nitrated Na pumps and that tissue from sedentary high fat fed pigs contain nitrated proteins.

A denitrase activity capable of modifying 3-nitrotyrosine back to tyrosine would have implications for cell signaling/repair mechanisms as well as overall 3-nitrotyrosine levels. The red blood cell, due to its lack of nucleus and long life span, represents an ideal cell type that may or may not contain a denitrase activity. Shown here are results of just two experiments that might suggest a denitrase activity in RBCs. However, other

experiments (not shown) seemed to lack any denitrase activity and ultimately the results from all experiments neither clearly demonstrated nor ruled out an obvious denitrase activity in red blood cells.

Also, presented here is a study investigating some basic aspects of Na pump functioning. Specifically, we demonstrate that terbium is a non-competitive inhibitor of rubidium uptake suggesting it does not bind to the outside transport site of the Na pump. In contrast we show that chrysoidine competes with sodium and potassium for ATPase activity suggesting it binds to the inside transport site. Together these results support that chrysoidine, but not terbium, might be a useful probe for the transport site. We also show that the outside transport site is very specific for monovalent cations over divalent cations.

Also, presented here is a study investigating the effects of perivascular adipose tissue on coronary artery reactivity and the influence of diet and exercise. Results from this study suggest that perivascular adipose tissue blunts contraction induced by endothelin-1 in coronary arteries from normal fat and high fat fed pigs. While exercise abolished this effect normal fat fed pigs, exercise did not alter the anti-contractile effect in the high fat fed pigs.

CHAPTER 1

Introduction

Part I: Membrane Transport - A Necessity for Life

Millions of years ago in the primordial soup something remarkable occurred. Cells developed. Concurrent with this was 1) development of a partition that separated and defined the intracellular space from the extracellular space, 2) basic processes for transporting molecules across this partition and perhaps 3) an intracellular environment unique from the extracellular environment. These developments likely fostered cell growth, survival and reproduction; indeed these characteristics are still found in today's cells.

We recognize today that cells and intracellular organelles are partitioned by a bilayer membrane, composed of phospholipids, cholesterol and proteins, which defines the outer boundary of the cell and compartmentalizes the inside of the cell into discrete functional regions. The lipid nature of these membranes prevents polar compounds and ions such as Na^+, K^+, Cl^-, Ca^{2+} , $HCO3^-$, glucose and amino acids to freely enter the cell. Based on this alone one would predict to find very little if any of these compounds in the intracellular space. Yet we know that the intracellular fluid composition not only contains these molecules but sometimes in higher concentrations than the extracellular environment (see table 1.1)! For example, in mammalian cells under basal conditions, potassium is roughly 40 fold higher inside the cell relative to outside the cell. So how then does the cell accomplish this remarkable feat? The cell accomplishes this by utilizing membrane spanning transport proteins that provide an accessible path into the

cell, much like a door or window provides access into a room. Specifically, membrane spanning transport proteins provide a pathway for polar, charged solutes into the cell by bypassing the hydrophobic interior of the lipid membrane, effectively lowering the required activation energy. It is membrane transport proteins that by exquisitely regulating what enters and exits the cell, generate and maintain an internal environment unique from the external environment which is so essential for cell function and homeostasis. Indeed many diseases such as Cystic fibrosis, Liddle's Disease, Type II diabetes, Wilson's Disease, and Hartnup's Disease are caused by malfunctions in membrane transport processes.

Molecule	Intracellular (mM)	Extracellular (mM)
Na^+	12	145
K^+	155	4.0
Ca^{2+}	0.0001	1.5
Cl^-	4.0	120

Table 1.1. Intracellular and extracellular concentrations of selected solutes.

1.2 Channels and Carriers

Membrane transport proteins historically have been divided into 2 categories: channels and carriers. The defining characteristic of an open channel is a continuous membrane spanning domain that forms a selective, aqueous pore through which substances such as Na^+, Ca^{2+}, K^+ and Cl^- can pass. When channels open they allow molecules to pass through the aqueous pore region of the channel and are thus able to bypass the lipid environment of the membrane. The directional movement of solutes

through channels is determined by the electrochemical gradient for each particular solute. For example, the inward movement of sodium through sodium channels occurs because 1) extracellular sodium concentrations are ~10 fold higher than intracellular sodium concentrations and 2) the inside of the cell is negatively charged which attracts positively charged sodium ions. Channels are gated by specific stimuli such as voltage, mechanical stretch and specific ligands and when channels open they allow molecules to pass through at a rate of ≈ one million ions/second. Notably, channels generally do not have binding sites for their respective substrate.

Carriers on the other hand never form a full pore across the membrane. Instead carriers alternate between several conformations that expose a transport binding site to one side of the membrane and then to the other and in doing so selectively transport their substrate across the membrane. Because of these alternating conformational changes carriers transport their substrates at much slower rates compared to channels. Carriers, like channels, allow for facilitated diffusion but unlike channels can also support active transport. For example, glucose transporters (GLUT) allow facilitated diffusion of glucose down its concentration gradient into the cell. Active transporters on the other hand utilize energy derived from ATP to move substrates against (uphill) their concentration gradient.

Active transporters are further divided into primary and secondary transporters. Primary active transporters directly use energy from ATP hydrolysis to move substrates against (uphill) their respective gradients while secondary active transporters use energy associated with existing ion gradients (generated by the primary active transporters) to move substrates uphill. Secondary active transporters include the Na/glucose

cotransporters, Na/Ca exchangers and Na/amino acid cotransporters to name a few. Examples of primary active transporters, also known as pumps, are the H, K pump, Ca, H pump and the Na, K pump. These primary active transporters will be discussed further.

1.3 Primary Active Transport ATPases - Nature's Pumps

There are 4 classes of primary active transport ATPases: P-type, V-type, F-type and multidrug. However, only the P-type class will be considered here. The P-type ATPase superfamily of proteins is a group of membrane spanning proteins that couple the hydrolysis of ATP to the movement of cations across biological membranes. Proteins in this family use the energy released upon ATP hydrolysis to "pump" ions, against their concentration gradients, into or out of cells and cellular organelles. P-type ATPases are present across the spectrum of life from single celled archaea and bacteria to complex, multicellular eukaryotes (animals, plants, fungi). P-type ATPases can be classified into five sub-groups based upon the transported substrate (see table 1.2) (Axelson et al 1998; Okamura et al 2003). Proteins belonging to the P-type ATPases undergo a unique transient phosphorylation (hence P-type) whereby the terminal phosphate of ATP gets transferred to an aspartate (D) residue found in the conserved sequence *DKTGT* (Horisberger 2004). This sequence (*DKTGT*) is necessary for inclusion into the P type ATPase family. This inclusion criterion emphasizes the genetic code over protein function - in this case pumping ions. I am unaware of a protein that contains the DKTGT sequence and is phosphorylated but is not an active transporter. If one exists in nature this might confuse the inclusion issue. However, in my mind this protein would still deserve to be classified as a P-type ATPase even though it is not involved with active transport.

There is some president for this scenario. For example, the cystic fibrosis transmembrane regulator (CFTR) is a chloride channel but because it contains two intracellular ATP binding cassettes it actually belongs to the ATP Binding Cassette (ABC) transporter family (chen et al 2008). Thus even though CFTR is a channel it belongs in a family of active transporters.

Group	Sub-Group	Substrate	Example Protein
1	A*	K+	KDP
	B	Heavy Metals (Cu2+, Ag+, Cd2+, etc)	Menkes / Wilson
2	A	Ca2+	SERCA
	B	Ca2+	PMCA
	C	Na+, K+, H+	Na,K / H,K pump
3*		Protons, Mg2+	
4		Aminophospholipids ?	Atp8a1
5		Unknown	unknown

Table 1.2. P-Type ATPase protein family: Bacterial high affinity K transporting ATPase (KDP) ; Menkes/Wilsons copper transporters ; Sarco/endoplasmic reticulum ATPase (SERCA); Plasma membrane calcium ATPase (PMCA); Na ,K- ATPase (Na,K Pump); H,K-ATPase (Proton pump); Aminophospholipid "flippase" (Atp8a1).

P-type ATPases are the site of action of some pharmacological therapeutics. For example, digoxin and other cardiac glycosides, have been used to treat heart failure for over 200 years (Gheorghiade et al 2006). These compounds work by inhibiting the Na pump which in cardiac tissue has a positive inotropic effect. Interestingly, the Monarch butterfly and plants in the genus *Digitalis* (common name Foxgloves) contain cardiac

glycosides and if consumed in large enough quantities both the Monarch butterfly and Foxglove kill potential predators presumably by at least in part inhibiting the Na pump. Other drugs such as omeprazole (Prilosec) and rabeprazole (Aciphex) are used to treat heart burn and peptic ulcers. These drugs work by inhibiting stomach H^+, K^+ pumps.

1.4 The Na,K-ATPase

The Na,K-transporting adenosine triphosphatase (Na,K-ATPase), also referred to as the Na pump, is perhaps the most famous and most studied of the P-type ATPases. The Na pump is a membrane bound protein/enzyme that couples the hydrolysis of ATP to the movement of 3 sodium (Na^+) ions out of the cell and 2 potassium (K^+) ions into the cell. The Na pump was discovered by Jens Christain Skou who while working in Aarhus, Denmark in 1957 discovered that crab nerve membrane preparations exhibited Mg-ATPase activity that was greatly activated by the presence of sodium and potassium (Skou 1957). In 1997 Skou was awarded a Nobel Prize for his seminal discovery and lifetime of work on the Na pump. However, the existence of a Na pump had been postulated for many years prior to 1957. Working with frog muscle in 1902 Ernest Overton demonstrated that adding sodium to an isotonic solution of sucrose restored frog muscle excitability. Overton concluded that sodium not only plays an important role in muscle excitability but also that for a short time the plasma membrane becomes permeable to sodium. Further, he theorized that:

> if some sodium ions enter and some potassium ions leave during each contraction, then the differences between internal and external cation concentrations would

> gradually be levelled out unless there is some mechanism at work which opposes this equilibration. In actual fact, our muscles contain, so far as I am aware, just as much potassium and as little sodium in old age as they do in early youth (Glynn 2002).

In essence Overton had just predicted the existence of the Na pump. A remarkable prediction for its time!

Remarkably, the seemingly straightforward action of the Na pump, transporting sodium out of the cell and potassium into the cell, has many functional consequences. First, by transporting sodium ions out of the cell and potassium ions into the cell, the Na pump is directly involved with regulating/maintaining relatively low intracellular sodium (~12 mM) and high intracellular potassium (~155 mM) concentrations which in turn maintain the inwardly directed sodium gradient and outwardly directed potassium gradient. Maintaining these gradients is absolutely essential for the function of many if not all cells. For example, in excitable tissue such as muscle and nerve these gradients provide the basis for excitation. In epithelial cells such as those in the kidney, lung and intestine the inwardly directed sodium gradient allows co-transport of molecules such as glucose and amino acids. Secondly, the Na pump is electrogenic meaning it moves a +1 (3 Na^+ ions out – 2 K^+ ions in = + 1 out) net charge out of the cell and thus contributes slightly to a cell's negative resting membrane potential (Hoffman et al 1979). Thirdly, the Na pump helps maintain osmotic stability and thus regulates cell volume (Tosteson et al 1960). Fourthly, it has been shown that inhibiting pump activity leads to increases in intracellular pH and Ca^{2+} concentrations by reversing Na/H and Na/Ca exchangers respectively (Williams et al 1971; Barry et al 1985). In fact, the rise in intracellular Ca^{2+}

concentration that accompanies Na pump inhibition is the basis for over 200 years of digitalis and ouabain-like compounds for treatment in heart failure (Gheorghiade et al 2006). Lastly, the Na pump requires energy (ATP) to continue working and thus contributes to basal metabolic rate and presumably heat production. Estimates in sheep, guinea pig and human suggest that Na pump activity accounts for between ≈ 20 – 40% of total body oxygen consumption (Kelly et al 1990). However, tissue specific variations in Na pump energy expenditures exist. For example, in the kidney and brain the Na pump accounts for 50 – 70 % of energy expenditure while 30 % in G.I tract and between 5 – 10 % in liver, cardiac and skeletal muscle (Clausen et al 1991). Taken together the Na pump is very important for maintaining many aspects of cellular homeostasis.

1.5 Na pump structure and subunits

The Na pump is comprised of 3 subunits termed alpha (α), beta (β) and FXYD (see figure 1 and Horisberger et al 2004 for review). The alpha subunit is the largest of the three subunits containing about 1,000 amino acids (100 kD). It is the catalytic subunit and thus I consider it the "action" subunit. The alpha subunit contains binding sites for Na^+, K^+, $Mg^{2+,}$ ATP and cardiac glycosides such as ouabain and digitalis. The alpha subunit also contains the site for ATP hydrolysis and regulatory and catalytic phosphorylations. The alpha subunit contains 10 transmembrane spanning domains, five extracellular loops, four intracellular loops and both the N and C terminus are located intracellularly (Hu et al 2000). Based on predictions from Toyoshima's crystal structure of SERCA and the newly crystallized Na pump the alpha subunit contains three important domains termed the N (nucleotide) domain, P (phosphorylation) domain and A

(actuator) domain which are found in the intracellular loops (Toyoshima et al 2000; Sweadner et al 2001; Morth et al 2007). Both the N and P domains are found in the large cytoplasmic loop between transmembrane segments 4 and 5. The N domain contains the ATP binding pocket while the P domain contains the catalytically phosphorylatable aspartate (D376) in the conserved sequence *DKTGT*. The A domain is comprised of the cytoplasmic loop between the 2nd and 3rd membrane spanning domains and part of the N terminus. The A domain is thought to play a role in transmission of conformational changes that occur during the Na pump cycle. Four alpha isoforms (α1, α2, α3, α4) have been identified and they are expressed in a tissue specific distribution. The α1 isoform is considered the "housekeeper" because it is found in most if not all tissue types. The α2 isoform seems to be limited primarily to muscle (skeletal, smooth, cardiac) and glial cells whereas the α3 isoform is located primarily in neurons. Lastly, the α4 isoform is found in sperm.

The beta (β) subunit is comprised of about 370 amino acids, is about 55 kD and contains a single transmembrane spanning domain, a short cytoplasmic tail (≈ 30 amino acids) containing the N terminus and a larger extracellular domain containing the C terminus (Kaplan 2002). The extracellular domain contains disulfide bridges, glycosylation sites and is thought to interact with the α subunit between transmembrane segments 7 and 8. The β subunit is thought to help stabilize the α subunit and has been shown to act as a chaperon that allows proper membrane insertion and maturation of the alpha subunit (Geering et al 1989; Ackermann et al 1990; Geering et al 1996). Further, the β subunit determines some of the transport properties of the Na pump. For example, β has been shown to affect K and Na affinity as well as formation of Na-dependent

phosphoenzyme (Jaiseer et al 1994; Hasler et el 1998; Eakle et al 1995). Interestingly, of the 300 or more known P-type ATPases only the Na,K ATPase and H,K ATPase have thus far been shown to contain a β subunit. Three beta isoforms (β1, β2, β3) have been identified.

The 3rd subunit, FXYD, belongs to the FXYD (pronounced FIX-ID) family of proteins which contains seven members (Geering et al 2005). FXYD proteins span the membrane once and contain a conserved phenylalanine (F) X, Y and aspartate (D) (hence FXYD) motif in their extracellular domain. Further, they contain 2 conserved glycine sites and 1 serine site. FXYD2 (also known as γ) was the first FXYD protein demonstrated to interact and alter Na pump activity (Forebush et al 1978; Mercer et al 1993). More recently, FXYD 1, 3, 4 and 7 also have been shown to interact and affect Na pump activity (Beguin et al 2001, Beguin et al 2002, Crambert et al 2002, Crambert et al 2005). In general these proteins have been shown to effect Na pump activity by altering Na, K and ATP affinity. Further, FXYD proteins are located in a tissue specific manner (Geering et al 2003). Taken together, current dogma suggests that the various FXYD proteins regulate Na pump activity in a tissue specific manner.

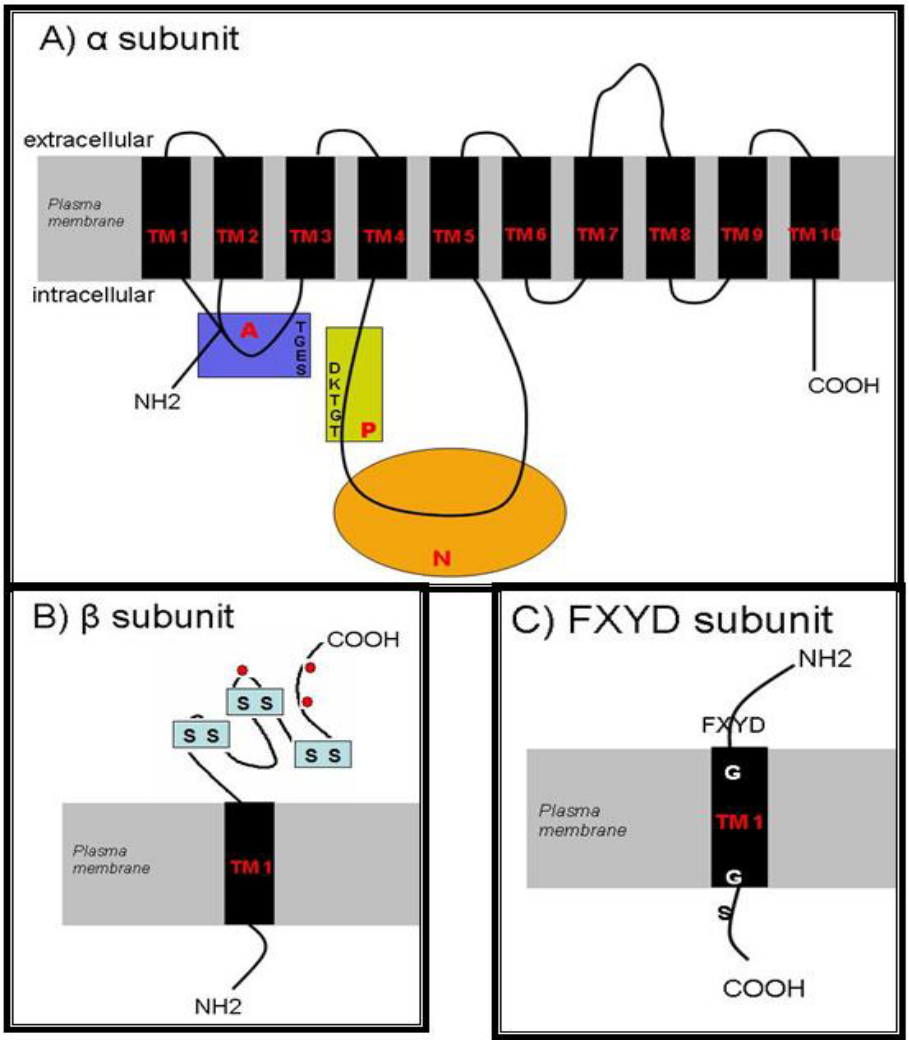

Figure 1.1 Diagram depicting α, β and FXYD subunits of the Na pump. (A – top) the alpha subunit contains 10 transmembrane spanning segments (TM1-10), an actuator (A) domain, phosphorylation (P) domain and nucleotide (N) domain. ATP binds to the N domain, upon hydrolysis an aspartate residue in the P domain becomes phosphorylated. (B – Bottom left) The beta subunit contains one transmembrane spanning domain and contains multiple disulphide bridges and glycosylation sties. (C-bottom right) The FXYD subunit has one transmembrane spanning domain and a conserved FXYD motif in the etracellular domain. This figure is adapted from Horsiberger et al.

1.6 How does the Na pump pump? The Na pump enzymatic cycle

Understanding exactly how the Na pump transports sodium and potassium in opposite directions at the expense of ATP has been a challenge. However, 40 years of experiments have shed considerable light on this subject. One current model of the Na pump enzymatic cycle is the Post-Albers model (Albers 1967**,** Post et al 1972) which accommodates many of the observed experimental kinetic and biochemical data and links ATP hydrolysis to the transport of sodium and potassium. The Post-Ablers model suggests the Na pump cycles through two different conformations historically and commonly referred to as E1 and E2. Several differences between the E1 and E2 conformations exist in regard to intrinsic fluorescence, substrate affinities, directionality and proteolysis. Typically the E1 conformation is defined as exhibiting a high affinity for sodium and ATP, low affinity for potassium and inward facing. The E2 conformation exhibits low affinity for sodium and ATP, high affinity for potassium and outward facing. However, the exact definition of E1 and E2 to a certain degree is ambiguous and differs depending on the exact conditions. For me, the E1 and E2 nomenclature, while initially confusing, has helped understand the enzymatic cycle and accompanying pump conformations described below. It will be interesting to see if this nomenclature endures as crystal structures of the Na pump become available. This nomenclature is still commonly used with SERCA which was originally crystallized in 2000. Needless to say at this moment and into the foreseeable future the E1, E2 nomenclature will be commonly used to describe the Na pump reaction cycle. The Na pump reaction cycle proceeds as follows (see figure 1.2): with ATP bound to the N domain (from previous cycle) three intracellular sodium ions bind to the inward facing transport site forming

E1ATP3Na. ATP is hydrolyzed, ADP is released and the terminal phosphate is transferred to D376 altering the pump conformation such that sodium becomes occluded forming E1P(3Na). Another conformation change occurs exposing the transport site to the outside which allows sodium to be released to the outside. Next, 2 potassium ions bind to the outside transport site forming E2P2K. The phosphate on D376 is released intracellularly which allows potassium to become occluded forming E2(2K). A new molecule of ATP binds to the N domain forming E2ATP(2K). ATP binding stimulates the Na pump to face inward once again and release potassium to the inside. At this stage the cycle is complete and the pump is in the E1ATP conformation ready to accept sodium again.

One remarkable aspect of the Na pump is the ability of its extracellular binding site to select potassium over sodium despite 1) the remarkable similarity between sodium and potassium and 2) there being roughly 40 fold more sodium than potassium in the extracellular space. Interestingly, the inside transport site exhibits the exact opposite selectivity. That is the transport site selects intracellular sodium over potassium despite there being approximately 10 fold more potassium than sodium in the intracellular space. The transport site is able to discriminate monovalent cations from similarly sized divalent cations. Exactly, how the transport site does this is a puzzle. In chapter 6 I present a paper looking at selectivity of the outside transport site as well as characterizing a potential probe for the transport site that could shed light on these selectivity issues.

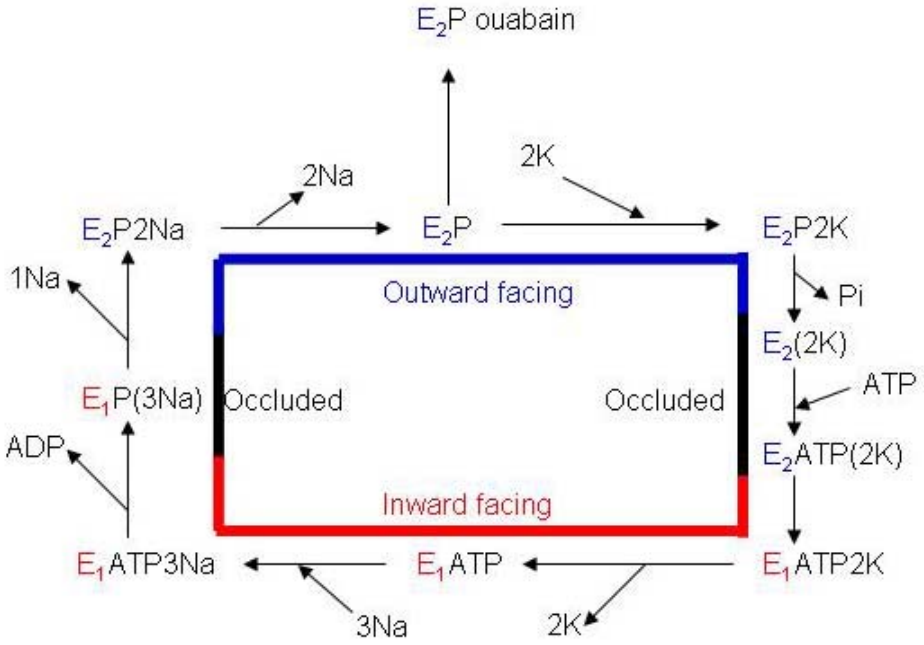

Figure 1.2. Na pump reaction cycle. The transport site is either in the E1 (inwardly facing – red), E2 (outwardly facing – blue), or occluded (black) conformation as indicated on the inside of the box. Shown on the outside is the enzymatic cycle of the Na pump. Interestingly, It has been shown that the Na pump can run "backwards" generating ATP. However, this does not occur under physiological conditions (Lew et al 1970).

1.7 Na pump crystal structure

In December of 2007 the Nissen group reported the first crystal structure of the Na pump (Morth et al 2007). While other P-type proteins have been previously crystallized, (notably SERCA, see Toyoshima et al 2000), this is significant because it is the first reported crystal structure of the Na pump. Using Rb^+ as a K^+ analog, and MgF_4^{2-} as a phosphate analog, Morth et al were able to crystallize the Na pump (including all three subunits) in the occluded E2 conformation. Specifically the $[Rb_2]E2 \cdot MgF_4^2$ conformation. The Na pump crystal structure confirmed the suspected high degree of homology with SERCA including in the cation transport site. This will certainly lead to more research investigating how cation selectivity is achieved. It also provided insight into how the α, β and FXYD subunits complex with each other. It will be interesting to see to what extent, if any, the orientation of the β and FXYD subunits change with respect to the α conformation, especially considering biochemical data suggest that both of these subunits can influence sodium and potassium affinities.

Part II: Reactive oxygen species: A Consequence of Aerobic Respiration

Perhaps around the same time cells in the primordial soup were developing increasingly complex membrane transport processes they also developed the ability to use molecular oxygen for respiration and metabolism. In other words they became aerobic. This was beneficial because it greatly increased the amount of energy (ATP) derived from nutrients such as glucose. For example, during anaerobic metabolism (glycolysis) a net gain of two ATP molecules (14.6 Kcal/mol) are generated from the breakdown of one glucose molecule into two pyruvate molecules. During aerobic metabolism, which requires oxygen as the final electron acceptor, a net gain of 36 ATP molecules (262.8 Kcal/mol) is generated from the complete oxidation of glucose. The increased ATP production that aerobic metabolism provided likely fueled an increasingly complex and diverse set of reactions. Among them, perhaps, was the Na, K-ATPase which, as previously aforementioned, can use ~ 50% of total energy in certain cells (Kelly et al 1990; Clausen et al 1991). However, using oxygen for respiration and metabolism also allowed production of reactive oxygen species (ROS) and has had some interesting consequences.

Reactive oxygen species are metabolites of molecular oxygen (O_2) that exhibit a higher reactivity than oxygen. ROS includes unstable oxygen radicals such as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), and nonradical molecules like hydrogen peroxide (H_2O_2) (see figure 3). ROS generation is the consequence of using molecular oxygen as a substrate for enzymes such as such as NADPH oxidase, xanthine oxidase and nitric oxide synthase and as the final electron acceptor in the electron transport chain.

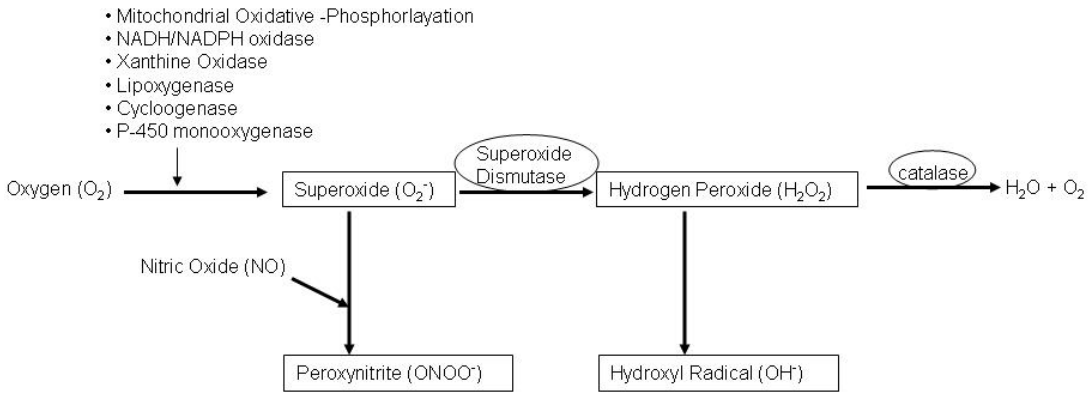

Figure 1.3. Diagram of common reactive oxygen species (shown in squares) and antioxidant enzymes (shown in circles).

ROS have the ability to oxidize biological macromolecules such as DNA, lipids and proteins (Balasubramanian 1998; Alvarez et al 2003; Keyer et al 1996; Radi et al 1991). As a consequence, ROS have traditionally been considered solely as unwanted byproducts of aerobic respiration. Indeed there is plenty of evidence in the literature that suggest ROS contribute to the pathophysiology that accompanies cancer, inflammation, diabetes, cardiovascular diseases, neurodegenerative diseases, and aging (Ames et al 2003; Andreassi 2003; Butterfield et al 2001; Golden et al 2002; Nathan 2002; Rao et al 2002; Taniyama et al 2003). Interestingly, accumulating evidence also suggests that beyond their damaging effects ROS are also involved in cell signaling (Rhee 2006, D'Autreaux et al 2007, Nathan 2003). This area will be discussed further in chapter 7.

One area in which ROS could either directly act as signaling molecules or influence other signaling pathways is in the vasculature. In chapter 5 I report on the effects of perivascular adipose tissue on coronary artery reactivity. This is work I

completed during a 5 month rotation in Dr. Harold Laughlin's lab. In chapter 5 we report that the presence of perivascular adipose blunts agonist-induced constriction of coronary arteries. This finding, which has also been observed in other vascular beds, has lead some to speculate on the existence of an adipose derived relaxing factor (ADRF) (Gollasch et al 2004; Verlohren et al 2004). The specific chemical nature of ADRF is unknown, and we did not specifically explore the mechanism accounting for the blunted constriction. However, one possibility is that ADRF is really a ROS/RNS; this intriguing possibility will be further discussed in chapter 7.

2.2 Peroxynitrite is a reactive nitrogen species derived from superoxide and nitric oxide

Superoxide (O2-) is the "gateway" ROS molecule that can ultimately lead to the generation of other ROS and reactive nitrogen species (RNS). As shown in figure 1.3 superoxide is generated by the partial reduction of molecular oxygen. This can happen either enzymatically by oxidases such as NADH/NADPH, xanthine oxidase, nitric oxide synthase and cycologenase or non-enzymatically via the electron transport chain in mitochondria. (Tanyama et al 2003; Wolin et al 2005; Stuehr et al 2001; Brand et al 2004)

One fate of superoxide (O_2^-) is to react with nitric oxide (NO) forming the reactive nitrogen species peroxynitrite ($ONOO^-$) (see Szabó et al 2007**;** pacher et al 2007; Beckman et al 1996 for reviews). Besides producing peroxynitrite this reaction also decreases the bioavailability of nitric oxide which could be deleterious in and of itself. The non-enzymatic reaction between superoxide and nitric oxide is rapid and has been

estimated to occur at near diffusion-limited rates ($1x10^{10}$ M/sec) (Pacher et al 2007; Koppenol et al 1992). In fact peroxynitrite production *in vivo* in specific compartments has been estimated to be as high as 50–100 μM per minute (Szabo et al 2007; Alvarez et al 2004). Peroxynitrite is short lived (≈ 10 ms) at physiological pH but peroxynitrite has been shown to diffuse through cell membranes suggesting that it can affect neighboring cells and molecules (Denicola et al 1998).

The short life of peroxynitrite makes it difficult if not impossible to measure in vivo in real time. However, one documented effect of peroxynitrite is to react with tyrosine residues producing 3-nitrotyrosine residues (see figure 4) (Pacher et al 2007). Interestingly, elevated levels of 3-nitrotyrosine have been shown to accompany several disease states and conditions including cardiovascular diseases, ischemia-repurfusion injuries, sickle cell disease, hyperglycemia and neurological disorders (Viappiani et a; 2006; Quijano et al 2007; walker 2006; Kooy et al 1997; Reynolds et al 2007; Aslan et al 2003). This has been widely interpreted as suggesting that peroxynitrite levels are elevated in these conditions and that 3-nitrotyrosine levels serve as a gauge for peroxynitrite levels. However, it should be noted that other mechanisms exist for generating 3-nitrotyrosine. For example, peroxidases (horseradish peroxidase, myeloperoxidase, lactoperoxidase) in the presence of nitrite (NO2-), a stable byproduct of NO metabolism and hydrogen peroxide, can form 3-nitrotyrosine (Eiserich et al 1998; van der Vliet 1997). The literature typically downplays this latter mechanism of generating 3-nitrotyrosine and instead focuses on peroxynitrite induced generation of 3-nitrotyrosine. However, from my reading of the literature it is really not known to what extent each mechanism contributes to the total pool of 3-nitrotyrosine nitration.

Moreover, this is a particularly difficult issue to address because both peroxynitrite and nitrite are byproducts of nitric oxide metabolism. If peroxidases in the presence of nitrite leads to 3-nitrotyrosine, as the literature suggests, perhaps creating a peroxidase knockout and looking for changes in 3-nitrotyrosine levels would shed light on the contribution of this mechanism to overall 3-nitrotyrosine levels.

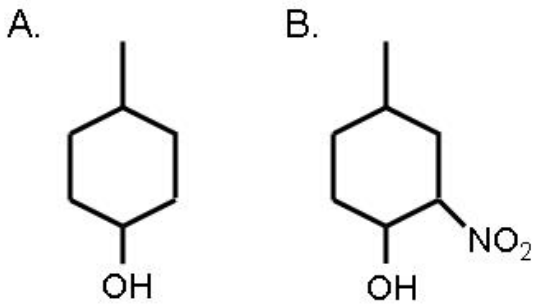

Figure 1.4. (A-left) tyrosine; (B-right) 3-nitrotyrosine

2.3 Effects of peroxynitrite on protein function and signaling

As with other ROS and RNS, considerable attention has been given to understanding how and to what extent peroxynitrite is able to effect protein structure and function and influence signaling pathways (Pacher et al 2007; Klotz et al 2002; Ferdinandy 2006). For example, it has been shown that peroxynitrite is a potent inhibitor of the antioxidant enzyme superoxide dismutase (SOD) and that this inhibition is the result of exclusive nitration of tyrosine 34 (Yamakura et al 1998; MacMillan-Crow et al 1998). Inhibiting SOD activity could have interesting implications for peroxynitrite concentrations. For example, inhibiting SOD activity could lead to elevated superoxide

levels which in turn could lead to elevated peroxynitrite levels, especially if nitric oxide was elevated. Elevated peroxynitrite could in turn inhibit more SOD, leading to yet higher superoxide levels and peroxynitrite levels. In this way inhibiting SOD activity produces a feed forward mechanism that might contribute to elevated peroxynitrite levels. One way to test this theory could be to incubate cells with a membrane permeant SOD inhibitor (if one exists) and measure resulting 3-nitrotyrosine levels. Interestingly, Xu et al have shown that SOD nitration is elevated with aging and in cardiovascular disease (Xu et al 2006).

One way in which peroxynitrite influences protein function and signaling pathways, is by reacting with tyrosine to form 3-nitrotyrosine. 3-nitrotyrosine can inhibit or mimic regulatory tyrosine phosphorylations or alter protein-protein interactions (Gow et al 1996; Kang et al 2007). All of which can alter protein function, activity and/or trafficking. Interestingly, a denitrase activity capable of converting 3-nitrotyrosine back to native tyrosine and thus analogous to phosphatase activity has been reported further suggesting a possible role of peroxynitrite and 3-nitrotyrosine in signaling (Smallwood et al 2007). If the reported denitrase activity can be verified and shown to be enzyme mediated this would suggest that addition and removal of NO_2 groups onto tyrosine residues is regulated. With this in mind, in chapter 4 I present data from experiments that attempt to detect a denitrase activity in red blood cells. We chose red blood cells to look for a denitrase activity because of their long life time (~ 120 days) and because they circulate throughout the vasculature. As a result they might be constantly exposed to low levels of peroxynitrite and therefore might accumulate 3-nitrotyrosine, in much the same

manner as RBCs accumulate glycoslated hemoglobin. As such we thought red blood cells might be an ideal cell type to look for a denitrase activity.

It has also been shown that SERCA nitration is elevated with aging and cardiovascular disease prompting some to suggest that SERCA is a cellular "sensor" of reactive nitrogen species (Xu et al 2006; Bigelow 2008). However, in my opinion the more proteins found to contain nitrated tyrosine residues the less likely any specific one protein acts as a sole "sensor" of RNS. Regardless, there has been interest in understanding the effects of peroxynitrite on SERCA activity and function in physiological and pathological states (Adachi et al 2004; Grover et al 2003; Gutiérrez-Martín et al 2004). Evidence presented by Adachi et al suggests that peroxynitrite in the presence of glutathione forms an S-glutathiolation (GSS) modification at cysteine residue 674 which increases SERCA activity. Mutating cys 674 to serine abolished this effect. Interestingly, during atherosclerosis, cys 674 was shown to be irreversibly modified to sulfonic acid. This modification to sulfonic acid prevented the regulatory S-glutathiolation from occurring and thus prevented the s-glutathiolation induced SERCA activation. Incidentally, sulfonic acid is a ROS induced modification. In effect Adachi et al have identified "a functional thiol switch that normally regulates protein function by reversible S-glutathiolation, but is prevented from doing so by irreversible thiol oxidation in the setting of chronic exposure to oxidants." (Adachi et al 2004). This mechanism of regulation could occur during pathological conditions leading to altered intracellular calcium homeostasis and therefore dramatically impact cell function.

Very little is known about the effects peroxynitrite has on the Na pump. The work on SERCA along with evidence suggesting the Na pump is affected by ROS, and perhaps

peroxynitrite, in some cell types prompted me to directly test the effects of peroxynitrite on purified Na pump (Comellas et al 2006; White 2008; Zhang 2002). In Chapter 2 I present the findings from these experiments investigating the effects of peroxynitrite on purified renal Na,K-ATPase activity. I also examine some of the resulting peroxynitrite induced amino acid modifications and address to what extent these modifications account for the loss of ATPase activity. In chapter 3 I present data from preliminary experiments where I attempted to detect nitrated Na pumps from LLC-PK1 cells and from tissue samples from a sedentary Yucatan pig fed a high fat diet. These studies were performed because they would indicate if the Na pump was a "target" of peroxynitrite in vivo.

In summary, membrane transport proteins like the Na pump are vitally important in maintaining cellular homeostasis and anything that alters their ability to properly function can seriously compromise cellular health. It is being increasingly recognized that ROS/RNS can alter protein function, including membrane transport proteins, and cell signaling pathways that can in turn contribute to cell dysfunction. Peroxynitrite is one such RNS. Levels of 3-nitrotyrosine, a modification induced by peroxynitrite, are elevated in number of different conditions including cardiovascular diseases, ischemia-repurfusion injuries, sickle cell disease, hyperglycemia and neurological disorders suggesting that peroxynitrite is being generated and reacting with proteins (Viappiani et a; 2006; Quijano et al 2007; walker 2006; Kooy et al 1997; Reynolds et al 2007; Aslan et al 2003). The effects of peroxynitrite on purified Na pump activity are reported in chapters 2 and 3. In chapter 4 I present data on a possible denitrase activity in red blood cells and in chapter 5 I present data investigating the role of perivascular adipose tissue in porcine coronary artery reactivity. In chapter 6 I present data on some basic functional

aspects of the Na pump including possible probes for the Na pump transport site and on selectivity of the outside transport site for monovalent cations over divalent cations. Lastly, in chapter 7 I discuss recurring themes of my work, future experiments and mechanisms whereby ROS and RNS could act as signaling molecules.

CHAPTER 2

The Reactive Nitrogen Species Peroxynitrite is a Potent Inhibitor of Renal Na,K-ATPase Activity

Matthew S. Reifenberger, Krista L. Arnett, Craig Gatto and Mark A. Milanick. The Reactive Nitrogen Species Peroxynitrite is a Potent Inhibitor of Renal Na,K-ATPase Activity**.** Submitted to *AJP-Renal* May 6th 2008**.**

ABSTRACT

Peroxynitrite (ONOO) is a reactive nitrogen species produced when nitric oxide (NO) and superoxide react. In vivo studies suggest that reactive oxygen species and perhaps peroxynitrite can influence Na,K-ATPase function. However, the direct effects of peroxynitrite on Na,K-ATPase function remain unknown. We show that a single bolus addition of peroxynitrite inhibited purified renal Na, K – ATPase activity with an IC_{50} of 107 ± 9 μM. To mimic cellular/physiological production of peroxynitrite a syringe pump was used to slowly release (≈ 0.85 μM / second) peroxynitrite. This treatment was similarly effective at inhibiting Na,K-ATPase activity as a single bolus addition of equal cumulative concentration. Peroxynitrite treatment produced 3-nitrotyrosine residues on the α, β and FXYD subunits of the Na pump. Interestingly, the flavonoid epicatechin, which prevented tyrosine nitration was unable to blunt peroxynitrite induced ATPase inhibition suggesting that tyrosine nitration is not required for inhibition. Peroxynitrite lead to a decrease in iodoacetamidofluorescein labeling implying cysteine modifications

were induced. Glutathione was unable to reverse ATPase inhibition. The presence of Na^+ and low Mg ATP during peroxynitrite treatment increased the IC_{50} to 145 ± 10 μM while the presence of K^+ and low Mg ATP increased the IC_{50} to 255 ± 13 μM. These results suggest that the E-PNa conformation of the pump is slightly more sensitive to peroxynitrite than the E (K) conformation. Taken together these results show that peroxynitrite is a potent inhibitor of Na,K-ATPase activity and that peroxynitrite can induce amino acid modifications to the pump.

INTRODUCTION

The Na,K-ATPase, also known as the Na pump, is an integral membrane enzyme that couples the hydrolysis of ATP to the movement of 3 Na^+ ions to the extracellular space and 2 K^+ ions to the intracellular space (Horisberger 2004; Kaplan 2002 for reviews). The Na pump plays a key role in renal cell homeostasis by maintaining the electrochemical gradients for both Na^+ and K^+ which in turn contributes to the maintenance of cell membrane potential, cell pH, cell volume and Na^+-coupled transport of amino acids and glucose. The Na pump contains three subunits alpha (α), beta (β) and FXYD (Morth et al 2007). The α subunit contains the ATP binding site, cationic transport site and ouabain binding site. The β subunit helps in trafficking and stabilization while the FXYD subunit modifies Na^+ and K^+ affinities in a tissue specific manner (Geering K 2006; Garty et al 2006; Arystarkhova et al 2007).

The Na pump undergoes a coordinated reaction sequence currently described by the Post-Albers model which suggests the Na pump cycles through different

conformations commonly referred to as E1 and E2 (Albers 1967; Post et al 1969; Jorgensen et al 2003; Skou et al 1992). Whereas several definitions of E1 and E2 exist we use the following definitions: the E1 conformation exhibits a high affinity for Na^+ and ATP, low affinity for K^+ and the transport site is inwardly facing while the E2 conformation exhibits low affinity for Na^+ and ATP, high affinity for K^+ and the transport site is outward facing. The Na pump reaction cycle proceeds as follows: with ATP bound to the nucleotide binding (N) domain from the previous cycle, 3 Na^+ ions bind to the transport site, ATP is hydrolyzed and the terminal phosphate is transferred to aspartic acid residue 376. This forms the E1PNa conformation. The Na^+ ions are then released and 2 K^+ ions bind to the transport site and become occluded forming the E(K) conformation. The phosphate on D376 is released which allows a new molecule of ATP to bind and the K^+ ions to then be released. With a new molecule of ATP bound and K^+ gone, the cycle starts over again.

The Na pump, besides regulating cell Na^+ and cell volume, also may play a role in signaling (Pierre et al 2006; Xie et al 2003). For many years it has been appreciated that alterations in cell Na^+ alter cell Ca^{2+} because of the influence of cell Na^+ on the Na-Ca exchanger (Blaustein et al 1999; Reuter et al 2002). More recently, it has been suggested that ouabain and potential endogenous ouabain-like compounds can alter cell signaling (Schoner et al 2007; Smith 1988; Xie 2003).

Elevated production of reactive oxygen and nitrogen species (ROS and RNS respectively) are increasingly recognized as contributing to cell dysfunction in a variety of disease states by inducing oxidative damage to cell macromolecules such as lipids, DNA and proteins (Pacher et al 2007; Radi et al 1991; Keyer et al 1996; Alvarez et al

2003). Peroxynitrite is a damaging reactive nitrogen species produced in vivo when nitric oxide (NO) and superoxide react (see Beckman et al 1996; Szabo et al 2007; Pacher et al 2007 for reviews). The reaction rate between nitric oxide and superoxide is nearly diffusion limited which is 3-4 fold higher than the reaction rate between superoxide and superoxide dismutase (SOD) (Ferdinandy et al 2003; Pacher et al 2007; Radi 2004). Thus, even if the NO concentration is as low as the SOD concentration, peroxynitrite is likely to form even in the presence of SOD.

The short half life (< 1s) and reactivity of peroxynitrite at pH 7.4 makes it difficult to measure in vivo (Denicola et al 1998; Pacher et al 2007; Koppenol et al 1992). However, peroxynitrite can react with tyrosine residues to generate 3-nitrotyrosine which is stable. 3-nitrotyrosine levels are elevated in a number of conditions including cardiovascular diseases, hyperglycemia and renal ischemic-reperfusion injuries (Viappiani et al 2006; Quijano et al 2007; Walker et al 2000). The presence of 3-nitrotyrosine in studies has been used to conclude that peroxynitrite is produced and that it modifies some proteins. However, it should be noted that another mechanism could account for elevations in 3-nitrotyrosine. For example, myeloperoxidase and lactoperoxidas, enzymes found in neutrophils and monocytes, have been reported to produce nitrating agents (H_2O_2, NO_2^-) that in turn produce 3-nitrotyrosine (Gaut et al 2002; Van der Vliet et al 1997).

The effect of peroxynitrite on protein structure and function is of interest because of the potential to impact cell behavior and contribute to disease processes. For example, Gutierrez-Martin et al have shown that peroxynitrite inhibits the sarco-endoplasmic Ca ATPase (SERCA) which could lead to pathological increases in cell Ca^{2+} (Gutierrez-

Martin et al 2004). SERCA shares a high degree of sequence and structural homology to the alpha subunit of the Na pump (Morth et al 2007; Sweadner et al 2001). Interestingly, alterations to the Na pump also can influence cell Ca^{2+} because the Na pump regulates cell Na^{+} which in turn regulates the Na-Ca exchanger (Blaustein et al 1999; Reuter et al 2002; Schoner et al 2007; Smith et al 1988; Xie 2003).

Several lines of evidence in different tissue types, including the kidney, suggest that ROS and perhaps peroxynitrite play a role in regulating Na,K-ATPase activity and function (White et al 2008; Comellas et al 2006; Zhang et al 2002). It is likely that peroxynitrite is produced in kidney cells since they have been reported to produce both nitric oxide and superoxide (Herrera et al 2005; Geiszt et al 2000; Li et al 2002; Traylor et al 1997; Walker et al 2000; Zhang et al 2002). Zhang et al show that nanomolar concentrations of angiotensin II in the proximal renal tubule inhibit Na,K ATPase activity compared to picomolar concentrations and that this inhibition was abolished in the presence of superoxide and peroxynitrite scavengers implying that superoxide and/or peroxynitrite were responsible for the inhibition (Zhang et al 2002). Further, they show that angiotensin II treatment increased total levels of 3-nitrotyrosine suggesting that peroxynitrite levels were elevated. However, the direct effects of peroxynitrite on Na pump activity and function were not tested.

Zolotarjova et al and Huang et al have shown that superoxide, hydrogen peroxide and hydroxyl radical irreversibly inhibit ATPase activity of purified renal Na pump and that these ROS could induce fragmentation and crosslinking of the Na pump (Zolotarjova et al 1994; Huang et al 1992). Further, they showed that oxidized Na pump was more

susceptible to proteolytic degradation by trypsin and chymotrpysin. However, they did not investigate peroxynitrite.

The exact consequence of peroxynitrite on renal Na pump activity and function remain unknown but could have important and detrimental ramifications. For example, peroxynitrite (and tyrosine nitration) could influence ATPase activity and thus renal Na^+ transport. Peroxynitrite also could alter Na pump ubiquitination, resulting in degradation by inducing damage (unfolding) of the pump. In support of this, Comellas et al have shown that hypoxia induced ROS induced ubiquitin mediated degradation of the Na pump in A549 cells and alveolar epithelial type II cells (Comellas et al 2006). Lastly, peroxynitrite might alter proper trafficking of the Na pump by nitrating tyrosine residues thought to play a role in trafficking. For example, peroxynitrite could alter Na pump trafficking by nitrating Y-10 and thus preventing or mimicking Y-10 phosphorylation (Caversasio et al 1999; Feraille et al 1999).

Because previous studies at the cellular level have shown that ROS and perhaps peroxynitrite might be involved in regulating the Na pump and studies on purified Na pump have shown that the pump is sensitive to superoxide, hyroxyl radical and hydrogen peroxide we investigated the functional effects of peroxynitrite, a reactive nitrogen species, on purified renal Na pump activity. Our results show that peroxynitrite is a potent inhibitor of Na,K-ATPase activity and that administration of a single bolus addition of concentrated peroxynitrite inhibits Na,K ATPase activity to a similar extent as administration of a constant, low concentration of peroxynitrite. We show that Na pump conformation has an influence, though small, on peroxynitrite induced inhibition. Further, we provide evidence that peroxynitrite modifies tyrosine and cysteine residues of the Na

pump. We present evidence that the presence of epicatechin, a flavonoid which prevents nitration, did not blunt peroxynitrite induced ATPase inhibition and that the endogenous redox regulator glutathione was unable to reverse peroxynitrite induced ATPase inhibition.

METHODS

Peroxynitrite reactivity and concentration

Peroxynitrite (Calbiochem catalog # 516620) was aliquoted under nitrogen gas and stored at – 70° C. Immediately prior to use, an aliquot of peroxynitrite was thawed and 2.5 μLs of peroxynitrite were diluted into 1mL of 5 M NaOH, the absorbance measured at 302 nm (A_λ) and the concentration was calculated using the molar extinction coefficient of peroxynitrite (1670 cm^{-1} M^{-1} (ε)).

To verify further that peroxynitrite exhibited the appropriate ROS activity we incubated peroxynitrite with aminophenyl fluorescein (APF), a ROS fluorescent probe. APF is non-fluorescent until it reacts with a ROS, particularly peroxynitrite or hydoxl radical. As expected, incubating APF with increasing concentrations of fresh peroxynitrite caused a dose-dependent increase in APF fluorescence (data not shown). However, there was very little, if any, fluorescence when APF was incubated with increasing concentrations of decomposed peroxynitrite (data not shown). Peroxynitrite was decomposed by storing it at room temperature and exposing it to both light and the atmosphere for a minimum of 48 hours.

Peroxynitrite treatment

Purified renal Na pump was prepared by following the Jorgenson technique with slight modifications (Gatto et al 2005; Jorgensen 1974). 10-40 ug of purified Na pump in 100 mM TRIS-HCl pH 7.4 was treated with either fresh or decomposed peroxynitrite administered as a single, bolus addition or with a syringe pump that slowly released perxoynitrite. Treatments were performed at 37° C. For single bolus additions a pipette was used to dispense peroxynitrite. For the constant, slow release of peroxynitrite, a Harvard Apparatus Pico Plus syringe pump was set to release peroxynitrite at a rate of 0.85 μM / minute for 18 minutes. Syringe pump parameters (0.85 μM / second for 18 min) were chosen because if peroxynitrite could accumulate it would be equivalent to the single bolus concentration used in the paired condition. In a separate experiment, the release rate of the syringe pump was validated by measuring the total volume of colored water released over a given period of time. When indicated, purified renal Na pump was placed into either the EP Na or E(K) conformations by incubating in the presence of 130 mM Na^+, 50 μM Mg ATP or 30 mM K^+, 50 μM Mg ATP respectively during peroxynitrite treatment. Also, when indicated, reduced glutathione (GSH) (Acros Organics) was present during the peroxynitrite treatment or was introduced only after peroxynitrite treatment for 30 minutes.

Na,K-ATPase activity

Ouabain sensitive Na, K – ATPase activity was measured via a previously described ascorbic acid-molybdate colometric assay capable of detecting inorganic phosphate (Gatto et al 1997; Brotherus et al 1981). Briefly, 1 – 4 ug of non-treated or

peroxynitrite treated Na pump was diluted into ~ 600 - 700 uL of assay media and incubated for 10 minutes at 37°C. The assay media contained ATP, NaCl, KCl and MgCl (see figure legends for concentrations). After the 10 minute ATPase incubation period the ascorbic acid-molybdate colorimetric assay was performed. For each experiment an ouabain condition was included in which the Na pump ATPase reaction was done in the presence of 1 mM ouabain. The resulting background value was subtracted from all other experimental conditions which yielded ouabain sensitive Na,K ATPase activity. Further, for all ATPase experiments, each condition was performed in quadruplicate and data were normalized to the activity in the absence of peroxynitrite.

Iodoacetamidofluorescein (IAF) labeling of free thiol groups

40 ug of purified renal Na pump in 300 uL of 200 mM Tris-HCl pH7.4 was incubated for 5 minutes at 37° C in the presence or absence of fresh or decomposed peroxynitrite. Samples were then incubated with 0.5% SDS for 15 minutes at room temperature and then 500 μM IAF for 30 minutes in the dark at room temperature. Cysteine (5 mM) was then added to quench any non-reacted IAF.

SDS PAGE/ Western

Na pump samples were incubated with Lane Marker Reducing Sample buffer (Pierce product # 3900) at 1 volume sample buffer to 4 volumes protein sample. Equal protein concentrations from each sample were then loaded into 4-20% Tris-HCl Ready Gels (Biorad catalog # 161-1159) and run for approximately 40 minutes at 200 V. A 12 uL volume of Precision Plus Protein Dual Color Standards (BioRad catalog # 161-0374)

were used for molecular weight markers. Gel running buffer consisted of 1x Tris/glycine/SDS buffer (BioRad catalog # 161 0732).

Protein was then transferred from the gel to Immobilon PVDF, 0.45 μM pore size transfer membrane (Millipore IPVH10100) in 100 mM CAPS/10% MeOH pH 11.0 (Matsudaira et al 1987). The protein transfer took place over 1 hour 15 minutes at 150 mA. The membrane was then blocked for 1 hour at room temperature with Superblock Dry Blend (TBS) Blocking Buffer (Pierce product # 37545).

3-nitrotyrosine residues were detected using 1 mg/mL rabbit anti-nitrotyrosine (Molecular Probes a21285) diluted 1:10,000 into 1x TBS with 0.05% Tween 20 (Fisher). IAF labeled thiol groups were detected using 1 mg/ml mouse anti-fluorescein / Oregon Green (molecular probes A6421) diluted 1:5,000 into 1x TBS with 0.05% Tween 20. Membranes were incubated with the appropriate primary antibody for 1 hour at room temperature and then washed five times. Each wash was five minutes and was done using fresh 1xTBS with 0.05 % Tween 20. Membranes were then incubated for 1 hour with goat anti-rabbit or goat anti-mouse HRP secondary antibody (Pierce product # 31460 and #31430 respectively) diluted 1:50,000 into 1x TBS with 0.05% Tween 20 and again washed five times, five minutes each, with fresh 1xTBS with 0.05 % Tween 20. Membranes were then incubated for 5 min with SuperSignal West Pico Chemiluminescent Substrate: 5 mL each of Super Signal West Pico Stable Peroxide Solution and the Luminol/Enhancer solution (Pierce, Thermo Scientific product # 34080). The membrane was placed in a plastic sheet cover, and excess Pico West Solution was wiped away. Classic Blue Autoradiography Film (Midsci) was exposed to the membrane and developed with an AFP Mini-Medical X-Ray Film Processor.

Methods for statistical analysis

All values are reported as means ± standard deviations. When appropriate a Students t-Test was used to determine significance between groups. A P value < 0.05 was considered significant.

RESULTS

Figure 2.1 shows that single bolus additions of fresh peroxynitrite decreased Na pump ATPase activity with an IC_{50} of 100 μM. On the other hand, single bolus additions of decomposed peroxynitrite had a minimal effect ($IC_{50} > 4$ mM).

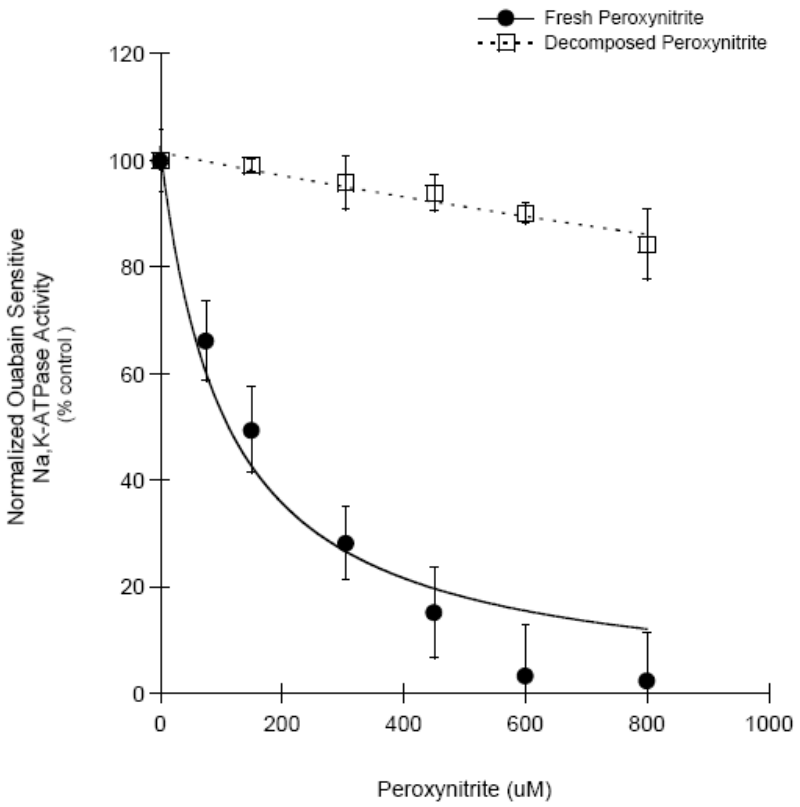

Figure 2.1: Peroxynitrite is a potent inhibitor of purified renal Na,K ATPase activity in 100 mM Tris-HCl pH 7.4. Purified renal Na pump was treated with indicated concentrations of fresh peroxynitrite (filled circles) or an equivalent volume of decomposed peroxynitrite (open circles) at 37° C and then incubated for five minutes. Ouabain sensitive Na,K-ATPase activity was next measured in the presence of 3.0 mM ATP, 4.0 mM MgCl2, 100 mM NaCl and 12 mM KCl. Data were fit to the equation $V = V_0 \times IC_{50} / (IC_{50} + P)$ where P is [peroxynitrite] and V_0 = maximum velocity in the absence of peroxynitrite (i.e P=0). The IC_{50} when treated with fresh peroxynitrite was 107 ± 9 μM whereas the IC_{50} when treated with decomposed peroxynitrite was 4,480 ± 603 μM. These are paired experiments and data represent N=3 experiments and values are reported as mean ± standard deviation.

We are unaware of reports that examined if peroxynitrite can be produced in high concentrations within cells. Although, under pathological conditions, a constant, low concentration of peroxynitrite may be produced intracellularly. Certainly red blood cell Na pumps are likely to be exposed to (fluctuating) low concentrations of peroxynitrite as both the pumps and the red blood cell itself must last 120 days. Moreover, red blood cells are faced with high oxygen concentrations and are also likely scavengers of excess "spillover" NO from vascular cells. To mimic this type of in vivo condition, a syringe pump was used to inject peroxynitrite into samples containing purified Na pump at a rate of less than 1.0 μM per second for 18 minutes. Results in figure 2.2 show that a constant exposure to low concentrations of peroxynitrite inhibited Na pump ATPase activity to a similar extent as a single bolus addition of peroxynitrite at much higher concentrations. Decomposed peroxynitrite administered either as a single bolus addition or with the syringe pump had minimal effect.

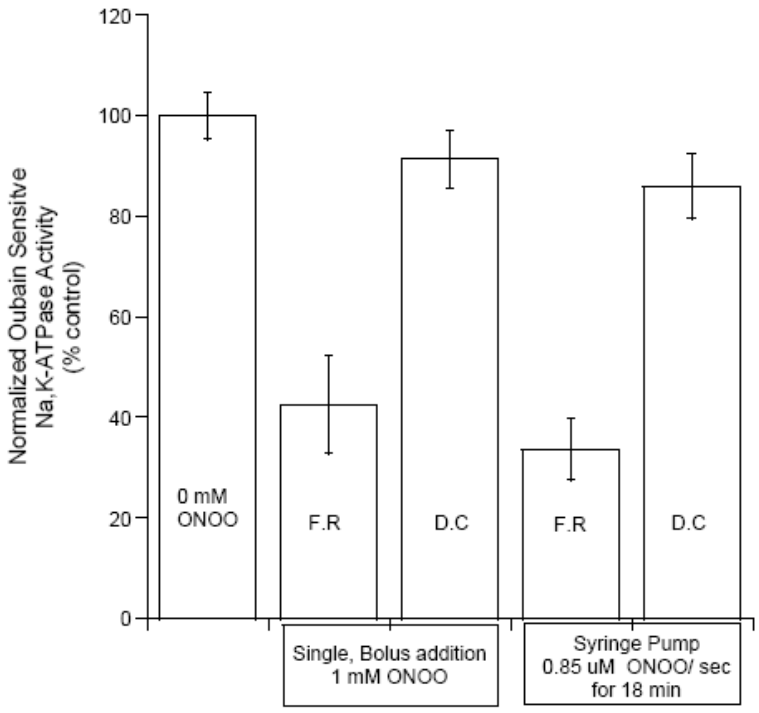

Figure 2.2: Administration of a single bolus of concentrated peroxynitrite inhibits Na,K ATPase activity to a similar extent as administration of a constant, low concentration of peroxynitrite. Purified renal Na pump was treated with either fresh (F.R) or decomposed (D.C) peroxynitrite administered as a single, bolus addition or via a syringe pump to generate a constant, low concentration of peroxynitrite as might be produced intracellulary. Syringe pump parameters (0.85 μM / second for 18 min) were chosen because if peroxynitrite could accumulate at pH 7.4 it would yield a equal cumulative concentration as that used in the single bolus conditions. Following peroxynitrite treatment, ouabain sensitive Na,K-ATPase activity was next measured in the presence of 2.5 mM ATP, 7.0 mM MgCl2, 95 mM NaCl and 15 mM KCl. Experiments were done as paired experiments and data represent N = 4 experiments and are reported as mean ± standard deviation..

The Na pump undergoes several conformational changes during its cycle and it is likely that changes in cell Na^+ change the predominant conformation. Furthermore, Na pump conformation has been shown to alter aspects of its regulation. For example, conformation has been shown to alter regulatory phosphorylation of the Na pump (Feschenko et al 1994). Therefore, we examined if Na pump conformation would alter the ability of peroxynitrite to inhibit the pump. In the presence of Na^+ and low concentrations of MgATP, the Na pump is catalytically phosphorylated. We wanted to compare this conformation to that which occurs in the presence of K^+ but the absence of Na^+. Because there is the potential for MgATP to scavenge peroxynitrite we chose to have the same MgATP concentrations in both conditions so that any scavenging would be equivalent. The effect of Na pump conformation on ONOO induced inhibition was determined by incubating the purified pump with 50 μM MgATP and either Na^+ or K^+ in paired experiments. As shown in figure 2.3 in the presence of Na^+ the IC_{50} for ONOO induced inhibition of ATPase activity was 145 ± 10 μM whereas the IC_{50} in the presence of K^+ was 255 ± 13 μM. It should be pointed out that the experiments represented in Figure 2.3 were done as paired experiments. The IC_{50} values did vary some day-to-day and thus direct comparisons of IC_{50} values in different experiments is difficult.

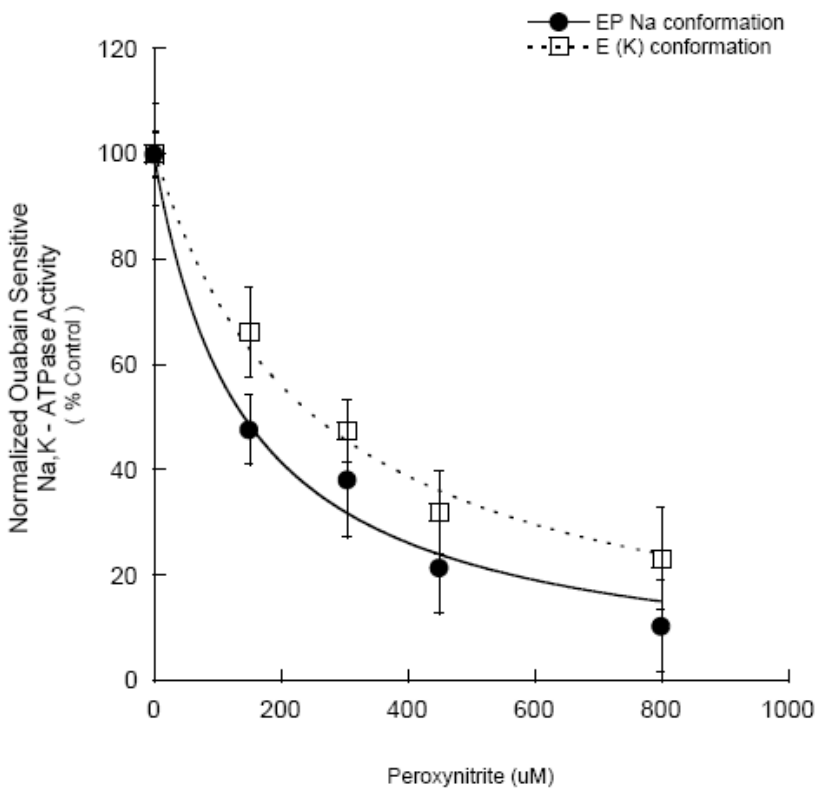

Figure 2.3: The EP Na confirmation is more sensitive to peroxynitrite than the E(K) conformation. Purified renal Na pump was placed in EP Na (filled circles) or E(K) (open squares) conformations by incubating in the presence of 130 mM Na, 50 μM Mg ATP or 30 mM K, 50 μM Mg ATP respectively during peroxynitrite treatment. The solutions for the ATPase were identical. Data were fit to the equation $V = V_0 \times IC_{50} / (IC_{50} + P)$ where P is [peroxynitrite] and V_0 = maximum velocity in the absence of peroxynitrite (i.e. P=0). The IC50 for the EP NA conformation is 145 ± 10 μM while the IC50 for the EP(K) conformation is 255 ± 13 μM. These are paired experiments and data represent N=5 experiments and are reported as mean ± standard deviation. A Students T-Test P-value comparing these two IC_{50}s was 0.007.

To test if peroxynitrite promotes tyrosine nitration of the Na pump, purified Na pump was exposed to increasing concentrations (0, 50, 250, 500 and 750 μM) of peroxynitrite. Figure 2.4 is a representative Western blot demonstrating that peroxynitrite induces tyrosine nitration of the 100 Kd α subunit, 50 kd β subunit and 15 kd FXYD subunit (LANES 1-5). It has been reported that the flavonoid epicatechin selectively prevents nitration but not other peroxynitrite induced oxidative reactions (Schroeder et al 2001). We confirmed that the presence of either 25 or 100 μM epicatechin prevents peroxynitrite induced tyrosine nitration even at the highest concentration (750 μM) of peroxynitrite (LANE 6, 7). We also showed that DMSO alone, used as the epicatechin vehicle control, does not prevent nitration (LANE 8, 9). Figure 4 bottom blot confirms equal Na pump loading in all lanes.

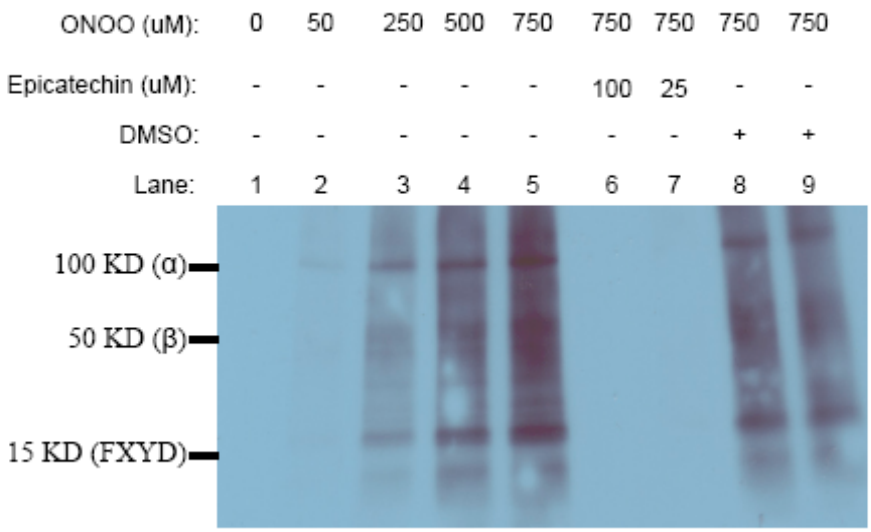

Figure 2.4: Western blot showing an increase in peroxynitrite induced tyrosine nitration of α, β, and FXYD subunits and that epicatechin prevents nitration. Equal amounts of purified renal Na pump were treated with 0, 50, 250, 500 or 750 μM peroxynitrite and probed with an anti-nitrotyrosine antibody. Prominent bands at 100, 50 and 15 KD represent α, β, and FXYD respectively (lanes 1 - 5). 100 or 25 μM epicatechin prevents tyrosine nitration at the highest concentration of peroxynitrite (lanes 6 and 7). DMSO, a vehicle control for epicatechin, did not prevent tyrosine nitration (lanes 8 and 9).

Having verified that epicatechin prevents tyrosine nitration we next determined if tyrosine nitration accounts for loss of ATPase activity with peroxynitrite. To test this we treated purified Na pump with 0, 275, or 490 μM peroxynitrite in both the presence and absence of 100 μM epicatechin or DMSO (vehicle control). Figure 2.5 shows that in the absence of peroxynitrite, 100 μM epicatechin alone does not affect Na pump ATPase activity. However, at 275 μM and 490 μM peroxynitrite, the presence of 100 μM epicatechin enhanced ATPase inhibition. At both concentrations of peroxynitrite, DMSO, used as a vehicle control, had a slight protective effect.

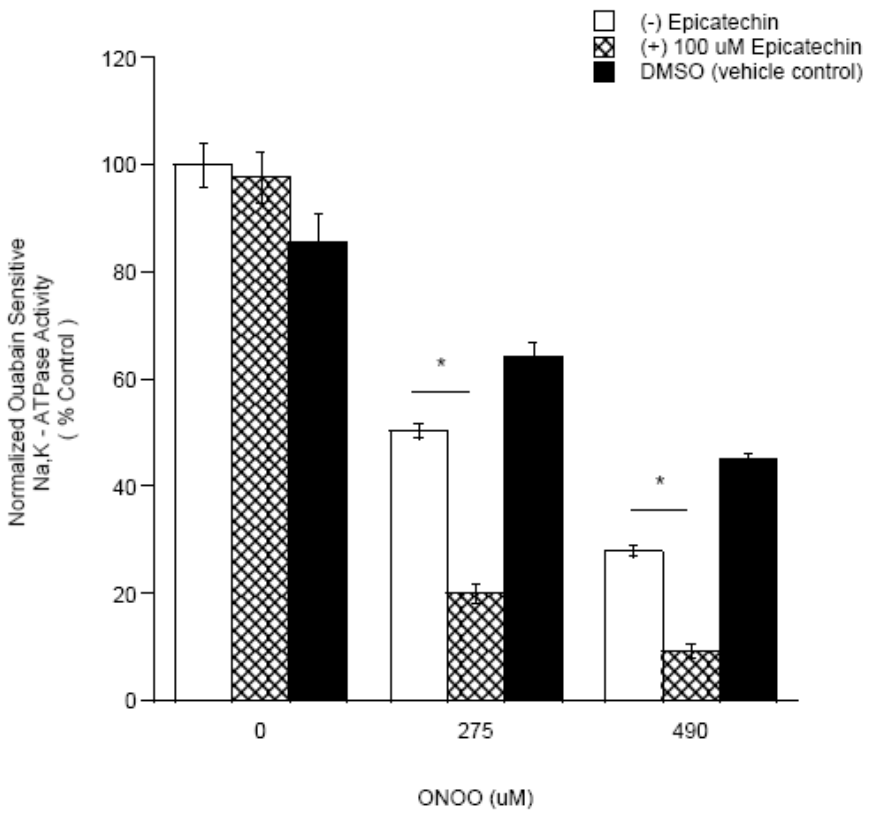

Figure 2.5: The presence of the flavonoid epicatechin enhances peroxynitrite induced Na,K-ATPase inhibition. Purified renal Na pump was treated with 0, 275 or 490 μM peroxynitrite in the absence (white bars) or presence of 100 μM epicatechin (hatch bars) or DMSO (black bars). After peroxynitrite treatment, ouabain sensitive Na,K-ATPase activity was measured in the presence of 2.5 mM ATP, 7.0 mM MgCl2, 95 mM NaCl and 15 mM KCl. Data represent N=3 experiments and are reported as mean ± standard deviation. * indicates P value < 0.001.

Thiol groups on cysteine residues are particularly sensitive to oxidative modifications (Eaton P 2006). To determine if peroxynitrite induced cysteine modifications we labeled non-treated and peroxynitrite or decomposed peroxynitrite treated Na pump free thiol groups with iodoacetamidofluorescein (IAF). Figure 2.6 shows peroxynitrite treated pump exhibited a decrease in IAF labeling compared to non-treated pump and decomposed peroxynitrite treated pump. These results demonstrate that peroxynitrite modifies Na pump cysteine groups. N-ethylmaleimide (NEM), a known thiol blocking agent, was used as a control to verify blocking thiol groups decreased IAF labeling (data not shown).

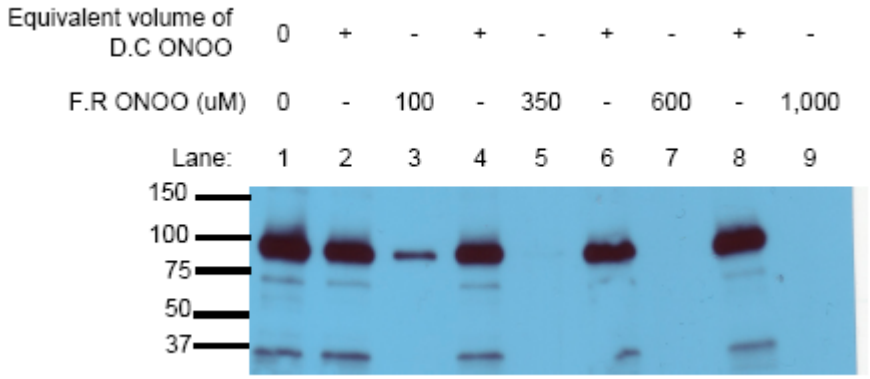

Figure 2.6: Peroxynitrite treatment decreased IAF labeling implying that peroxynitrite modified cysteine thiol groups. Purified Na pump was either non-treated (lane 1) or treated with increasing concentrations of fresh (F.R) peroxynitrite (lanes 3, 5, 7, 9) or an equivalent volume of decomposed (D.C) peroxynitrite (lanes 2, 4, 6, 8).

Having verified that cysteine thiol groups are modified by peroxynitrite we next asked if glutathione (GSH), an intracellular tri-peptide capable of reducing some ROS induced thiol modifications, was able to reverse peroxynitrite induced ATPase inhibition. Figure 2.7 shows that 0.5, 2.5 and 5.0 mM GSH present during peroxynitrite treatment had protective effects on ATPase activity. That is, in the presence of increasing concentrations of GSH, ATPase activity returned to normal. However, GSH introduced only after peroxynitrite treatment did not reverse ATPase inhibition.

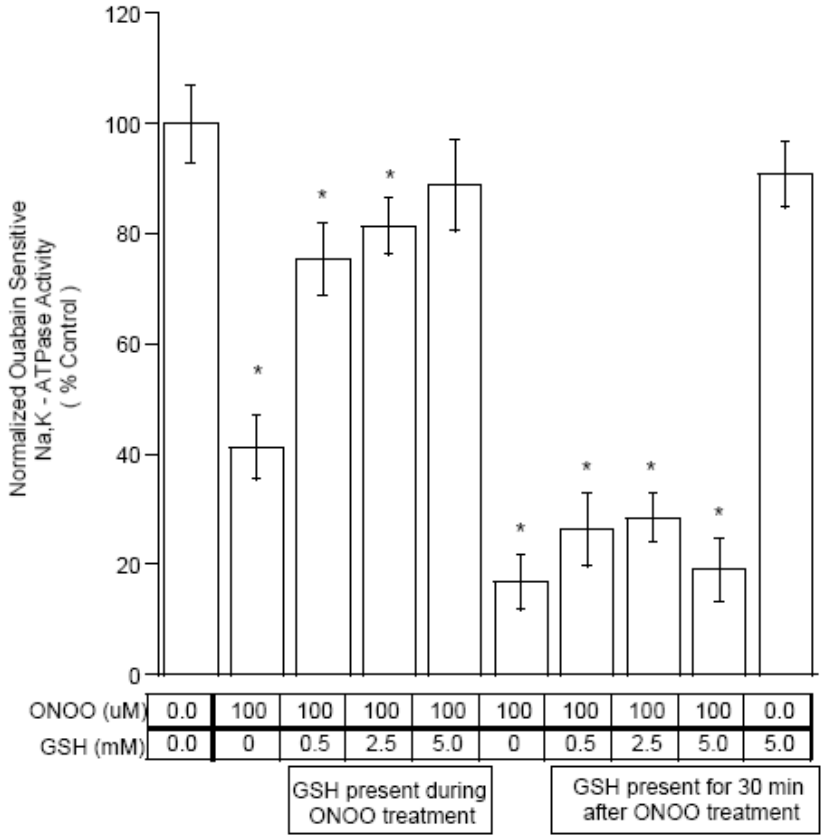

Figure 2.7: Glutathione present during peroxynitrite treatment confers protection while glutathione present only *after* peroxynitrite does not reverse ATPase inhibition. Purified renal Na pump was either non-treated or treated with 100 μM peroxynitrite and with indicated concentrations of glutathione present during treatment or introduced only after peroxynitrite treatment. After treatment, ouabain sensitive Na,K-ATPase activity was measured in the presence of 3mM ATP, 4 mM MgCl2, 100 mM NaCl and12 mM KCl. Data represent N = 2 experiments and are reported as mean ± standard deviation. * < 0.001 compared to control (ATPase activity at 0.0 ONOO and glutathione).

DISCUSION

In the present study we investigated the effects of the peroxynitrite on purified renal Na pump activity with the goal of characterizing the effects of peroxynitrite on Na,K-ATPase activity. In this discussion two different types of intracellular peroxynitrite production are considered: 1) Peroxynitrite concentrations could be elevated in localized areas ("hot spots") likely centered around sites of superoxide production because of the difference between nitric oxide and superoxide reactivity and diffusion distances. 2) Peroxynitrite might also be produced at low concentrations over a long time course. We also discuss if peroxynitrite modified tyrosine and cysteine residues and whether these account for the observed ATPase effects. Lastly, we consider how these modifications might contribute to altered Na pump signaling which could in turn lead to disturbed degradation and trafficking.

Our data provide direct evidence that peroxynitrite is a potent inhibitor of Na,K-ATPase activity. However, decomposed peroxynitrite had minimal effect on ATPase activity. The lack of effect that decomposed peroxynitrite exhibited indicates that stable breakdown products don't account for inhibition and confirms that the buffer capacity is more than adequate for the NaOH in the peroxynitrite stock (for stability reasons peroxynitrite is stored in 4% (1.1 M) NaOH). The results in figure 2.1 suggest that pH changes do not account for the inhibition seen with fresh but not decomposed peroxynitrite. Gutiérrez-Martín et al found the K0.5 (IC_{50}) of peroxynitrite on SERCA ATPase activity to be between 200-300 μM (Gutierrez-Martin et al 2004). Given the high degree of structural and sequence homology between SERCA and the Na pump alpha

subunit our results fit well with the idea that peroxynitrite in general is a potent inhibitor of P-type ATPases and a potent inhibitor of Na,K ATPase activity. Although these are not paired experiments, the results suggest that the Na pump might even have a lower IC_{50} than SERCA.

In this study we also addressed how alterative models of peroxynitrite production might differentially influence Na,K- ATPase inhibition. Specifically, we modeled two extremes of intracellular peroxynitrite production by administrating peroxynitrite either as a single bolus of concentrated peroxynitrite or as a constant, low concentration of peroxynitrite using a syringe pump. We show that a single bolus addition of concentrated peroxynitrite inhibits Na,K-ATPase to a similiar extent as Na pump treated constantly with lower concentrations of peroxynitrite. This result is significant because it provides evidence that a constant low concentration of peroxynitrite, as might be produced intracellularly, could over time, induce significant inhibition of Na pumps.

The precise rate and concentration of renal peroxynitrite production in vivo has not been determined. For example, peroxynitrite production very well might occur such that it is constantly produced in a chronic manner at low concentrations (nM – pM) but it is also possible that local "hot-spots" exist where subcellularly isolated concentrations of peroxynitrite are substantially higher (μM) than surrounding areas. This seems feasible considering that nitric oxide is fairly stable and able to diffuse relatively long distances wherease superoxide is much more reactive and has a much shorter half life. Therefore, peroxynitrite could be produced in discrete localized areas within the cell most likely centered around sites of superoxide production such as mitochondria, NAD(P)H oxidase, xanthine oxidase and other superoxide production sites. In either case peroxynitrite could

lead to inhibition of cell Na pumps if our purified preparation behaves like cell Na pumps.

We hypothesized that peroxynitrite was nitrating Na pump tyrosine residues and might account for loss of ATPase activity. We show here that all three subunits (α, β, FXYD) of the Na pump contain 3-nitrotyrosine residues after treatment with peroxynitrite. The sheep alpha, beta, and FXYD subunits contain 25, 20 and 3 tyrosine residues respectively and clearly one or more tyrosine residue on each subunit is being nitrated. Tyrosine nitration itself has been implicated in altering phosphotyrosine dependent signaling, protein-protein interactions and enzymatic activity (Gow et al 1996; Elsasser et al 2007; Kang et al 2007; Yamakura et al 1998). To test if tyrosine nitration was the sole reason for inhibition of ATPase activity we used epicatechin. Epicatechin is a polyphenol flavonoid found in dark chocolate, green tea and red wine and is a reported selective inhibiter of nitration but not other peroxynitrite induced oxidation reactions (Schroeder et al 2001; Scalbert et al 2000). Shroeder et al have hypothesized that the selectivity of epicatechin is a result of its ability to scavenge the tyrosyl radical, a peroxynitrite intermediate, rather than directly peroxynitrite. We verified that the presence of 100 μM epicatechin prevents nitration.

Having confirmed that epicatechin prevented nitration we predicted that the presence of epicatechin would blunt peroxynitrite induced inhibition. This would suggest that tyrosine nitration partially or fully accounts for the loss of Na,K-ATPase activity. Contrary to our prediction, the presence of epicatechin did not blunt inhibition and actually the presence of epicatechin enhanced ATPase inhibition. That is, even when tyrosine nitration was prevented with epicatechin, ATPase inhibition was still present and

actually enhanced. This result shows that tyrosine nitration is not required for inhibition. However, we cannot conclude that solely nitrating Na pump tyrosines does not cause inhibition as we have not been able to nitrate tyrosine without also altering other residues.

In contrast to the Na pump, in SERCA, nitrotyrosine does account for some of the inhibition. Gutiérrez-Martín et al found that epicatechin reversed a sizeable fraction of the inhibition caused by peroxynitrite-whereas we found that epicatechin actually increased the inhibition (Gutierrez-Martin et al 2004). In both studies, epicatechin decreased the amount of nitrotyrosine formation. Work on SERCA has shown that it is nitrated in-vivo and that it might act as a "sensor" for reactive nitrogen species (Bigelow 2008). For example, aging and cardiovascular disease are associated with increased nitration of SERCA residues Y294 and Y295 (Xu et al 2006). Both of these residues are located in the M4 segment of SERCA which, as in the Na pump, serves an important role in the structural-coupling mechanism that links ATP hydrolysis, phosphorylation and transport. Bigelow et al have hypothesized that nitrating SERCA Y294 and/or Y295 might significantly perturb helix–helix interactions at the M4–M5 interface and inhibit the coordinated movements of membrane helices required for active transport by the Ca^{2+}-ATPase (Bigelow 2008). Given the high degree of homology between SERCA and the Na pump it would be tempting to think that a similar mechanism of inhibition might occur with the Na pump. However, our epicatechin data suggests otherwise. Interestingly, the Na pump has two adjacent Y residues at 146 and 147 but these are located in M2, not M4; indeed, the Na pump has no tyrosine residues in M4. Whether or not the Na pump also accumulates nitrated residues under similar conditions is unknown, but because we have now shown that peroxynitrite inhibits the Na pump, we are now

looking for nitrotyrosines in the Na pump in vivo. If the Na pump is modified by peroxynitrite in vivo, then the Na pump, SERCA and perhaps P-Type ATPases in general, might act as RNS "sensors" as has been suggested (Bigelow 2008). If the Na pump is not modified by peroxynitrite in vivo, under conditions where other proteins (e.g., SERCA) are, then that would raise a puzzle since peroxynitrite can modify the pump in vitro. Two solutions to this potential puzzle are local production of peroxynitrite that is spatially constrained (e.g., near the SR/ER and away from the PM) or local interactions or scavengers that protect the Na pump from damage.

Given our results that tyrosine nitration doesn't account for loss of activity we next checked to see if cysteine modifications accompany peroxynitrite treatment. Cysteine thiol groups are particularly sensitive to ROS/RNS mediated modifications and there are a number of RNS/ROS induced thiol modifications including inter/intra-disulfide formation, nitrothiol, sulfenic / sulfinic / sulfonic acid. The decrease in IAF labeling that accompanied peroxynitrite treatments indicate that peroxynitrite is indeed modifying cysteine residues.

Glutathione is an intracellular tri-peptide. It is able to scavenge many reactive species, including peroxynitrite (Whiteman et al 1996). Thus, the reduction in ATPase inhibition in the presence of glutathione probably reflects this scavenging activity and thus cannot provide definitive information about what residue(s) is modified to cause Na,K-ATPase inhibition. In addition to scavenging, glutathione is also capable of reducing certain kinds of RNS/ROS induced thiol modifications under some conditions. Glutathione was unable to reverse peroxynitrite induced ATPase inhibition. This result is consistent with two interpretations: 1) glutathione led to recovery of cysteines but not

activity, thus the inhibition is the result of modifying an amino acid besides cysteine (and the epicatechin data suggest that it is not tyrosine). Other amino acids that react directly with peroxynitrite include methionine, tryptophan, phenylalanine and histadine (Alvarez et al 2003). The second interpretation is that cysteine modifications do account for the inhibition however the peroxynitrite-cysteine product is not reduced by glutathione under our conditions.

It is well known that the Na pump undergoes a very coordinated series of conformation changes between the E1 and E2 conformations during the pump enzymatic cycle (see Kaplan 2002 for review of Na pump ATPase cycle). Interestingly, there is evidence that conformation-dependent processes exist. For example, Sweadner et al have reported that PKC and PKA regulatory phosphorylation occurs in rat Na pumps in a conformation-dependent manner (Fescheneko 1994). Further, compounds such as 2-[4'-maleimidylanilino]naphthalene 6-sulfonic acid (MIANS) and fluorescein isothiocyanate (FITC) react with specific Na pump residues, cys 577 and lys 501 respectively, in a conformation-dependent manner (Gatto et al 1999; Farley et al 1984). If peroxynitrite damage of the Na pump were to play a signaling/sensor function, the signaling might be conformation dependent and thus a way for the cell to respond differently to low and high cell Na^+ concentrations. We investigated if peroxynitrite inhibition was conformation dependent by treating the Na pump with peroxynitrite in two distinct conformations: E1PNa and E(K). The presence of Na^+, low MgATP was used to place the Na pump in E1PNa conformation while K^+, low MgATP was used to place the Na pump in the E(K) conformation. Our results show that conformation had small effects on peroxynitrite induced inhibition. However, results demonstrate that the E1PNa conformation is slightly

more sensitive to peroxynitrite than is the E(K) conformation. This could be due to residues that are exposed in the E1PNa conformation but not in the E(K) conformation.

In summary we have shown that peroxynitrite is a potent irreversible inhibitor of Na,K ATPase activity. Peroxynitrite also leads to nitration of α, β, and FXYD subunits and modification of cysteine residues. Glutathione did not reverse the ATPase inhibition. While our results show that tyrosine nitration is not required for ATPase inhibition, nitration could alter trafficking and degradation of the Na pump.

CHAPTER 3

Detecting 3-nitrotyrosine in LLC-PK1 Cells and Porcine Tissue

unpublished experiments and observations

INTRODUCTION

In Chapter 2 I presented results from experiments on purified Na pump. Experiments on purified Na pump were advantageous because they allowed me to directly test the effects of peroxynitrite on Na pump activity. These experiments also allowed me to probe for peroxynitrite induced amino acid modifications, such as 3-nitrotyrosine and cysteine modifications. However, these experiments did not and could not address if peroxynitrite might react with the Na pump under cellular conditions in vivo. To what extent the Na pump is a "target" of peroxynitrite in vivo is unknown.

Several lines of evidence suggest it plausible to hypothesize that the Na pump is a "target" of peroxynitrite in vivo. First, SERCA, which shares a high degree of homology with the Na pump, contains nitrated tyrosine residues in cardiovascular disease and with aging (Xu et al 2006). This has lead some to speculate that SERCA acts as a cellular sensor for RNS (Bigelow 2008). Given the high degree of structural similarity could the Na pump also be a "target" of peroxynitrite in vivo? Second, our own evidence on purified pump shows that the Na pump can be nitrated in vitro when directly exposed to peroxynitrite. If the Na pump can be nitrated in vitro it seems reasonable to predict that it also happens in vivo. Thirdly, several lines of evidence in different tissue types, including the kidney, heart and lung suggest that ROS and perhaps peroxynitrite play a role in regulating Na,K-ATPase activity and function (White et al 2008; Comellas et al 2006;

Zhang et al 2002). White et al have shown that pharmacologically uncoupling NOS and thus shifting the balance of production from nitric oxide to superoxide decreased Na pump current in cardiac myocytes as measured by whole cell patch clamping (White et al 2008). Interestingly, superoxide dismutase abolished the inhibition induced by uncoupling NOS suggesting that superoxide and/or subsequent compounds formed from superoxide, like peroxynitrite, accounted for the observed inhibition. In the rat proximal tubule Zhang et al have shown that picomolar concentrations of angiotensin II stimulate Na,K ATPase activity however at nanomolar concentrations the stimulation is abolished (Zhang et al 2002). Interestingly, nanomolar concentrations of angiotensin II in the presence of ROS scavengers, such as superoxide dismutase, deferoxamine and uric acid, returned the stimulatory effect. This also suggests that superoxide and/or peroxynitrite is influencing Na pump function in cells. One difficulty in examining the effects of ROS at a cellular level is that it becomes difficult to tease out direct effects from indirect effects. For instance peroxynitrite could alter Na pump trafficking or intracellular Na both of which influence Na pump activity.

Two different approaches were used to determine if the Na pump is a "target" of peroxynitrite in intact cells and in vivo. To determine if the Na pump is nitrated under cellular conditions, LLC-PK1 cells were treated with peroxynitrite and Na pumps were immunoprecipitated and then probed for the presence of 3-nitrotyrosine. LLC-PK1 cells (ATCC Cl-101), a porcine renal epithelial cell line, were used because they exhibit good Na pump expression and have been used for Na pump trafficking studies. To detect nitrated Na pumps in vivo, tissue from a sedentary adult male Yucatan miniature swine fed a high fat diet was obtained. These pigs were used because they have a similar

phenotype as ApoE $^{-/-}$ knockout mice which have been shown to exhibit elevated levels of 3-nitrotyrosine (Upmacis et al 2007). ApoE is a protein involved in the global transport of lipids and cholesterol and also serves as a ligand for LDL receptors (Mahley 1988). Apolipoprotein E (ApoE) $^{-/-}$ knockout mice develop spontaneous hypercholesterolemia, endothelial dysfunction, atherosclerosis and chronic inflammation (Zhang et al 1992; Upmacis et al 2007). Interestingly, heart, lung, liver and kidney tissue of ApoE$^{-/-}$ knockout mice all contained proteins with elevated levels of 3-nitrotyrosine (Upmacis et al 2007). In a similar manner sedentary Yucatan pigs fed a high fat diet develop elevated plasma levels of cholesterol, triglycerides, LDL cholesterol as well as exhibit impairments in endothelium-dependent relaxation (Thomas et al 2002 , Thompson et al 2004). However the extent of protein nitration is unknown in these animals. Ideally, it would have made most sense to look for nitrated Na pumps in tissue from ApoE $^{-/-}$ knockout mice however, these animals were not immediately available to us. As a best alternative we attempted to detect nitrated proteins and Na pump from a sedentary adult male Yucatan miniature swine fed a high fat diet (tissue courtesy of Hamilton / Laughlin labs).

METHODS

Immunoprecipitation of Na pumps from LLC-PK1 cells treated with peroxynitrite

LLC-PK1 cells (ATCC Cl-101), a porcine renal epithelial cell line, were grown at 37 °C, 5% CO2 / 95% O_2 in 6 well plates in 1x Dulbecco's Modified Eagle Medium until approximately 80% confluent. Cells were washed three times with 1.5 mL ice cold

1x PBS immediately prior to use. After the final wash 450 uL of ice cold 100 mM Tris HCl pH 7.4/150 mM NaCl were added to each well. Cells in two of the wells were treated with peroxynitrite such that every quadrant was treated with ~ 200 μM peroxynitrite. Cells in the other four wells were not treated with peroxynitrite and were used as controls. Cells in each well were then lysed by incubating in 0.1% SDS for 30 minutes on ice with gentle rocking. Following this, cells were scraped and placed into micro-centrifuge tubes with 50 uL of 10x nonidet P40 and incubated 30 minutes on ice with rocking. Next, 25 uL anti—Sodium/Potassium ATPase alpha 1 mouse mono-clonal antibody (1:40 stock) (Affinity Bioreagents MA3-929) were added to the peroxynitrite treated samples and two of the control non-peroxynitrite treated samples. Cell lysate material was incubated with the Na pump antibody for 60 minutes on ice with rocking. Samples were then transferred to new micro-centrifuge tubes containing 50 uL immobolized protein G beads (Thermo Scientific prod #20399) and incubated an additional 30 minutes on ice with continuous rocking. Samples containing the protein G beads were then centrifuged at 4,000 RPM at 4° C for 2 minutes. Samples were then washed and re-suspended in 1 mL ice cold PBS with 0.05% tween 20 for 15 minutes on ice with rocking. This step was repeated two more times. Samples were incubated with 100 uL 1:5 sample buffer + TCEP (800 uL 50 mM Tris pH 8.0 + 0.0143g TCEP + 200 uL 5x sample buffer) for 45 minutes at 37° C to eluate Na pumps from protein G beads. Lastly, the beads were centrifuged at 4,000 RPM and 4° C for 2 minutes and 25 uL of the resulting supernatant was used for SDS/PAGE and Western protocols.

Yucatan swine tissue homogenization and processing

Plasma, liver, deep deltoid skeletal muscle and heart tissue were collected from an adult sedentary high fat fed Yucatan swine plasma courtesy of Dr. Hamilton and Dr. Laughlin Labs. Rat liver tissue from our own lab was also used. Each tissue type was weighed and homogenized in a ten fold volume of homogenization buffer that consisted of ice cold 25 mM imidazole, 250 mM sucrose and 1 mM EDTA titrated with 4 M HCl to pH 7.4. A 10 uL volume of the tissue homogenate or plasma was then diluted into 90 uL of 50 mM Tris-HCl pH 7.4 and 25 uL non-reducing sample buffer. Non-reducing (DTT-Free) sample buffer consisted of 300 mM Tris HCl pH 8.0, 5% SDS, 50% glycerol and 0.025% bromophenol blue. Further dilutions (100 fold and 1,000 fold) of the plasma samples also were prepared. Non-reducing (DTT free) sample buffer was used at all times because of reports suggesting that nitrotyrosine can be non-enzymatically reduced to aminotyrosine in the presence of heme and thiol reducing agents such as DTT (Balabanli et al 1999). Samples were then loaded into a 4-20% Tris HCl precast ready made gel and subjected to electrophoresis and western blotting as reported in chapter two. Resulting PVDF membranes were incubated with anti-nitrotyrosine antibodies (see methods section of chapter 2 for more specific details).

To address the binding specificity of the secondary antibody the gel was run such that lanes 1 through 5 were identical to lanes 6 through 10, in the sense that they were loaded with the same volume from the same sample. After the gel was run and the protein transferred to PVDF membrane and blocked with superblock the membrane was cut in half between lanes 5 and 6. The membrane containing protein from lanes 1 through 5

was exposed to both the primary and secondary antibodies while the membrane containing protein from lanes 6-10 was exposed to the secondary antibody ONLY.

RESULTS

To test if Na pumps are nitrated under cellular conditions LLCPK1 cells were treated with peroxynitrite and Na pumps from these cells were then immunoprecipitated and probed for the presence of 3-nitrotyrosine. Results shown in figure 3.1 suggest that Na pumps from LLC-PK1 cells treated with peroxynitrite contain 3-nitrotyrosine residues. This experiment was repeated a total of 4 times and figure 3.1 represents the best looking result. While the 3-nitrotyrosine signal wasn't nearly as crisp and as clean as preferable there is a broad dark signal at ~100 kd suggesting that Na pumps contain 3-nitrotyrosine. The dark smudge region at 100 kd present in cells treated with peroxynitrite was a consistent finding in the four independent experiments. The presence of a 3-nitrotyrosine signal from non-treated cells was not consistent between the independent immunoprecipitation experiments. However the experiment shown in figure 3.1 does appear to contain nitrated Na pumps from the non-treated cells. Image J analysis of this Westernblot is shown in figure 3.2

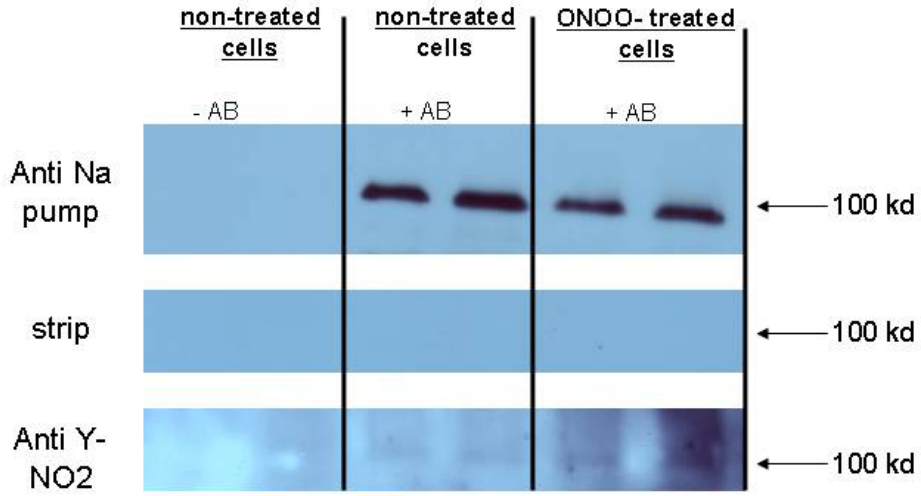

Figure 3.1 – Na pumps from LLC-PK1 cells treated with peroxynitrite contain 3-nitrotyrosine residues. LLCPK1 cells were treated with peroxynitrite (far right panel) or left untreated (far left and middle panels). Cells were then lysed and Na pumps were immunoprecipitated (see methods). A no antibody control (far left pane; - AB) was included as a negative control to ensure specificity of the pulldown assay. (TOP) Probing with an anti-Na pump antibody yields a band at 100 kd consistent with the Na pump. As expected bands are only present in middle and right panels but not the –AB control suggesting the pull down assay worked. (MIDDLE) Membrane was stripped, as confirmed by lack of signal, to remove all antibodies prior to re-probing for 3-nitrotyrosine. (BOTTOM) Probing with an anti-3nitrotyrosine antibody yields a broad band at 100 kd from cells treated with peroxynitrite. Each condition was done in duplicate.

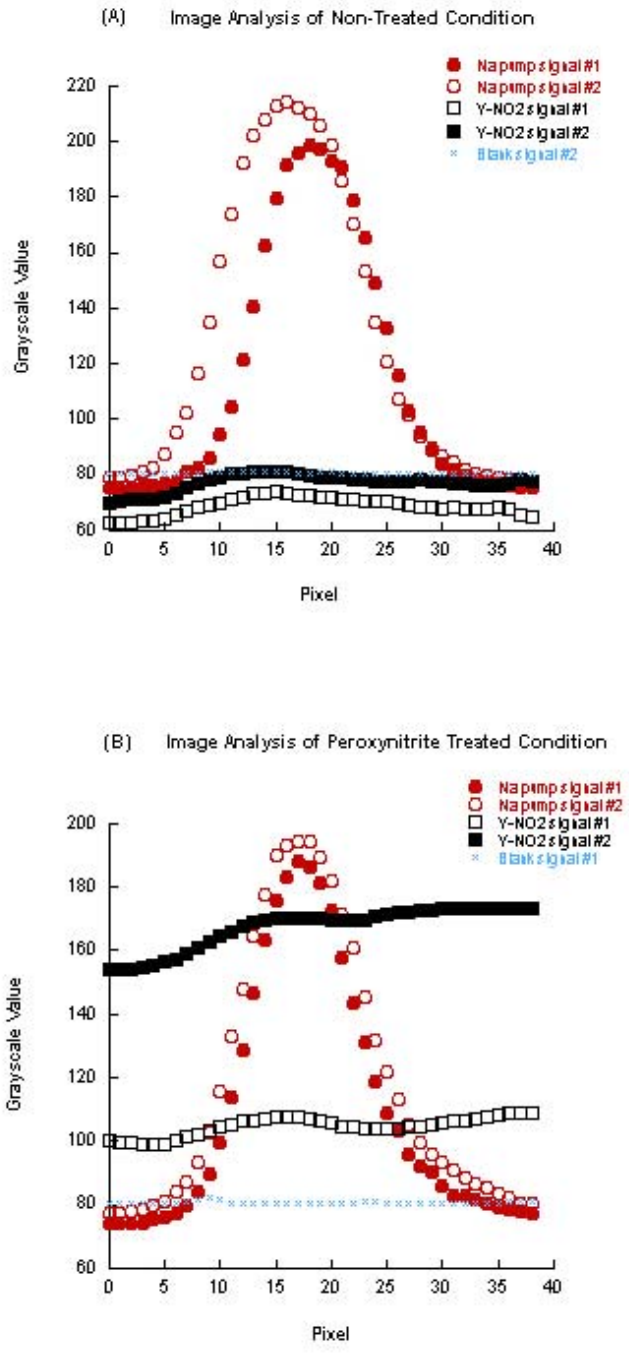

Figure 3.2 – Gray scale analysis of Westernblot shown in figure 3.2. Top (A): Image J analysis of non-treated condition. Bottom (B): Image J analysis of peroxynitrite treated condition. Both (A) and (B) show a clear Na pump signal (red circles). Both (A) and (B) show a much smaller 3-nitrotyrosine signal (black boxes). For comaparison a blank signal was also analyzed (blue x).

Tissues from a sedentary Yucatan pig fed a high fat diet were homogenized and probed for the presence of 3-nitrotyrosine to test if Na pumps are nitrated in vivo. Figure 3.2 suggests that liver, heart and skeletal muscle homogenates from these animals exhibited 3-nitrotyrosine reactivity. To ensure that this signal was not due to non-specific binding of the secondary antibody, the PVDF membrane was cut in half and one half was incubated with both primary and secondary antibodies while the other half was incubated ONLY with the secondary antibody. As seen in Figure 3.2, the 3-nitrotyrosine signal was much more pronounced when incubated with both primary and secondary antibodies (figure 3.2 left) as opposed to secondary antibody only (figure 3.2 right).

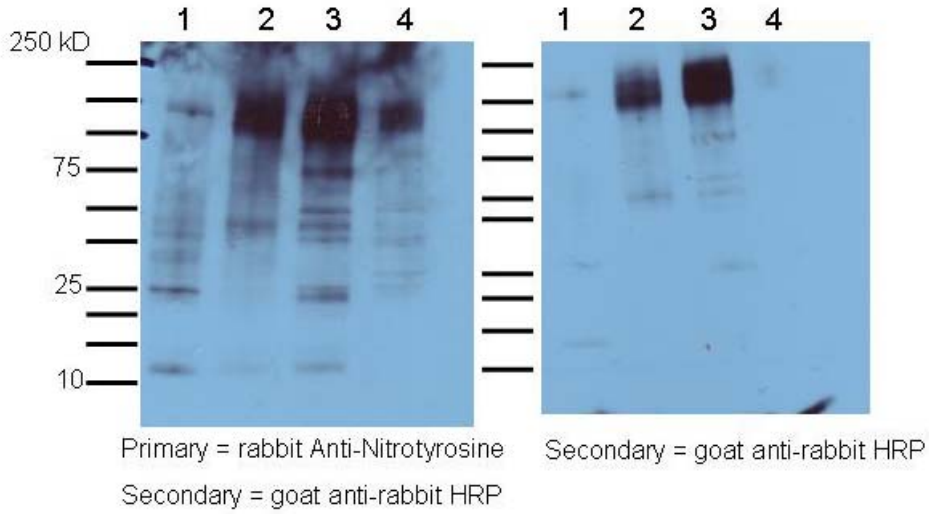

Figure 3.3: Tissue homogenates from a sedentary Yucatan pig fed a high fat diet exhibit 3-nitrotyrosine reactivity. (LEFT) 3-nitrotyrosine reactivity from rat liver and Yucatan liver, heart and skeletal muscle homogenates (lanes 1-4 respectively). As indicated the membrane was probed with both primary and secondary antibodies. (RIGHT) As a control for non-specific secondary antibody binding the membrane was probed with secondary antibody ONLY. Exposure times for the two membranes occurred simultaneously and for exactly the same time.

A plasma sample was also tested for the presence of nitrated plasma proteins. Results shown in Figure 3.3 show a large signal between 75 and 250 kD. However this large signal was also present in the secondary antibody only control suggesting that it is due to non-selective reactivity of the secondary antibody and not a nitrotyrosine signal. Bands present at 50 kD and 22 kD suggest the presence of nitrated plasma proteins. These bands were noticeably lighter or absent altogether in the secondary antibody only control.

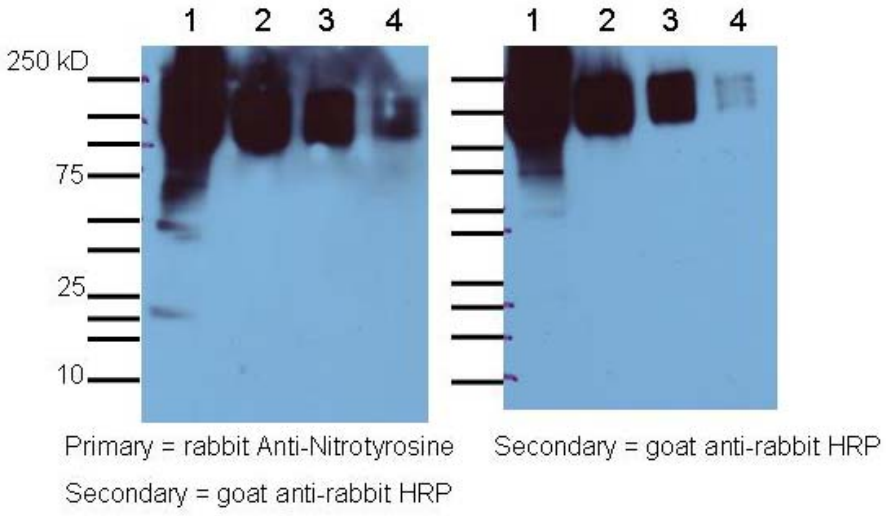

Figure 3.4 - 3-nitrotyrosine detection in sequential dilutions of Yucatan plasma. Lanes 1-4 were loaded with sequential 10 fold sample dilutions. (LEFT): Membrane probed with both primary and secondary antibodies. Bands present at ~50 kd and 22 kd suggest the presence of nitrated plasma proteins. (RIGHT) membrane probed with secondary antibody ONLY. Exposure times for the two membranes occurred simultaneously and were the exact same time.

To confirm the presence of Na pump from tissue homogenate samples the membrane used to generate figure 3.2 was stripped and re-probed with an anti Na pump antibody. The two bands present in Figure 3.4 at ~ 100 kd represent a Na pump signal from Yucatan liver and heart homogenates.

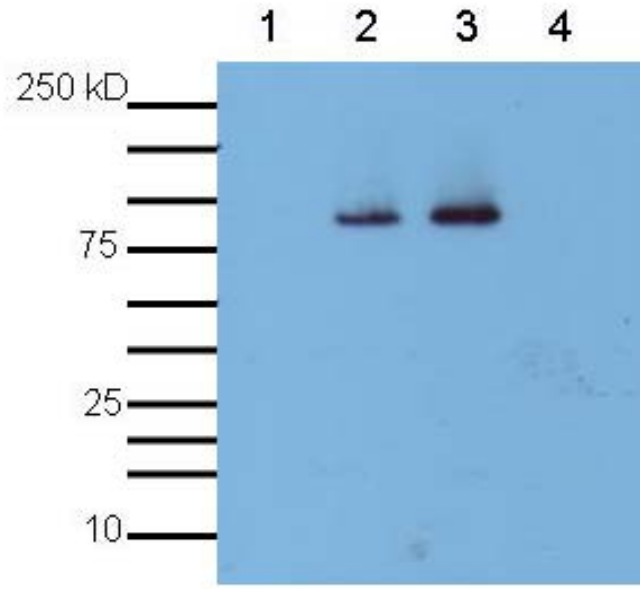

Figure 3.5 – Yucatan liver and heart homogenates contain Na pump. Membrane used to generate figure 3.2 was stripped and re-probed with an anti-Na pump antibody.

DISCUSION

In this chapter I attempted to address two questions; 1) can the Na pump be nitrated under cellular conditions and 2) is the Na pump nitrated in vivo. Detecting nitrated Na pumps would suggest that peroxynitrite is reacting with the Na pump. However, as mentioned in chapter one there is at least one alternative to generating 3-nitrotyrosine. For example, peroxidases (horseradish peroxidase, myeloperoxidase, lactoperoxidase) in the presence of nitrite (NO2-), a stable byproduct of NO metabolism

and hydrogen peroxide, can form 3-nitrotyrosine (Eiserich et al 1998; van der Vliet 1997). Experiments on LLCPK1 cells treated with peroxynitrite suggest that the Na pump can be nitrated under cellular conditions. More experiments are needed to say whether or not the Na pump is nitrated in vivo. However, it appears that tissue samples from a high fat-fed sedentary Yucatan pig contain both 3-nitrotyrosine containing proteins and Na pumps (see figures 3.2 and 3.3). However, at the moment I can not say if Na pumps from these tissues contain 3nitrotyrosine. To confirm if Na pumps are nitrated Na pumps should be immunoprecipitated and probed for the presence of 3-nitrotyrosine. Identifying nitrated Na pumps in vivo would suggest that peroxynitrite is reacting with Na pumps which would increase the relevance of the work I have done on peroxynitrite and purified Na pumps presented in chapter 2. It also would prompt future experiments (see chapter 7).

To address binding specificity of the secondary antibody one half of the membrane was incubated with both primary and secondary antibodies while the other half of the membrane was incubated with ONLY secondary antibody. In this way any signal that was the same between both membranes was attributed to non-specific binding of the secondary. Alternatively, any signal that was present only in the membrane incubated with both primary and secondary was interpreted as a true 3-nitrotyrosine signal. As seen in both figures 3.2 and 3.3 there appear to be bands that are only present when incubated with both primary and secondary suggesting a true 3-nitrotyrosine signal.

Given our data on purified pump, LLC-PK1 cells and the data by Shanqin et al suggesting SERCA is nitrated in vivo, not finding nitrated Na pumps in these tissue samples would be a bit of a surprise. One explanation for this could be due to spatial

differences in the cellular localization of SERCA and the Na pump relative to peroxynitrite production sites. For instance, SERCA is localized to the endoplasmic reticulum/sarcoplasmic reticulum while the Na pump is localized to the plasma membrane and intracellular vesicles. If peroxynitrite production is closer in proximity to SERCA this would account for the observation that SERCA but not the Na pump is nitrated. This finding would also suggest that peroxynitrite is produced in localized "hot spots" within the cell. There is independent evidence supporting that the Na pump, NOS and NADPH are all localized to caveolin (Xie et al 2003; Liu et al 2003; Wang et al 2003; Shaul et al 1996; Garcia-Cardena et al 1996; Hilenski et al 2004) which supports the possibility that localized production of nitric oxide, superoxide and peroxynitrite might influence Na pumps spatially organized in the same area (more in chapter 7).

In summary these studies were undertaken to address if the Na pump is a "target" of peroxynitrite under cellular conditions and in vivo. The results suggest that LLCPK1 cells treated with peroxynitrite contain nitrated Na pumps and that porcine tissue contains both nitrated proteins and Na pumps. However, more experiments are needed to determine if these Na pumps are themselves nitrated.

CHAPTER 4

Possible Denitrase Activity in Red Blood Cells

Unpublished results and observations

INTRODUCTION

As red blood cells (RBCs) circulate throughout the cardiovascular system they are exposed to a myriad of bioactive factors including nitric oxide and superoxide produced from endothelial cells, platelets, macrophages and perhaps red blood cells themselves (Sase et al 1995, Fialkow et al 2007; Kleinbongard et al 2006). In fact, RBCs act as a nitric oxide "sink" because of iron found in hemoglobin (Sonveaux et al 2007). Thus over their 120 day lifetime, RBCs and RBC proteins could be continuously exposed, albeit at low and perhaps fluctuating concentrations, to nitric oxide, superoxide and peroxynitrite. In support of this, evidence suggests that plasma proteins such as VLDL, fibrinogen, plasminogen and HDL and LDL (see Pacher et al 2007 Table 1 and Parastatidis et al 2007) contain 3-nitrotyrosine residues.

RBC exposure to peroxynitrite over 120 days could lead to accumulation of peroxynitrite induced protein modifications such as 3-nitrotyrosine which could disrupt RBC function. Accumulation of other types of post translational modifications in RBCs is known to occur. For example, RBCs accumulate glycosylated hemoglobin (HbA1c) which has been a useful maker of long term regulation of glucose particularly important with regards to diabetes management (Koenig et al 1976). In a similar manner, over the life span of a RBC, proteins might accumulate 3-nitrotyrosine residues as a result of being exposed to constant and/or fluctuating low concentrations of peroxynitrite. Further,

because RBCs contain no nucleus they are unable to synthesize new proteins and probably have a limited capacity to degrade damaged proteins. Allowed to accumulate over time 3-nitrotyrosine might cause significant protein and cell disruption. For example, tyrosine nitration of Band 3 (AE1), a bicarbonate / chloride exchanger and predominant RBC membrane protein, could inhibit not only its function as an exchanger but also its ability to regulate and organize RBC glycolytic enzymes. Interestingly, tyrosine phosphorylation of Band 3 (AE1) regulates the activity of glycolytic enzymes (Harrison et al 1991; Low et al 1987). What effect Band 3 tyrosine nitration has is unknown but presumably could either mimic or inhibit tyrosine phosphorylation.

Evidence suggests that some tissues and cell types contain a denitrase activity capable of nitrotyrosine modifications (Smallwood et al 2007; Kamisaki et al 1998; Kamisaki et al 1998). If enzyme mediated, as has been suggested, this activity would be analogous to phosphatase activity. However, it should be noted that thiol reducing agents in the presence of heme can cause non-enzymatic conversion of nitrotyrosine to aminotyrosine (Balabali et al 1999). Given the potential for 3-nitrotyrosine to alter tyrosine phosphorylation, a denitrase enzyme that removes NO2 groups could be an important signaling mechanism. It would further suggest that cellular 3-nitrotyrosine levels are determined not only by peroxynitrite and other nitrating agents but also by the activity of the proposed denitrase. Whether RBCs contain a similar denitrase activity is unknown and the following experiments were performed to determine if RBCs contain a denitrase activity. In these experiments we tested the hypothesis that RBCs contain a denitrase activity capable of reducing 3-nitrotyorisine levels. To test this hypothesis, RBC membrane proteins were nitrated with peroxynitrite and then incubated with either

RBC lysate or buffer (control) at 37° C or on ice. If RBCs contain a denitrase activity then incubating nitrated proteins with RBC lysate at 37C should lead to a time dependent decrease in 3-nitrotyrosine levels.

METHODS (see figure 4.1 for general overview)

RBC isolation

500 mL porcine blood (Pel Freeze) were centrifuged (Beckman Model J2-21) 10 minutes at 15,000 RPM and the plasma and buffy coat (WBCs) were removed. The remaining RBCs were re-suspended in isotonic 165 mM NaCl and centrifuged at 15,000 RPM for 1 minute. The supernatant was removed leaving behind packed RBCs.

RBC membrane preparation (july 26 2007)

RBC membranes were isolated by mixing 7 mL of washed/packed RBCs with 240 mL ice cold 1x lysis buffer. 10x Lysis buffer consisted of 10 mM $MgCl_2$, 20 mM EDTA, 50 mM Na3PO4, pH 7.4.

The RBC-lysis buffer mixture was kept on ice for 15 minutes with continuous stirring and then centrifuged in eight, 50 mL tubes for 20 minutes at 4° C at 15,000 RPM. For each tube the supernatant was removed and the pellet was suspended into 40 mL 1x lysis buffer and centrifuged for 15 minutes at 15,000 RPM at 4° C. The supernatant was removed and the pellet was placed in the freezer for 1.5 hours. The pellet was then thawed at room temperature and re-suspended in 35 mL of ice cold 1x lysis buffer and centrifuged 15 minutes at 15,000 RPM at 4° C. The supernatants were removed and the

pellet was suspended in 30 mL of 20 mM Tris HCL pH 7.6 and centrifuged 15,000 RPM for 15 minutes at 4° C. After this final spin the pellets (membranes) were suspended in approximately 250 uL 20 mM Tris HCl pH 7.6. The resulting membrane preparations were combined and then aliquoted.

RBC membrane treatments.

200 uL of RBC membranes were diluted into 2.1 mL of 100 mM Tris HCl pH 7.4. To induce 3-nitrotyrosine membranes were treated with 4 uL of peroxynitrite and incubated 5 minutes at 37° C. Peroxynitrite treated membranes were then incubated with either RBC lysate for 1-2 hours at 37° C or kept on ice. As a control some RBC membranes were incubated in RBC lysis buffer. Samples were then washed by centrifuging at 8,000 RPM (TOMY centrifuge) 4 minutes at 4° C, the supernatants were removed and each pellet suspended in ~ 800 uL of 20 mM Tris-HCl pH. This wash procedure was repeated three times or until the supernatant was clear at which time the supernatant was removed and the pellet was suspended in 170 uL of 50 mM Tris-HCl pH 7.4. Samples were then subjected to gel electrophoresis and then silver staining or western blotting procedures.

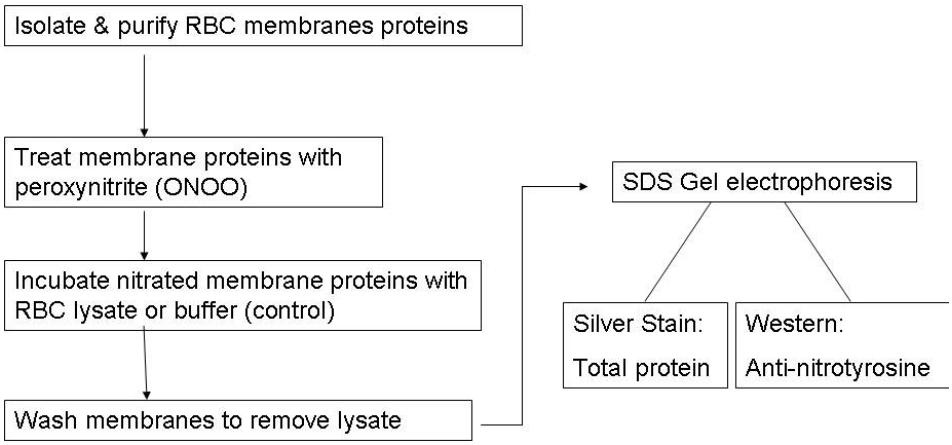

Figure 4.1: General outline of protocol used in detection of RBC denitrase

RESULTS

Figure 4.2 shows results of an experiment attempting to detect denitrase activity in RBCs. If a denitrase activity exists in RBCs there should be an attenuated 3-nitrotyrosine signal from peroxynitrite treated RBC membranes incubated with RBC lysate compared to buffer controls. Results shown in figure 4.2 seem to show this, albeit a small difference. The silver stain in figure 4.2 suggests equal protein concentrations were loaded within each group (100, 20, 4). Verifying this allows for fair comparison between groups within each protein concentration. The western blot in figure 4.2 suggests that incubating peroxynitrite treated RBC membranes with RBC lysate slightly decreased the overall 3-nitrotyrosine signal (compare lanes with red arrows to their appropriate control). This was also confirmed using ImageJ analysis (see figure 4.3).

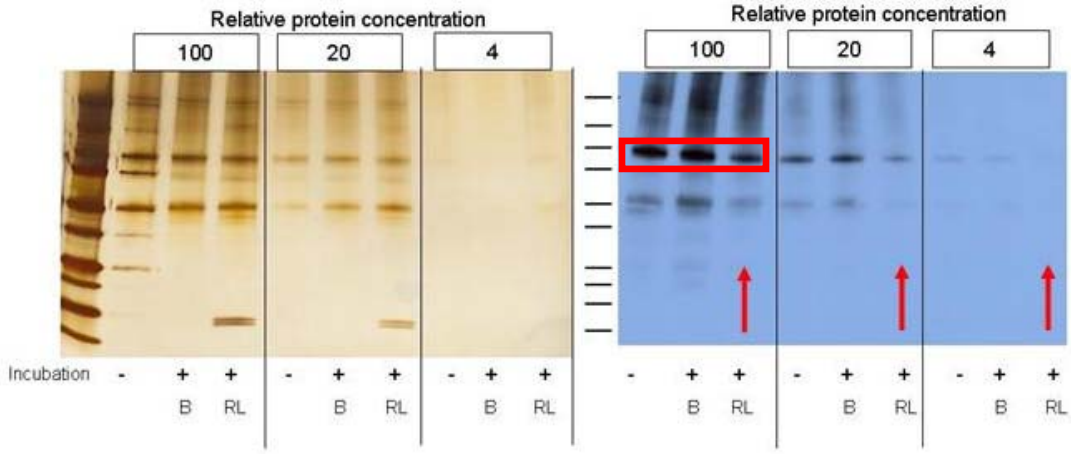

Figure 4.2: Possible RBC denitrase activity example 1. Three different relative concentrations of RBC membranes (100, 20, 4) were treated with peroxynitrite and incubated 1 hour at 37° C with either buffer (B) or RBC lysate (RL) or incubated with buffer on ice (-). (LEFT) silver stain showing equal protein staining within each given protein concentration group (100, 20, 4). (RIGHT) Western blot showing 3-nitrotyrosine signal from samples incubated with RBC lysate (see lanes with red arrows) compared to those incubated with buffer (B). While the difference is small it does appear that there is an attenuated 3-nitrotyrosine signal coming from samples incubated with RBC lysate compared to others. This difference could be due to a denitrase activity in RBC lysate.

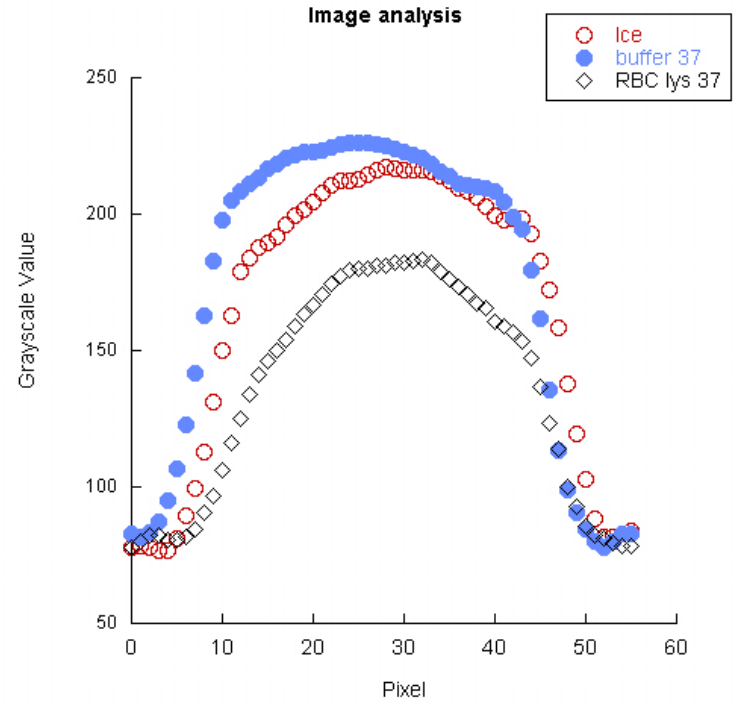

Figure 4.3 – Image J analysis of bands in red box region from Westernblot shown above in figure 4.2. The RBC lys 37 condition (open diamonds) corresponds to the lane with the red arrow beneath the "100". The open and filled circles correspond to the other bands in the box. Both by eye and by Image J it appears that incubating with RBC lysate at 37° C decreased 3-nitrotyrosine signal suggestive of a denitrase activity.

Figure 4.3 is another experiment showing a possible denitrase activity in RBCs. Again, RBC membranes were treated with peroxynitrite to induce 3-nitrotyrosine and then incubated as indicated with either buffer or RBC lysate at 37 ° C or ~ 4 C (ICE). 3-nitrotyrosine signal from bands at 25 kD and 100 kd appear to be attenuated compared to other conditions (see immunoblot). The silver stain suggests that protein concentrations were similar between conditions although there are some differences which might account for those seen in the immunoblot?

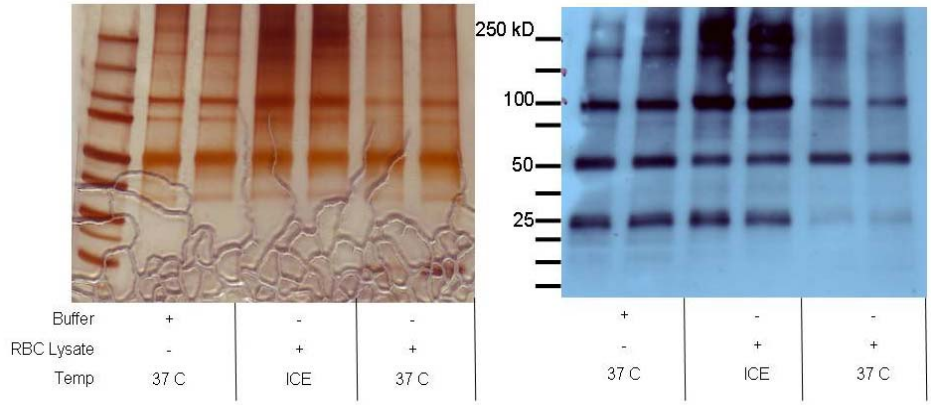

Figure 4.4: Possible RBC denitrase activity example 2. Peroxynitrite treated membranes were treated with peroxynitrite and then incubated with either buffer or RBC lysate at either 37° C or at ~ 4° C as indicated. (LEFT) Silver staining for total protein content appears to be somewhat similar across all lanes. (RIGHT) Western blot probed for 3-nitrotyrosine. Samples incubated with RBC lysate at 37 C appeared to have a somewhat lessened 3-nitrotyrosine signal (see bands at 100 kd and 25 kd). Each condition was done in duplicate.

DISCUSION

Shown here are results of just two experiments that suggest a denitrase activity in RBCs. However, other experiments (not shown) seemed to lack any denitrase activity. Accounting for and identifying the cause of these inconsistent results proved somewhat difficult. Multiple preparations and methodologies were used but they did not resolve this issue. The totality of the results from all of these experiments neither clearly demonstrated an obvious denitrase activity nor ruled one out. Ultimately, given the inconsistent results, the lack of a large obvious denitrase signal, time constraints and the high risk nature, this project was not pursue further.

An enzyme mediated denitrase activity would have several general implications. First, it would suggest that overall amount of 3-nitrotyrosine is the balance between nitration events and denitrase activity. We did not observe a 3-nitrotyrosine signal from native RBCs suggesting either that RBCs exhibit a high denitrase activity or that RBCs used in present experiments were not endogenously subjected to nitrosative stressors such as peroxynitrite. Secondly, a RBC denitrase activity might represent a new method of protein repair. It is possible that accumulation of 3-nitrotyrosine residues significantly impairs its ability to function. A denitrase activity capable of converting 3-nitrotyrosine back to native tyrosine might serve as a repair mechanism. This would be especially important in RBCs because they lack the ability to make new proteins and probably have a limited capacity to degrade damaged proteins.

The presence of a denitrase activity would suggest that tyrosine nitration, like other signaling modifications, is a reversible process. The presence of phosphatases, de-acetylases and SERCA/PMCA suggest that many signaling processes resulting from

phosphorylation, acetlyation, and Ca^{2+} are reversible. Indeed reversibility is a key aspect of signaling pathways because reversibility in many situations represents a method for turning a signal "off". Turning off a signal is just as important as turning one on because it allows signaling molecules to reset so that they can be ready for the next signal and because having a signaling pathway constitutively active would be inefficient. If nitration is involved in signaling pathways than denitrase activity would be an important regulatory mechanism to allow reversibility.

CHAPTER 5

Perivascular fat alters reactivity of coronary artery: effects of diet and exercise

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Reifenberger MS, Turk JR, Newcomer SC, Booth FW, Laughlin MH. Perivascular fat alters reactivity of coronary artery: effects of diet and exercise. *Med Sci Sports Exerc* 39(12):2125-34. 2007.

ABSTRACT

Perivascular adipose tissue (PAT) has been reported to blunt agonist induced arterial tone via a relaxing factor acting in a paracrine manner. The **PURPOSE** of this study was to test the hypothesis that PAT of porcine coronary artery blunts constriction similarly and that this anti-contractile effect of PAT is altered by diet and/or exercise training. **METHODS:** Fourteen adult male pigs were fed a normal-fat (NF) diet and ten adult male pigs were fed a high-fat/cholesterol (HF) diet. Four weeks after the initiation of diet, pigs were exercised (EX) or remained sedentary (SED) for 16 weeks, yielding four groups:1) NF-SED, 2) NF-EX, 3) HF-SED and 4) HF-EX . Left circumflex coronary artery (LCX) rings were prepared with PAT left intact or removed. LCX reactivity to acetylcholine (ACh), endothelin (ET-1), bradykinin (BK) and sodium nitroprusside (SNP) was assessed in vitro using standard techniques. **RESULTS** demonstrate that both ACh and ET-1 elicited dose dependent increases in tension from LCX rings from all groups. Removal of PAT had no significant effect on ACh-induced contractions in any group. In contrast, removal of PAT increased ET-1-induced tension in LCX from NF-

SED, HF-SED and HF-EX but not NF-EX. PAT had no significant effect on relaxation responses to BK except in HF-EX animals where removal of PAT increased BK-induced relaxation. PAT removal decreased SNP-induced relaxation in HF LCX, but not LCX from NF pigs, suggesting basal release of a relaxing factor LCX from HF pigs.

CONCLUSION: PAT blunts contraction induced by ET-1 in LCX from NF and HF pigs. While EX abolished this effect of PAT in NF pigs, exercise did not alter the anti-contractile effect in HF pigs.

INTRODUCTION

Perivascular adipose tissue surrounds nearly all blood vessels and is commonly removed for in-vitro studies of vasomotor function of arteries (Lohn et al 2002). Although perivascular adipose tissue was traditionally thought to provide structural support for arteries, increasing evidence suggests that perivascular adipose tissue also functions as an endocrine organ able to signal locally and/or systemically and as a paracrine tissue that can modulate the function of arterial vascular tone through release of a substance or substances that cause relaxation of vascular smooth muscle (Gao et al 2006; Lohn et al 2002). The yet to be identified substance(s), released by perivascular adipose tissue, referred to as adipocyte-derived relaxing factor (ADRF), seems to be a heat labile protein(s) (Gollasch et al2004). Available evidence indicates that ADRF causes smooth muscle relaxation through opening K^+ channels (Dubrovska et al 2004; Verlohren et al 2004). Lohn et al demonstrated that rat aortic rings with perivascular fat exhibited a blunted constriction in response to angiotensen II, serotonin and

phenylephrine compared to rings with perivascular adipose tissue removed (Lohn et al 2002). Similarly, Verlohren et al found that vessels with perivascular fat exhibited a blunted constriction in response to serotonin, endothelin-1 and phenylephrine (Verlohren et al 2004). Furthermore, the anti-contractile effect of perivascular fat was related to the amount of fat left on the ring preparation. Importantly, it is thought that differences in the function of perivascular adipose tissue in disease may contribute to vascular dysfunction (Gollasch et al2004).

On the basis of these observations, the experiments presented in this manuscript were designed to test three hypotheses. First, that coronary perivascular adipose tissue anti-contractile effect (i.e. releases ADRF) in a manner similar to that reported for rat aorta and visceral arteries (Gollasch et al 2004; Lohn et al 2002; Verlohren et al 2004) . Second, we hypothesized that coronary arteries with perivascular adipose tissue isolated from pigs on a high fat diet will exhibit greater vasoconstrictor responses and/or blunted vasodilator responses because of a decreased anti-contractile effect of perivascular adipose tissue (Henrichot et al 2005; Iacobellis et al 2003; Mazurek 2003). Third, given our interest in the ability of physical activity/exercise training to have beneficial effects on coronary vasomotor function in health and disease, we hypothesized that sustained physical activity through treadmill exercise training for 5 days/week for16 weeks will increase the anti-contractile effect of coronary perivascular adipose tissue in both normal diet and high fat diet fed pigs (Thompson et al 2004). The results reveal that porcine coronary perivascular adipose tissue blunts vasocontraction stimulated by endothelin-1 but not contraction stimulated by acetylcholine, and that arteries from pigs on the high fat diet did not exhibit greater vasoconstrictor responses and/or blunted vasodilator responses

associated with presence of perivascular adipose tissue. Finally, whereas exercise training seemed to decrease the anti-contractile effect of perivascular adipose tissue on contractions induced by ET-1 in NF diet pigs, exercise had no apparent effect on the anti-contractile effect of perivascular adipose tissue in coronary arteries from pigs on the high fat diet.

METHODS

Experimental animals and design

Approval from the Animal and Use Committee at the University of Missouri was obtained prior to the initiation of this study. Adult male Yucatan miniature swine (N= 24) used in this study were purchased from Sinclair Research Farm, Columbia MO and housed in the animal care facility at a temperature of 20 ^{o}C with a 12-h:12-h light-dark cycle. Twenty pigs were assigned into four groups based on diet (normal fat or high fat) and exercise (sedentary or trained). The normal fat (NF) diet consisted of Purina Lab Mini-pig Chow in which 8% of daily calories came from fat. The high fat (HF) diet was composed of pig chow supplemented in the following manner: cholesterol (2%), coconut oil (17.1 %), corn oil (2.3 %) and sodium cholate (0.7%) such that 46% of daily calories came from fat. Four weeks after the initiation of either the NF or HF diets, pigs were either exercise trained (EX) or remained sedentary (SED) for 16 weeks. Exercised pigs performed endurance running at ≈ 75% of max capacity on a motorized treadmill as reported previously (Laughlin et al 2006; Laughlin et al 1998; Laughlin et al 1989; Laughlin et al 2001; Thompson et al 2004). Sedentary pigs were restricted to their 2 x 4

m pens. After completion of the experiments with the first 20 pigs we noted that variability within the NF-SED group required that we do additional experiments. Therefore, we added four NF-SED pigs to the study. This design yielded four experimental groups Normal fat, sedentary (NF-SED)(N = 9), normal fat, exercise (NF-EX)(N = 5), high fat, sedentary (HF-SED)(N=5) and high fat, exercise (HF-EX) (N=5). Prior to and at the conclusion of training/cage confinement programs, both EX and SED pigs performed a graded intensity treadmill exercise test to exhaustion (Laughlin et al 2006; Laughlin et al 1998; Laughlin et al 1989; Laughlin et al 2001; Thompson et al 2004). The efficacy of the training protocol was evaluated from measurements of endurance time (from the treadmill exercise test), heart weight/body weight ratio, and oxidative capacity of skeletal muscle tissue was estimated from measures of citrate synthase activity, as reported previously (Laughlin et al 1989; Muller et al 1994; Thompson et al 2004).

Histology/ImmunoHistochemistry methods:

At the end of 20-24 weeks pigs were anesthetized with intramuscular atropine, ketamine, and xylazine and intravenous pentobarbital and the chest was opened to achieve euthanasia. Hearts were removed and placed in iced Krebs-bicarbonate buffer for dissection of coronary artery samples. Samples of left circumflex branch of the left coronary artery were taken for examination of vasomotor function as described below. Samples of epicardial LAD with periadventitial adipose tissue attached to the left ventricular myocardium were taken approximately midway between the base and apex of the left ventricle and fixed in neutral buffered 10% formalin for a minimum of 24 hours.

Formalin-fixed samples were processed routinely through paraffin embedment, sectioned at five microns and stained histochemically with hematoxylin and eosin and Verhoeff's method for elastin and immunhistochemically for SRA (http://exercisevascularcells.org/SOP_CoreB/43.htm); TNFα (http://exercisevascularcells.org/SOP_CoreB/48.htm), Interleukin-6 (http://exercisevascularcells.org/SOP_CoreB/28.htm), and MCP-1 (http://exercisevascularcells.org/SOP_CoreB/34.htm) . Sections were examined using an Olympus BX40 photomicroscope and photographed with a Spot Insight digital camera.

Citrate Synthase Activity:

Skeletal muscle oxidative capacity was estimated with a citrate synthase assay. After euthanasia, samples of accessory, medial, long and lateral heads of triceps brachii and of the deltoid muscle were isolated. Muscle samples were frozen in liquid N_2 and stored at -70 ^{o}C until processed. Citrate synthase activity was measured from whole muscle homogenate using the spectrophotometric method of Srere (Srere et al 1969).

In vitro assessment of vessel reactivity

Portions 3 to 4 mm lengths of the left circumflex coronary arteries (LCX) from pigs in each group were dissected. Perivascular adipose tissue was either removed (-fat) or left intact (+fat). Vessel segments were then mounted onto a force transducer able to measure grams of tension and placed into a 20 ml bath containing Krebs-bicarbonate buffer maintained at 37 ^{o}C with a gas mixture of 95% O2 and 5% CO2. Prior to dose response curves, coronary rings were stretched to a length that produced maximal force

stimulated by 80 mM KCl. Both acetylcholine (ACh; $10^{-9} – 10^{-4}$ M) and endothelin (ET-1;$10^{-10} – 10^{-8}$ M) were used to assess vasoconstriction. Bradykinin (BK;$10^{-11} – 10^{-6}$ M) was used to assess endothelium-dependent relaxation while sodium nitroprusside (SNP;10^{-10} - 10^{-4} M) was used to assess endothelium-independent relaxation. Change in force was measured from the force transducer in response to cumulatively increasing doses of agonist with washouts occurring between each dose-response protocol.

Solutions and Drugs

Krebs bicarbonate buffer solution contained 131.5 mM NaCl, 5.0 mM KCl, 1.2 mM NaH_2PO4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11.2 mM glucose, 20.8 mM $NaHCO_3$, 0.003 mM propranolol and 0.025 mM EDTA. Solution were aerated with 95% O_2 and 5% CO_2 (pH 7.4) and maintained at 37 ^oC. All drugs and chemicals were purchased from Sigma Chemical.

Statistical Analysis

All Values are means ± standard errors (SE). The vasomotor function experiments were in the form of a mixed linear model with fixed effects (Diet, Exercise, Dose, Fat) and random effects. The data analysis was done using the MIXED procedure in SAS V9 (SAS Institute Inc., Cary, NC, USA). The animal was considered a random effect and the GROUP option was used to model heterogeneous variances for different dose levels. In the analysis we included all interaction terms in the model and noted that several of the pair-wise interactions were significant. Consequently we were not able to look only at the main effect of fat since the effect of fat may differ depending on the level

of the other factors. Therefore we held each of the other factors constant and evaluated the effect of fat in each case by using Least Squares Means. For ACh, we evaluated each of the 2*2*6=24 factor combinations where diet, exercise, and dose were held fixed and asked if there was a significant difference in the response with and without fat. Since BK and SNP had more levels, there were more factor combinations for them but the data were analyzed as described above for ACh. In view of the large number of pairwise comparisons made in the dose-response curves, a significance level of 0.01 was used rather than the usual 0.05.

Differences between groups with respect to blood lipid data, heart weight, body weight, citrate synthase activity, treadmill performance data, ring characteristics, maximal responses, and half-maximal effective dose were determined via unpaired t-test. All data were analyzed in SuperANOVA.

RESULTS

Experimental Animals

Plasma lipids were measured as described previously and found to be similar to plasma lipids reported previously for Yucatan pigs (Thomas et al 2002). The high fat diet induced elevated plasma cholesterol; NF SED = 80 $\pm$ 5 mg/dl, NF EX = 86 $\pm$ 3 mg/dl, HF SED = 387 $\pm$ 28 mg/dl; HF EX = 342 $\pm$ 59 mg/dl; but not triglyceride, NF SED = 77 $\pm$ 3 mg/dl, NF EX = 68 $\pm$ 3 mg/dl, HF SED = 65 $\pm$ 2 mg/dl; HF EX = 67 $\pm$ 3 mg/dl. Thus, the diet produced similar changes in plasma lipids as reported previously and exercise training did not alter plasma lipids in NF diet or HF diet pigs (Thomas et al 2002).

There were no between group differences in body weights. Also, average heart weight-to-body weight ratios were greater in both groups of trained pigs (NF-SED = 4.3 $\pm$ 0.2 g/kg, NF-EX = 5.1 $\pm$ 0.3 g/kg, HF-SED = 4.0 $\pm$ 0.2 g/kg and HF-EX = 5.1 $\pm$ 0.1 g/kg). Further evidence of the effectiveness of the exercise training program is provided in that citrate synthase activity was increased in the muscles of both trained groups (i.e. the long head of the triceps brachii muscle: NF-SED = 16.6 $\pm$ 2.1 umol/min/g, N- EX = 31.5 $\pm$ 3.5 umol/min/g, HF-SED = 13.3 $\pm$ 1.8 umol/min/g and HF-EX = 21.5 $\pm$ 1.0 umol/min/g). In the long head of the triceps brachii muscle, HF groups had significantly lower citrate synthase activity than NF ($p = 0.01$). However, there was no effect of diet on citrate synathase activity of the other muscles sampled (accessory, medical, lateral heads of the triceps and the deltoid muscle). Also consistent with the conclusion that the training program was effective is that the duration of running in the stress test was significantly increased in both training groups ((NF-SED = 27.8 $\pm$ 2.4 minutes, NF-EX = 38.0 $\pm$ 0.5 minutes, HF-SED = 24.1 $\pm$ 2.3 minutes and HF-EX = 34.1 $\pm$ 1.0 minutes). The duration of running was significantly less in the HF-EX pigs than in the NF-EX pigs.

Coronary Perivascular Adipose Tissue

Histopathologic findings for coronary perivascular adipose tissue revealed that SRA-immunoreactive macrophages were numerous in HF-SED but not in coronary perivascular adipose tissue of other groups (Figure 5.1). Immunoreactivity was also documented for TNFα (Figure 5.2), IL-6, and MCP-1 in coronary perivascular adipose tissue. However, immunoreactivity for these markers was highly variable among the 4 groups.

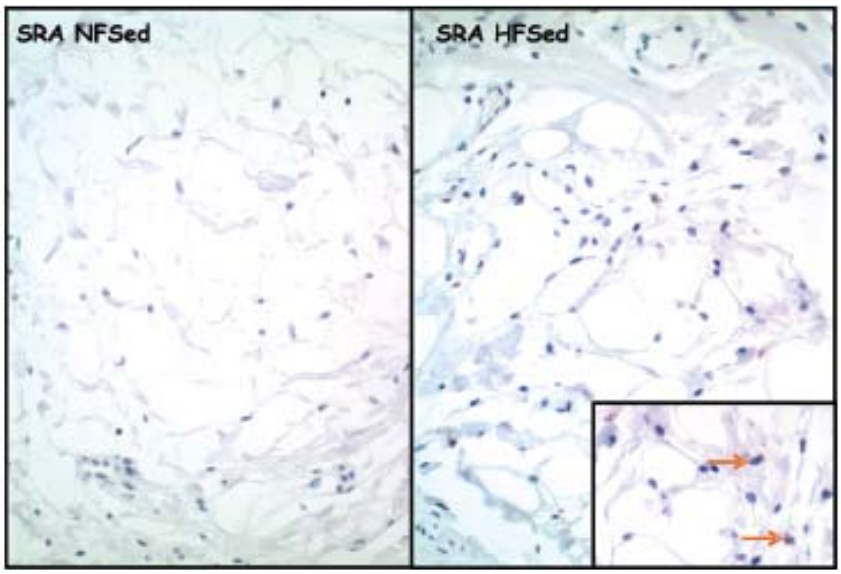


Figure 5.1: Representative photomicrographs of epicardial adipose tissue stained immunohistochemically for scavenger receptor-A (SRA). Immunoreactivity for macrophages in HFSed (right); inset shows higher magnification of immunoreactive macrophages (brown arrows).

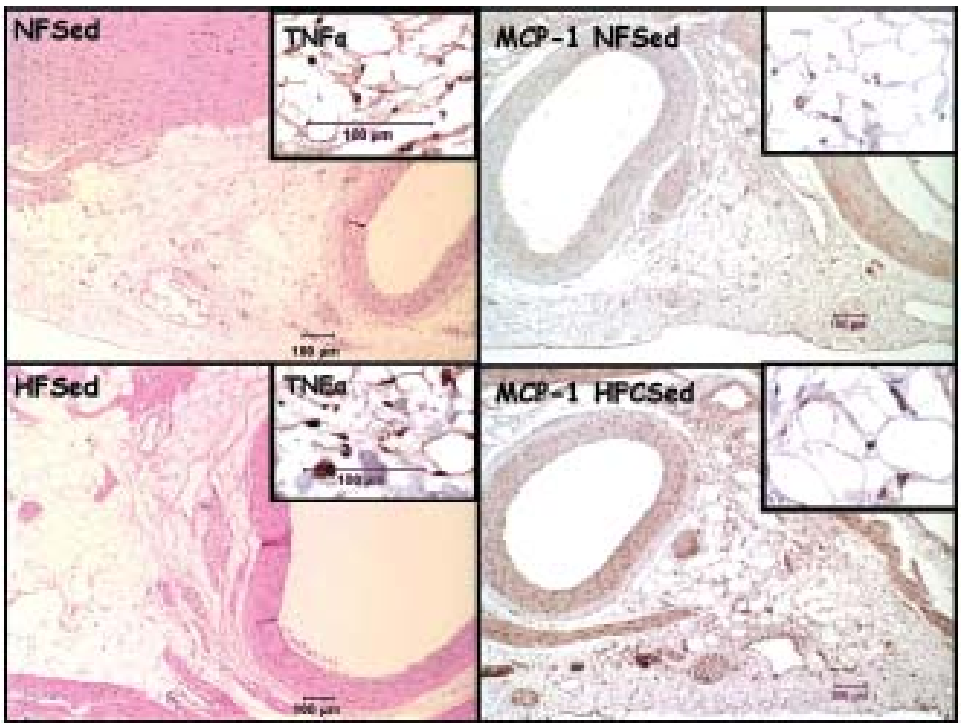


Figure 5.2: Representative photomicrographs of epicardial adipose tissue stained with hematoxylin and eosin (H&E) top and bottom left. Inset: Immunohistochemistry for tumor necrosis factor-alpha (TNFα). Representative photomicrographs of epicardial adipose tissue stained immunohistochemically for monocyte chemoattractant protein-1 (MCP-1) top and bottom right. Inset higher magnification of immunoreactivity for MCP-1.

During preparation of the coronary artery rings for vasomotor responsiveness measurements we estimated the amount of perivascular adipose tissue on each ring. The results reveal that there were no statistically significant differences among the 4 groups (NF-SED = 14.4 $\pm$ 7.1, NF-EX = 9.4 $\pm$ 6.3, HF-SED = 12.8 $\pm$ 7.0, and HF-EX = 14.9 $\pm$ 3.9; sqmm). Also, there were no statistically significant differences among the measured wall thickness, artery area, % stretch at Lmax, length, outside diameter or inside diameter of the artery segments isolated from the four groups of pigs used in these experiments. Thus, neither diet nor exercise significantly altered the structural characteristics of the circumflex branches of the coronary arteries and the size of arteries isolated was similar among groups.

In Vitro Vasocontraction Responses: Normal Fat Diet.

There were no differences among the outer or inner diameter or wall thicknesses of the left circumflex coronary artery segments used for these experiments (Table 5.1). However, the axial length of the segments with perivascular fat removed tended to be shorter than those segments with fat intact (Table 5.2). Resting tensions before vasoconstrictor experiments were not different among groups (Table 5.2). Both ACh and ET-1 elicited dose dependent increases in tension from LCX rings from NF pigs (Fig 5.3). Removal of perivascular fat from the LCX had no statistically significant effect on ACh-induced contractions, contrary to our hypothesis. In contrast, removal of perivascular fat resulted in increased contractile force produced in response to ET-1 in the NF-SED group at the two highest doses (Fig 5.3B). This effect of perivascular fat is

consistent with our hypothesis that predicted an increase in contractile force with fat removal. There was no significant difference between responses of SED and EX-LCX to ACh or ET-1. Exercise training did modify the effect of perivascular fat in that removal of perivascular fat had no effect on ET-1-induced contraction in LCX from exercise-trained NF pigs, whereas it did in SED.

	NF-SED	NF-EX	HF-SED	HF-EX
	(*N* = 9)	(*N* = 5)	(*N* = 5)	(*N* = 7)
Outer diameter (mm)	2.31 ± 0.11	2.08 ± 0.13	2.29 ± 0.13	2.47 ± 0.10
Inner diameter (mm)	1.51 ± 0.08	1.37 ± 0.11	1.43 ± 0.08	1.51 ± 0.08
Wall thickness (mm)	0.40 ± 0.02	0.36 ± 0.06	0.43 ± 0.03	0.49 ± 0.05

Table 5.1: Left circumflex coronary artery diameter and wall thickness. Means ± SEM are presented. NF, normal-fat diet; HF, high fat diet; SED, sedentary; EX, exercise trained; N, number of pigs. There are no statistically significant differences among groups for these variables.

	NF-SED	NF-EX	HF-SED
	−fat	−fat	−fat
	(*N* = 9)	(*N* = 5)	(*N* = 5)
Axial length (mm)	3.70 ± 0.22*	3.68 ± 0.26	3.61 ± 0.107*
Percent stretch to Lmax (%)	165.00 ± 4.50	166.00 ± 2.45	162.00 ± 2.00
Resting tension pre ACh (ST)	2.87 ± 0.23	2.63 ± 0.43	3.00 ± 0.37
Resting tension pre ET-1 (ST)	3.16 ± 0.29	2.76 ± 0.56	2.74 ± 0.27
BK precontraction tension (ST)	6.90 ± 0.76	6.10 ± 1.12	4.65 ± 1.06#
SNP precontraction tension (ST)	7.70 ± 0.65	6.23 ± 1.16	4.38 ± 1.06#
	NF-SED	NF-EX	HF-SED
	+fat	+fat	+fat
	(*N* = 9)	(*N* = 5)	(*N* = 5)
Axial length (mm)	4.09 ± 0.10	3.93 ± 0.34	4.05 ± 0.15
Percent stretch to Lmax (%)	166.11 ± 4.47	164.00 ± 4.00	160.00 ± 0.00
Resting tension pre ACh (ST)	3.37 ± 0.32	3.04 ± 0.73	3.04 ± 0.25
Resting tension pre ET-1 (ST)	3.80 ± 0.37	3.29 ± 0.94	3.07 ± 0.32
BK precontraction tension (ST)	6.92 ± 0.52	7.50 ± 1.31	4.55 ± 0.61#
SNP precontraction tension (ST)	6.931 ± 0.40	6.87 ± 1.10	4.51 ± 0.67#

Table 5.2 Ring Characteristics and Tensions at start of Dose-response Curves. Means ± SEMs are presented. NF = normal fat diet. HF = high fat diet. SED = sedentary. EX = exercise trained. *n* = number of pigs. −fat = perivascular fat removed. +fat = with perivascular fat intact. Lmax = % stretch of the artery ring that resulted in the greatest KCl-induced active tension. ST = specific tension in units of grams of tension per square mm of artery wall. Resting tension is the amount of tension present prior to addition of vasoconstrictor agents (ACh = acetylcholine and ET-1 = endothelin-1). Precontraction tension is the amount of tension (induced by 10-8 M ET-1) present prior to addition of vasodilator agents (BK = bradykinin and SNP = sodium nitropruside). * indicates that the −fat value is significantly different from the +fat value for this variable. # indicates that the HF value is significantly less than the NF value for this variable.

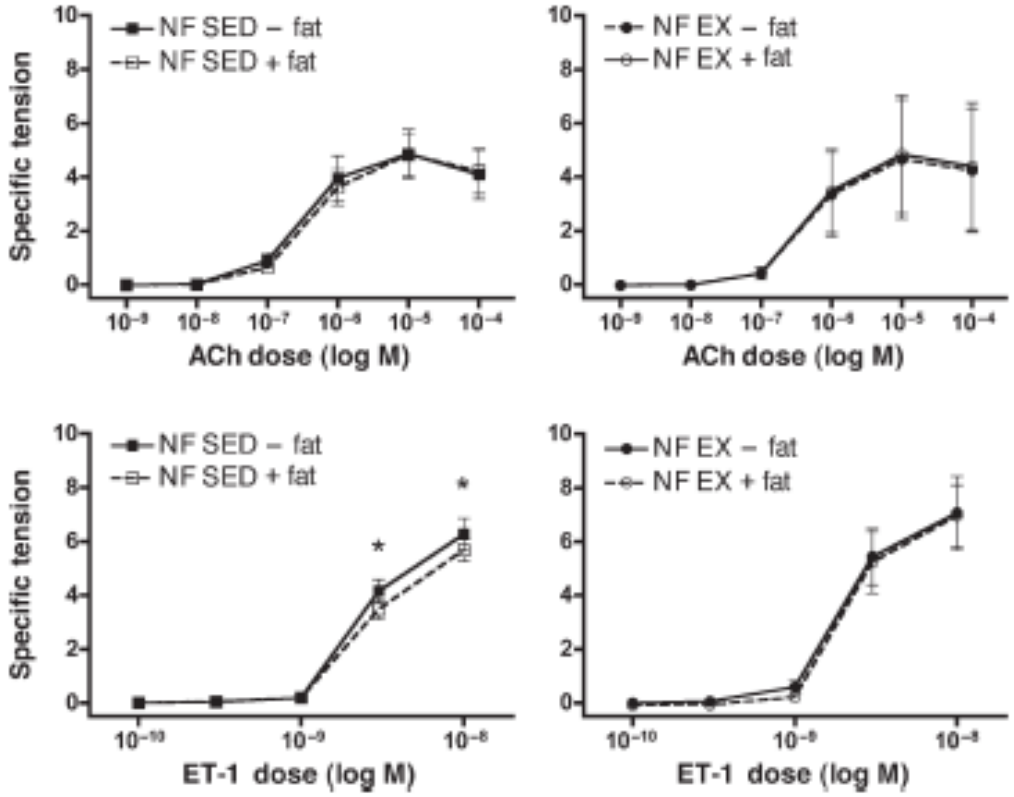


Figure 5.3: Acetylcholine (ACh) and endothelin-1 (ET-1) –induced contractile responses of circumflex coronary arteries sedentary (SED) and exercise trained (EX) from normal fat diet (NF) pigs. Values represent means ± SE in grams of tension per square mm of artery wall. N = 5-9 pigs per group as outlined in methods. ***Top (A):*** Dose/response curve for ACh. Normal Fat (NF), sedentary (SED) and exercised (EX) pigs, with perivascular fat left intact (+) or removed (-). ***Bottom (B):*** Dose/response curve for ET-1. * indicates a statistically significant difference between perivascular fat intact and removed.

In Vitro Vasocontraction Responses: HF Diet.

Both ACh and ET-1 elicited dose dependent increases in tension from LCX rings from HF pigs, and these increases were similar to responses in arteries from NF pigs (Fig 5.4). There was no statistically significant difference between the ACh dose response curve of LCXs from NF and HF diet pigs. In contrast, LCX from HF animals developed statistically significantly less specific tension than did arteries from NF animals. Also, there were no differences among groups for specific tension developed in response to 80 mM KCl (data not shown). As shown in Table 5.2, the ET-1-induced specific tension in arteries from NF pigs, which was greater than that found in arteries from HF pigs, was apparent in the ET-1-induced contractile tension measured before the BK and SNP vasorelaxation experiments.

Removal of perivascular fat from LCX of HF animals had no statistically significant effect on ACh-induced contractions (Figure 5.4). In LCX from HF-SED and HF-EX animals, removal of perivascular fat resulted in increased contractile force produced in response to ET-1 at higher doses. This effect of perivascular fat is consistent with our hypothesis that predicted an increase in contractile force with fat removal. Exercise training did not alter responses of LCX from HF diet pigs to ACh or ET-1. Thus, statistical analysis indicates that the removal of perivascular adipose tissue resulted in increased ET-1-induced contraction in LCX from sedentary and exercise trained HF diet pigs.

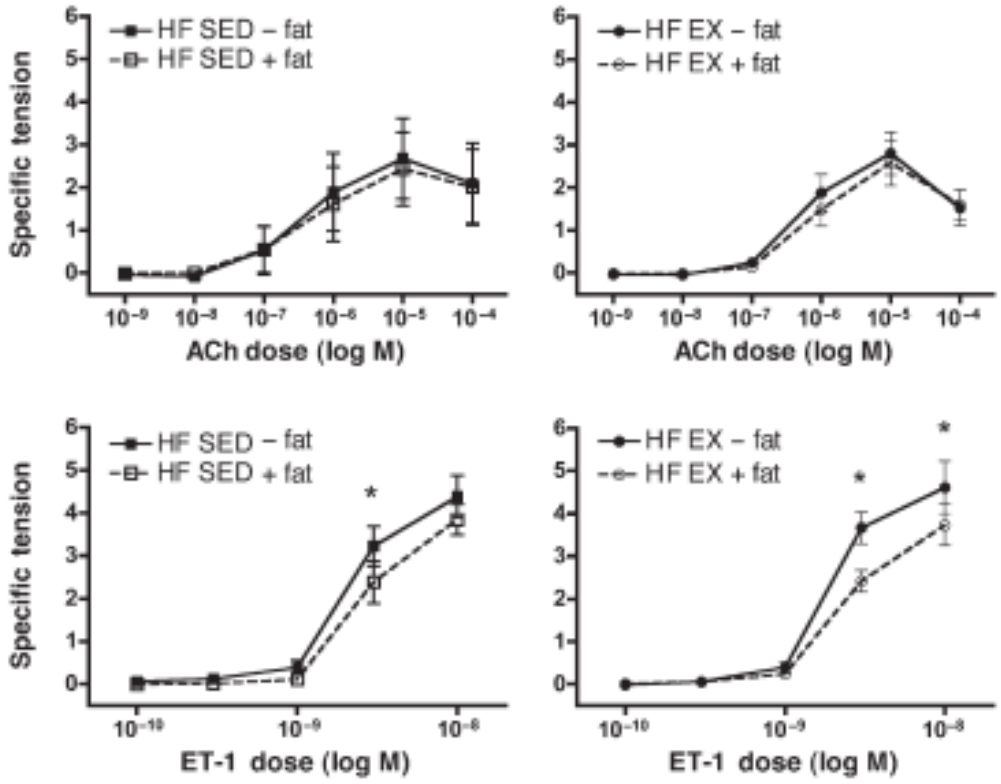


Figure 5.4: Acetylcholine (ACh) and endothelin-1 (ET-1) –induced contractile responses of circumflex coronary arteries sedentary (SED) and exercise trained (EX) from high fat diet (HF) pigs. Values represent means ± SE in grams of tension per square mm of artery wall. N = 5-9 pigs per group as outlined in methods. ***Top (A):*** Dose/response curve for ACh. High Fat (HF), sedentary (SED) and exercised (EX) pigs, with perivascular fat left intact (+) or removed (-). ***Bottom (B):*** Dose/response curve for ET-1. * indicates a statistically significant difference between SED perivascular fat intact and removed. + indicates a statistically significant difference between EX perivascular fat intact and removed.

In Vitro Vasorelaxation Responses

BK was used to assess endothelium-dependent relaxation. As expected, BK induced a concentration dependent increases in relaxation in LCX rings from all groups (Figs 5.5 and 5.6). HF-EX arteries tended to exhibit greater BK-induced relaxation than HF-SED arteries, similar to the effects of exercise on coronary responses in male pigs on this diet as reported by Thompson et al (Thompson et al 2004). however differences between HF-EX and HF-SED were not statistically significant in the present study.

Removal of perivascular fat altered responses to BK in LCX from HF-EX pigs were removal increased relaxation at one dose (Fig 5.5 & 5.6) relative to LCX with fat. Statistical analysis indicates that the absence of perivascular adipose tissue had no effect on BK-induced endothelium dependent vasorelaxation in NF-SED, NF-EX, and HF-SED.

Sodium nitroprusside was used to assess endothelium independent relaxation. SNP induced a concentration dependent relaxation in LCX rings isolated from all groups of pigs. Direct smooth muscle relaxation was similar in both NF groups (Fig 5.5). In arteries from HF-SED pigs we noted that removal of perivascular fat resulted in decreased relaxation at higher does (Figure 5.6).

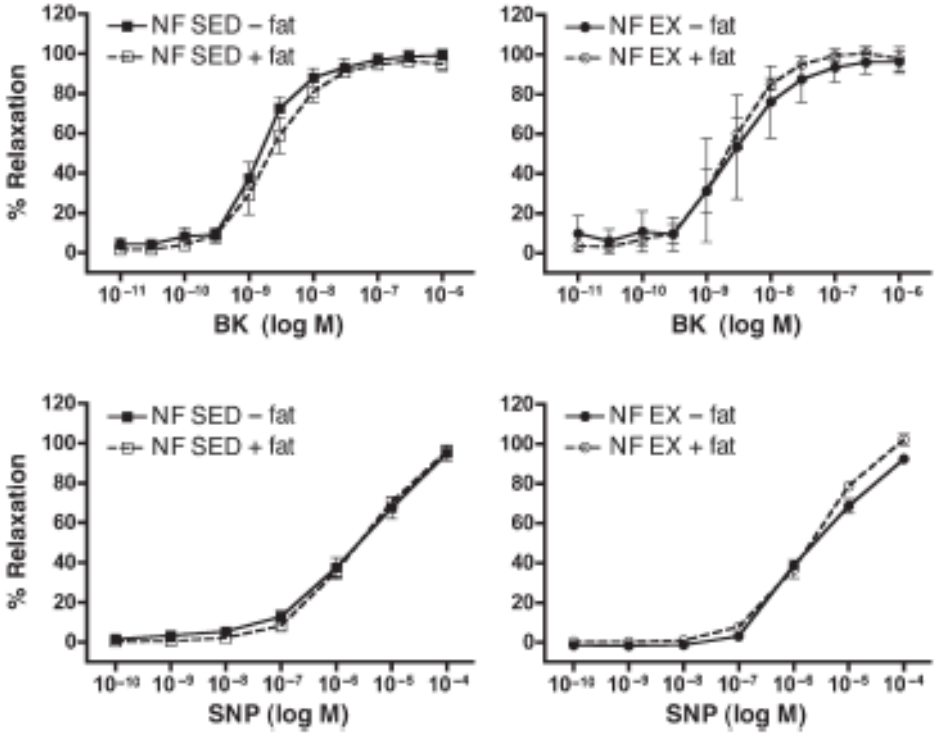


Figure 5.5: Bradykinin (BK) and Sodium-nitropruside (SNP) –induced relaxation responses of circumflex coronary arteries sedentary (SED) and exercise trained (EX) from normal fat diet (NF) pigs. Values represent means ± SE in % relaxation. ***Top (A):*** Dose/response curve for BK, n = 3-5 pigs per group. Normal Fat (NF), sedentary (SED) and Exercised (EX) pigs, with perivascular fat left intact (+) or removed (-). ***Bottom (B):*** Dose/response curve for SNP, n = 5-7 pigs per group. Normal Fat (NF), sedentary (SED) and exercised (EX) pigs, with perivascular fat left intact (+) or removed (-). There were no between group differences in responses among these groups.

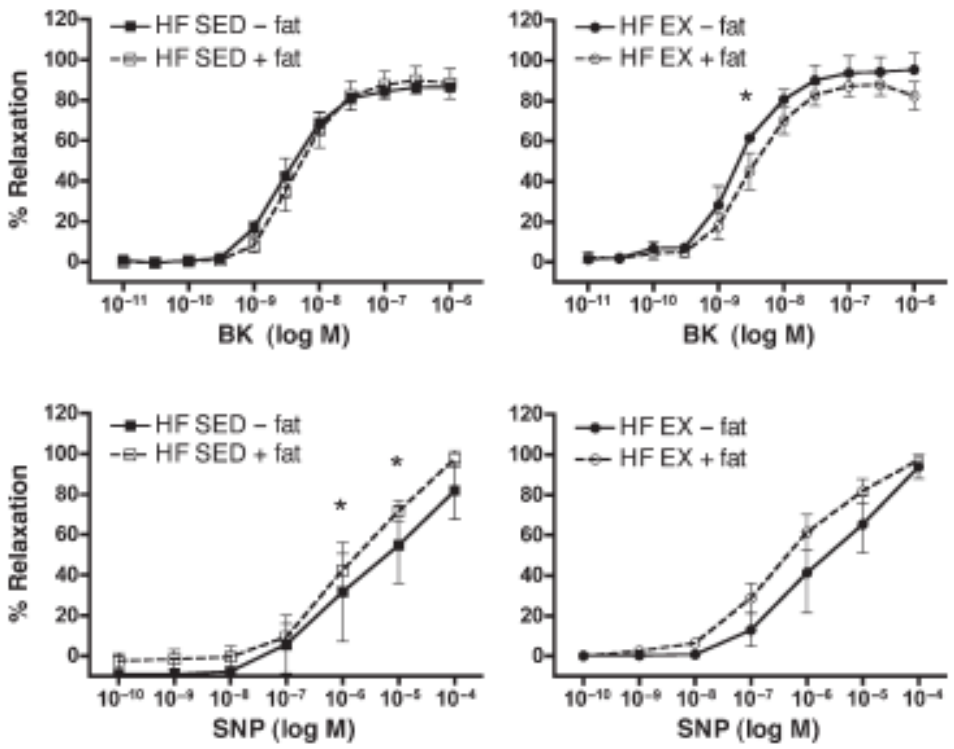


Figure 5.6: Bradykinin (BK) and Sodium-nitropruside (SNP) –induced relaxation responses of circumflex coronary arteries sedentary (SED) and exercise trained (EX) from high fat diet (HF) pigs. Values represent means ± SE in % relaxation. ***Top (A):*** Dose/response curve for BK, n = 3-5 pigs per group. High Fat (HF), sedentary (SED) and exercised (EX) pigs, with perivascular fat left intact (+) or removed (-). ***Bottom (B):*** Dose/response curve for SNP, n = 5-7 pigs per group. High Fat (HF), sedentary (SED) and Exercised (EX) pigs, with perivascular fat left intact (+) or removed (-). * indicates a statistically significant difference between perivascular fat intact and removed.

DISCUSSION

The purpose of this study was to test the hypothesis that coronary artery perivascular adipose tissue has an anti-contractile effect comparable to that reported for rat aorta and visceral arteries (Lohn et al 2002; Gollasch et al 2004; Verlohren et al 2004). We further proposed that coronary perivascular adipose tissue of pigs fed a high fat diet would exhibit less of a blunting effect on contraction (i.e. exhibit greater agonist induced constriction) than pigs on a normal diet and that physically active pigs would exhibit increased anti-contractile and/or enhanced dilator responses in coronary arteries from normal diet pigs as well as in pigs on a high fat diet. Results indicate that porcine coronary perivascular adipose tissue exhibits blunting of vasoconstrictor responses to ET-1 similar to those reported previously in rat aorta and visceral arteries (Figure 5.3 & 5.4) (Lohn et al 2002; Gollasch et al 2004; Verlohren et al 2004). Interestingly, porcine coronary perivascular adipose tissues did not appear to alter ACh-induced vasoconstrictor responses (Figures 5.3 and 5.4). Further, present results indicate that the high fat diet did not appear to alter the effects of coronary perivascular adipose tissue on contractions induced by ET-1. Sustained exercise training for 16 weeks appeared to abolish the effect of perivascular adipose on ET-1 contraction in NF pigs but had no effect on the effect of perivascular adipose tissue on ET-1 contractions in coronary arteries from high fat diet fed pigs.

We were surprised that our results indicate that perivascular adipose tissue of porcine coronary arteries has little to no effect on ACh-induced contraction, where as coronary perivascular adipose tissue had anti-contractile effects on ET-1 induced contractions similar to those reported by Lohn et al and Verlohren et al, in rat aortic and

rat mesenteric artery rings (Lohn et al 2002; Verlohren et al 2004). Lohn et al demonstrated that rat aortic rings with intact perivascular fat exhibited a blunted constriction in response to angiotensen II, serotonin and phenylephrine compared to rings with the adipose removed (Lohn et al 2002). Verlohren et al found similar effects in rat mesenteric arteries in that arteries with perivascular fat left intact exhibited a blunted constriction in response to serotonin, endothelin-1 and phenylephrine (Verlohren et al 2004). Of interest, Verlohren et al. reported that the anti-contractile effect depended on the amount of fat left on the ring preparation. It is interesting that perivascular adipose has different effects on contractions induced by ACh and ET-1. Our results do not allow us to explain this differential response.

Our ET-1 results suggest that there are no major species differences (rats versus pigs) in the effects of perivascular adipose tissue on artery vasomotor function (Lohn et al 2002; Verlohren et al 2004). We anticipated that our experiments might provide evidence of different results related to regional differences in the biochemical make-up/function of perivascular adipose tissue of different arteries (i.e. difference between coronary perivascular adipose tissue and perivascular adipose tissue of abdominal arteries) because both epidemiological and molecular characterizations of adipose tissue support the notion that regional fat depots have different phenotypes and functional effects. For example, epidemiological data suggests that central/visceral/intra-abdominal adiposity is more predictive of negative health outcomes, such as coronary heart disease and diabetes, than total and subcutaneous adipose tissue (Gao et al 2006; Henrichot et al 2005; Wellen et al 2003). So much so that increases in intra-abdominal adipose tissue are now recognized as a component of the "metabolic syndrome" (Grundy et al 1999).

Regional differences in adipose tissue also exist at the molecular level. In patients with coronary artery disease, epicardial adipose tissue near the proximal right coronary artery was found to have higher expression of chemokine (monocyte chemotactic protein [MCP]-1) and inflammatory cytokines (IL-6, IL-1ß, and TNF-α) compared to leg subcutaneous adipose (Mazurek et al 2003). Our results indicate that porcine perivascular adipose tissue also expresses these inflammatory markers (Figs 5.1 & 5.2). And the results suggest that there may be more inflammation in perivascular fat of high fat diet pigs (Fig 5.1).

We expected to find that the perivascular adipose tissue in the pigs on the HF diet would have deleterious effects (i.e. greater constrictor responses) on vasomotor function of the coronary arteries. Results indicate that circumflex arteries from pigs on the high fat diet developed less maximal tension in response to ACh and ET-1 than did arteries from normal diet pigs. Consistent with present results, Thompson et al. reported no effect of the high fat diet on ACh-induced contractions of porcine coronary arteries (Thompson et al 2004). However, present results are in contrast to the report that left anterior descending arteries from high fat diet pigs developed greater tension in response to KCl, serotonin (5-HT), and aggregating platelets. Importantly, present results also indicate that perivascular fat from high fat fed pigs exhibited a blunting effect on ET-1 contractions that was not significantly altered by exercise training.

Present results indicate that coronary perivascular adipose tissue has no effect on BK-induced, endothelium-dependent vasorelaxation in NF-SED, NF-EX, and HF-SED pigs, removal of vascular fat increased BK-induced relaxation in HF-EX pigs. Histopathology results indicate that some of the expected changes in inflammatory

cytokines were present in the perivascular adipose tissue of the high fat fed pigs, but at this early stage of disease, the changes are modest. This may also be related to the fact that obesity is not a feature of this model of coronary disease (Turk et el 2005). Although no significant increase in inflammatory markers were observed with this high fat diet, it is possible that perivascular adipose tissue in pigs with advanced coronary disease would exhibit greater signs of inflammation than observed at this stage of disease [17] and that therefore the pervascular fat effect would be altered.

Finally, the effects of perivascular adipose tissue on vasorelaxation responses of coronary arteries from the HF pigs are interesting. As shown in Figure 5.5, removal of perivascular adipose did not alter SNP-induced relaxation in NF pigs. In contrast, in HF pigs, removal of the perivascular adipose tissue appeared to decrease SNP-induced relaxation in both HF-SED and HF-EX (this was only statistically significant in the HF-SED pigs; Fig 5.6B). These results are consistent with the hypothesis that perivascular adipose tissue produces a vasorelaxing effect, perhaps through release of ADRFs. If this interpretation is correct it is not clear why this effect is not apparent in the responses of the HF arteries to BK. Whatever the explanation for this phenomenon, it is interesting that this effect of perivascular adipose tissue is greater in pigs on the atherogenic diet. This is contrary to our second hypothesis. Also, exercise training did not alter the effects of perivascular fat on coronary reactivity in the HF animals which is contrary to our third hypothesis.

In summary, the results of this study indicate that porcine coronary perivascular adipose tissue blunts ET-1 induced vasocontraction responses of coronary arteries but not ACh-induced contraction of these arteries. This effect of perivascular adipose tissue on

ET-1 coronary vasomotor responsiveness was not influenced by diet. Exercise training appeared to abolish the effect of perivascular adipose tissue on ET-1 induced vasocontraction in pigs on a normal diet but not in the pigs on the HF diet. Perivascular fat appeared to enhance direct, smooth muscle responses to SNP in pigs on the HF diet but not NF. Finally, perivascular adipose tissue appeared to blunt BK-induced relaxation only in LCX from HF-EX pigs, but not in NF pigs. Future studies should attempt to characterize regional differences in the biochemical make-up of perivascular adipose tissue to determine whether visceral perivascular adipose (aortic and mesenteric) tissue has effects on vasomotor reactivity that differ from those in coronary arteries. In addition, it is important in future work to determine whether coronary perivascular adipose tissue influences coronary vasoreactivity in obese subjects and/or in arteries with advanced coronary artery disease in contrast to the high fat diet model used in this study which results in very early stage disease (Stary stage 1-3).

CHAPTER 6

Erythrocyte and renal Na pump inhibition by chrysoidine and extracellular terbium

Reprinted from publication with permission from Elsevier: **Reifenberger MS, Arnett KL, Gatto C, Milanick MA**. Extracellular terbium and divalent cation effects on the red blood cell Na pump and chrysoidine effects on the renal Na pump. *Blood Cells Mol Dis* 39(1):7-13. 2007.

ABSTRACT

We examined the effect of extracellular terbium (Tb^{3+}) and divalent metal cations (Ca^{2+}, Sr^{2+}, and Ba^{2+}) on $^{86}Rb^{+}$ influx into rabbit and human red blood cells. We found that Tb^{3+} at 15 and 25 μM was a non-competitive inhibitor of $^{86}Rb^{+}$ influx suggesting Tb^{3+} is not binding to the transport site. This result reduces the usefulness of Tb^{3+} as a potential probe for the E_{out} conformation (the conformation with the transport site facing extracellularly). Ba^{2+}, Sr^{2+} and Ca^{2+}, at concentrations > 50 mM had minimal effects on Rb^{+} influx into red blood cells (1 mM Rb-out). This suggests that the outside transport site is very specific for monovalent cations over divalent cations, in contrast to the inside transport site. We also found that chrysoidine (4-phenylazo-*m*-phenylenediamine) competes with Na^{+} for ATPase activity and K^{+} for pNPPase activity suggesting it is binding to the E_{in} conformation. Chrysoidine and similar compounds may be useful as optical probes of the E_{in} conformation.

INTRODUCTION

The Na,K ATPase is a plasma membrane protein found in nearly all cells. The Na,K pump maintains the electrochemical gradients for both Na^+ and K^+ by coupling ATP hydrolysis with ion transport. Specifically, the Na,K pump actively extrudes 3 Na^+ ions to the extracellular space and recovers 2 K^+ ions from the extracellular space into the intracellular space per molecule of hydrolyzed ATP. The Na,K pump, by maintaining Na^+ and K^+ gradients, contributes to the maintenance of cell membrane potential, cell pH, cell volume and Na^+-coupled transport of amino acids and glucose.

The Na,K pump reaction cycle has been extensively studied and is currently described by the Post/Albers model (Albers et al 1967; Post et al 1969; Kaplan 2002; Jorgensen et al 2003; Horisberger 2004; Toyoshima et al 2004; Moller et al 2005). In our formulation, we define the E_{in} conformation as those conformations that allow intracellular cations access to the intracellular binding site while the E_{out} conformation allows extracellular cations access to the extracellular binding site. In the E_{in} conformation, 3 intracellular Na^+ ions bind to the transport site allowing for ATP hydrolysis to occur at the nucleotide binding site (N domain). The terminal phosphate of ATP is transferred to the cytosolic "P" domain forming a phosphorylated intermediate termed E-P. The release of the remaining ADP facilitates the translocation of Na^+ to the extracellular space resulting in a shift to the E_{out} protein conformation. With the transport site now facing the extracellular space, 2 K^+ ions bind and the phosphate is released

cytoplasmically. A conformational change of the protein occurs back to E_{in} allowing the release of K^+ to the inside.

In addition to hydrolyzing ATP the Na,K pump is also capable of hydrolyzing the phosphate containing molecule *para*-nitrophenyl phosphate (pNPP) (Berberian et al 1992; Robinson 1989). Na,K pump mediated breakdown of pNPP is stimulated by intracellular K^+ (Drapeau et al 1980). It is important to note that K^+ is also able to stimulate ATP breakdown, but this is an extracellular effect.

Optical probes have provided valuable insight into the structure/function of many proteins. With regard to the Na,K pump, for example, FITC has been shown to covalently label Lys 501 in the ATP binding site with a ratio of one fluorescein molecule per pump (Karlish 1980). The bound fluorescein acts as a convenient reporter group that has been used to monitor conformational changes (Karlish 1980). Another fluorescent probe used in Na,K studies is eosin, which, unlike FITC, binds non-covalently to the ATP site. Eosin has also been used to distinguish between conformations (Skou et al 1981).

Despite these probes for the ATP site, there have been few studies investigating fluorescent probes at the transport site. Finding such a probe would allow for studies that monitor the conformational changes at the transport site and would greatly contribute to our knowledge of pump structure/function. Peluffo et al have shown that benzyltriethylammonium is a useful extracellular probe for electrophysiological measurements (Peluffo et al 2004). The Karlish lab has identified a number of interesting organic cations that have served as useful probes for Na pump structure/function studies (Hoving et al 1995:Or et al 1993; David et al 1993; David et al 1992). They have also characterized the binding of lanthanides to the pump (David et al 1991). Because some

lanthanides are luminescent and David and Karlish have shown that they inhibit the Na pump in broken membranes, we were interested in determining additional interactions of the lanthanide, terbium, with E_{out} (David et al 1991). We were also interested in determining the interactions of divalent cations with E_{out}; while the inside transport site has been shown to bind not only monovalent cations but also divalent cations, few if any studies have investigated in detail the divalent cation effects on the outside transport site (Vasallo et al 1986; Forbush 1988; Schneeberger 2001; Gatto et al).

Diamines have been shown to interact with the inside transport site (Or et al 1993; Schuurmans et al 1988; Van der Hijden et al 1989; Schuurmans 1989; Forbush 1988; Gatto et al 2006). Chrysoidine is a blue dye diamine that structurally resembles propyl diamine. Because propyl diamine is a competitive inhibitor at the inside transport site, we sought to determine if chrysoidine might also go to the inside site (Gatto et al 2006).

Here we report on potential optical probes for both the inside and outside transport site. We investigated the effects of the trivalent, luminescent lanthanide terbium (Tb^{3+}), as a potential probe for the outside transport site by measuring Rb^+ influx into red blood cells. We also investigated the specificity of E_{out} for monovalent cations by measuring Rb^+ influx into red blood cells. Chrysoidine was tested as a potential probe for E_{in} using purified renal Na pumps.

METHODS

$^{86}Rb^+$ influx into erythrocytes

Ouabain-sensitive $^{86}Rb^+$ influx into intact rabbit or human red blood cells was determined, as described previously, in the presence of indicated concentrations of

divalent cations or Tb^{3+} (Milanick et al 2002). In all cases, the rate of influx of these divalent ions or Tb^{3+} is very low, so the cations only have access to extracellular sites. Also, red cells can tolerate high extracellular concentrations of these cations. The cells were washed three times in 110 mM $Mg(NO_3)_2$ and placed into flux media. Nitrate was used so that there would be no chloride-dependent rubidium flux, thus reducing the ouabain-insensitive component of the flux. The media contained 1.0 mM $^{86}Rb^+$, 62 mM $Mg(NO_3)_2$, or the indicated concentrations of divalent cations, and 20 mM N-methyl-D-glucamine HEPES, titrated to pH 7.4. The final concentration of red cells was <1%. The tubes were incubated at 37°C for 10 min. The flux was stopped by adding an 8 fold excess of ice cold 165 mM NaCl, 1 mM EDTA and the cells spun down. They were washed two additional times in the same media and the amount of $^{86}Rb^+$ in the pellet determined with a scintillation counter. For all reported fluxes, we determined the ouabain-sensitive flux as the difference between controls and samples containing 1 mM ouabain. (The rabbit red cells were obtained from Pel-freeze, AR and the human red blood cells were obtained from volunteers under a protocol approved by the UMC IRB).

Na,K-ATPase Activity

Purified sheep kidney was prepared following our previously described modification of the Jorgensen technique (Gatto et al 2005; Jorgensen 1974). Ouabain-sensitive, Na,K-ATPase activity was measured using a previously described colorimetric assay technique capable of detecting inorganic phosphate, see below (Gatto et al 1997; Brotherus et al 1981). For all ATPase experiments 1 – 2 ug of Na,K protein was

incubated in 600 ul of the appropriate assay media for 10 minutes at 37 ^{o}C. (The sheep kidneys were obtained from Pel-freeze, AR.)

For chrysoidine IC_{50} experiments, Na pump ATPase activity was assayed in the presence of 0.00, 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0 mM chrysoidine in assay media containing a final concentration of 2.7 mM KCl , 50 mM NaCl, 20 mM MgCl2 and 3 mM ATP at pH 7.4.

For the K^+ activation ATPase experiments, Na pump activity was assayed in the presence or absence of 1 mM chrysoidine in assay media containing 50 mM NaCl, 20 mM MgCl2, 3 mM ATP and increasing concentrations of K^+ (0.17, 0.5, 1.5, 3, and 6 mM) at pH 7.4.

For Na^+ activation experiments, Na pump ATPase activity was assayed in the presence or absence of 0.5 mM chrysoidine in assay media containing 5 mM KCl, 20 mM MgCl2, 3 mM ATP and increasing concentrations of Na^+ (4.0, 8.0, 16.0, 32.0 and 64 mM) at pH 7.4

Na,K-ATPase para-nitrophenylphosphatase activity

Purified sheep kidney, ouabain-sensitive, Na pump pNPPase activity was measured as previously described (Gatto et al 2005). For all pNPPase experiments 1 – 2 ug of Na,K protein was incubated with 600 ul of the appropriate assay media for 30 minutes at 37 ^{o}C.

For chrysoidine Na pump pNPPase IC_{50} experiments, Na pump pNPPase activity was assayed in the presence of 0.00, 0.3, 0.1, 0.3, 1.0, 3.0 or 6.0 mM chrysoidine in assay

media containing a final concentration of 2.4 mM KCl, 20 mM MgCl2, and 6 mM pNPP at pH 7.4. The chrysoidine was obtained from Acros.

For the K^+ activation pNPPase experiments, Na pump pNPPase activity was measured in the absence or presence of 2 mM chrysoidine in assay media containing a final concentration of 20 mM MgCl2, 6 mM pNPP and increasing concentrations of K^+ (0.75, 1.5, 3, 4.5, 13.5 mM) at pH 7.4.

Colorimetric assay (Gatto et al 1997; Brotherus et al 1981)

Na pump mediated ATP or pNPP hydrolysis was stopped by addition of 1 mL of an ice-cold stop solution, which consisted of 440 mM HCl, 325 mM ascorbic acid, 4 mM ammonium molybdate and 50 mM SDS . Samples were incubated with the stop solution for 10 minutes on ice. After 10 minutes, 1.5 mL of ACA solution was added to each sample. ACA solution consisted of 154 mM sodium-m-arsenite, 68 mM sodium citrate, and 340 mM acetic acid. Samples were then incubated for 5 minutes at 37 ^oC followed by a 20 minute room temperature incubation. Following the 20 minutes at room temperature the absorbance at 850 nm for each sample was measured in a cuvette in a Shimadzu spectrophotometer.

RESULTS

Studies on the extracellular site

To test if Tb^{3+} could competitively inhibit $^{86}Rb^{+}$ influx, we performed inhibition experiments using intact rabbit red blood cells. Competitive inhibition between $^{86}Rb^{+}$ and Tb^{3+} would indicate Tb^{3+} is able to gain access to the outside transport site. Figure 6.1 shows that in the presence of 15 and 25 μM Tb^{3+} there was a significant decrease in Vmax but no change in Km. Specifically, Vmax values for control (0 mM Tb), 15 μM and 25 μM Tb^{3+} were 1.08 ± 0.02 , 0.64 ± 0.01, and 0.27 ± 0.02 respectively, while Km values were 0.14 ± 0.02 , 0.13 ± 0.02 , and 0.08 ± 0.05. These data suggest that terbium is a non-competitive inhibitor of $^{86}Rb^{+}$ flux and not binding to the transport site.

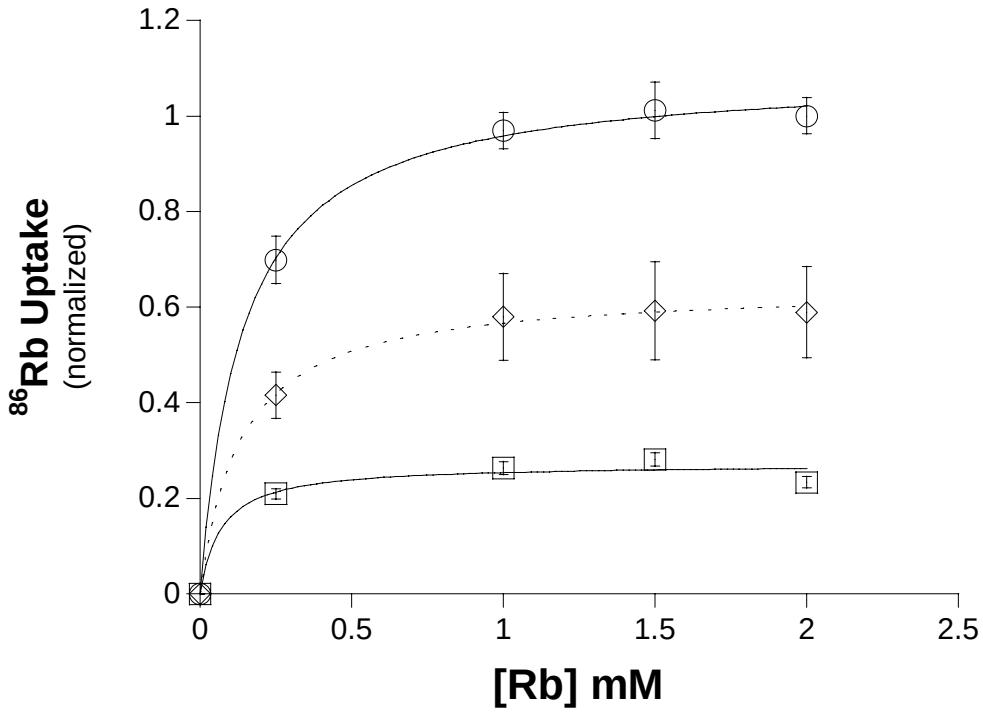
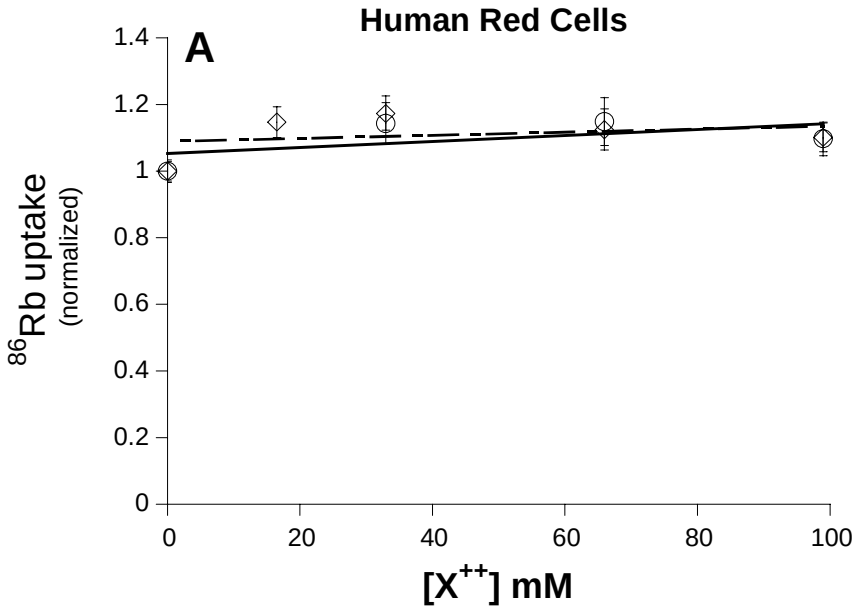
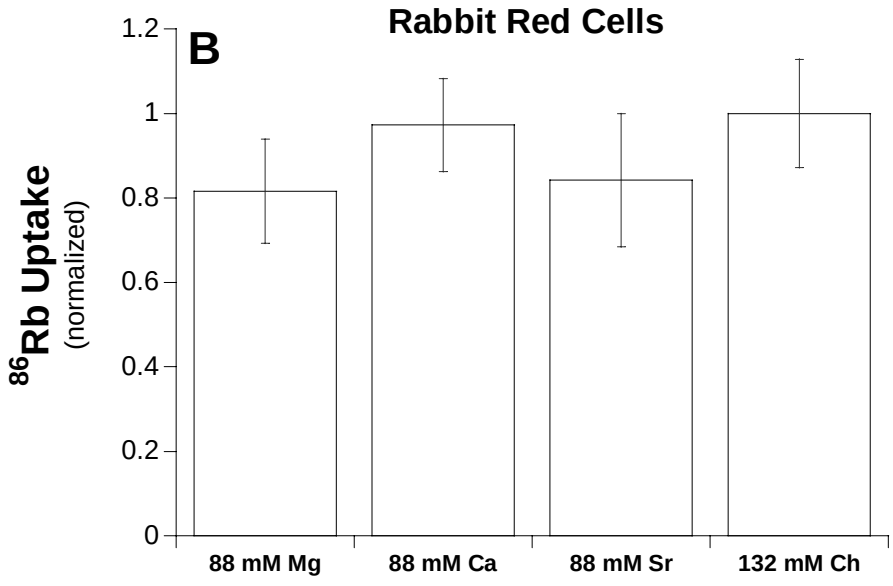


Figure 6.1: Terbium inhibition of Rb^+-uptake. Ouabain-sensitive ^{86}Rb flux was measured into rabbit red cells in the presence of the indicated $^{86}Rb^+$ concentrations and the absence (○) or presence of either 15μM (□) or 25μM (◊) Tb^{3+}. Increasing concentrations of $^{86}Rb^+$ did not decrease the inhibition by Tb^{3+} consistent with mutually exclusive binding for $^{86}Rb^+$ and Tb^{3+}. Control: Vmax = 1.08 $\pm$0.02; K_m = 0.14 $\pm$0.02 mM; 15 μM Tb^{3+}: Vmax = 0.64 $\pm$0.01, K_m 0.13 $\pm$0.02 mM; 25 μM Tb^{3+}: Vmax = 0.27 $\pm$0.02, K_m = 0.08 $\pm$0.05 mM. The data from at least three different experiments were normalized to the maximum $^{86}Rb^+$ flux measured in the absence of Tb^{3+}. Points represent means $\pm$ SEM.

To gain further insight into the binding specificity at the outside transport site we examined the ability of divalent cations to inhibit $^{86}Rb^{+}$ flux. The divalent cations Ba^{2+} and Ca^{2+} were investigated because they have ionic radii very similar to K^{+} (135 vs. 133) and Na^{+}, respectively (99 vs 95). We determined if Ba^{2+} and Ca^{2+} could inhibit $^{86}Rb^{+}$ flux into human red blood cells. Figure 6.2A demonstrates that even at exceedingly high concentrations neither Ba^{2+} nor Ca^{2+} were able to inhibit $^{86}Rb^{+}$ flux into human red blood cells. As shown in Figure 6.2B, divalent cations also had little effect on the influx into rabbit red blood cells. These results along with the Tb^{3+} data suggest that the outside transport site binds monovalent cations but not divalent or trivalent cations, and that the discrimination is based upon valence rather than size.





(See next page for legend)

Figure 6.2: Lack of inhibition of Rb^+-uptake by divalent cations. A) Ouabain-sensitive ^{86}Rb flux was measured into human red cells in the presence of 1mM $^{86}Rb^+$ and the indicated concentrations of Ca^{2+} (○) and Ba^{2+} (◊). Neither divalent cation was able to effectively reduce Na pump-mediated $^{86}Rb^+$ uptake. The data from at least three different experiments were normalized to the flux measured in the absence of divalent cations. Points represent means $\pm$ SEM. **B)** Ouabain-sensitive ^{86}Rb flux was measured into rabbit red cells in the presence of 1mM $^{86}Rb^+$ and the indicated concentrations of divalent cations. The data from at least three different experiments were normalized. Points represent means $\pm$ SEM.

Studies on the intracellular site

We next investigated the organic cationic dye chrysoidine as a potential probe for the inside transport site. Figure 6.3 demonstrates that chrysoidine inhibited purified renal Na,K ATPase activity in a dose dependent manner with an IC_{50} of 1.03 ± 0.15 mM. In order to investigate if chrysoidine would compete with K^+ for ATPase activity we performed K^+ activation experiments in the presence or absence of 1 mM chrysoidine and increasing concentrations of K^+. Note that, for ATPase activity, activation by K^+ occurs at the extracellular site. Figure 6.4 shows results from these experiments. In the absence of chrysoidine Vmax was 100.8 ± 3.45 and the Km was 0.155 ± 0.034 mM. In the presence of chrysoidine Vmax was 67.2 ± 2.13 and the Km was 0.215 ± .042 mM.

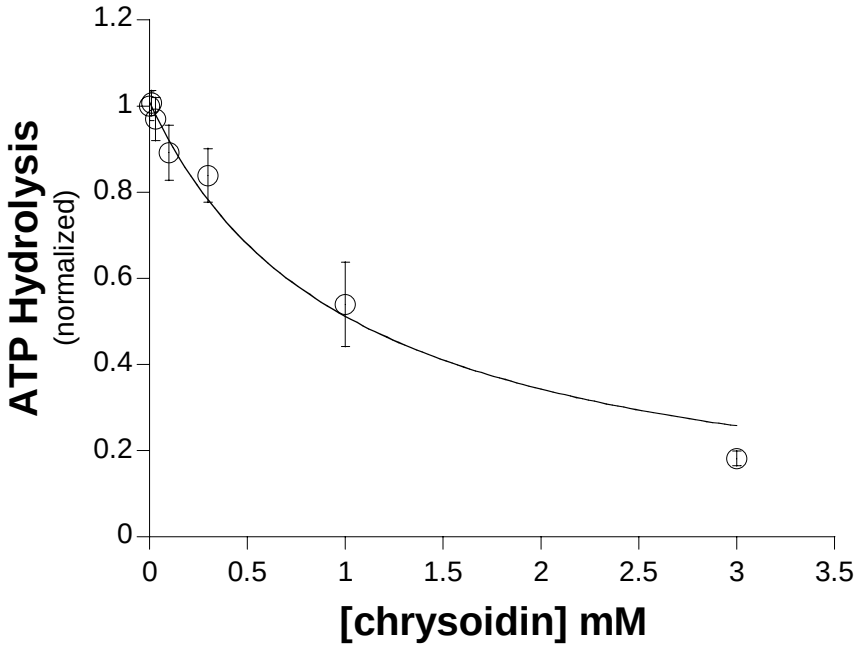


Figure 6.3: Chrysoidine inhibition of purified renal Na,K-ATPase. Ouabain-sensitive Na,K-ATPase activity from purified sheep kidney preparations was measured in the presence of 3 mM ATP, 50 mM Na, 2 mM K, 20 mM Mg and increasing concentrations of chrysoidine. The data were fit to the equation $v = Vo * IC_{50} / (IC_{50} + I)$ where Vo is the maximal velocity in the absence of chrysoidine (i.e. I=0), and I is [chrysoidine]. The IC_{50} was 1.03 $\pm$ 0.15 mM. Data represent n = 3 experiments and are reported as means ± SD. Data were normalized to the activity in the absence of chrysoidine.

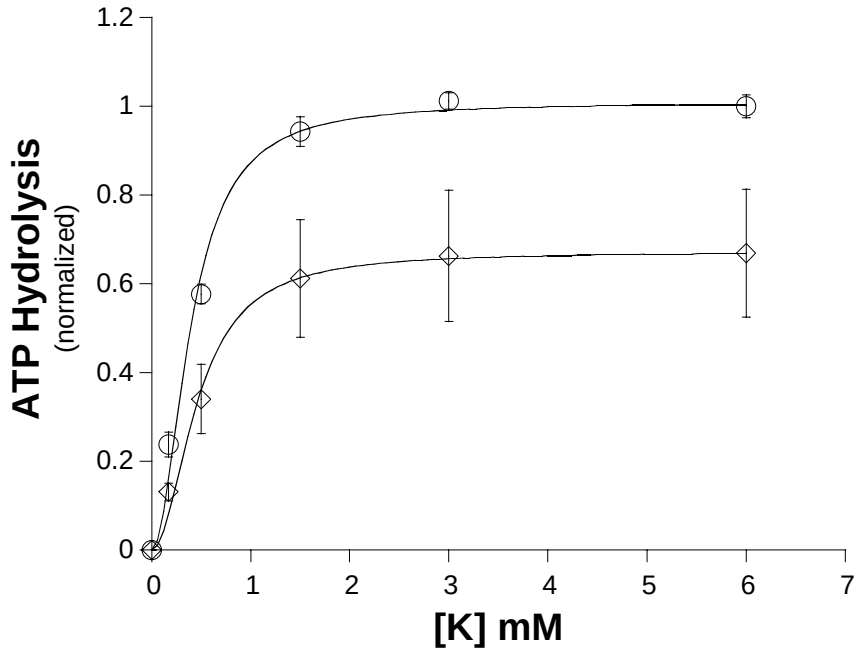


Figure 6.4: K^+ activation of ATPase in the presence and absence of chrysoidine. Ouabain-sensitive Na,K ATPase activity from purified sheep kidney preparations was measured in the presence of 3 mM ATP, 50 mM Na, 20 mM Mg, increasing concentrations of potassium, and the absence or presence of 1 mM chrysoidine. Data were fit to the equation $v = Vmax \times K \times K / (B \times B + K \times K)$, where K is the potassium concentration and B is, in the simplest case, the binding constant. In the absence of chrysoidine, Vmax was 100.8 ± 3.5 and B was 0.155 ± 0.034 mM. In the presence of 1 mM chrysoidine Vmax was 67.2 ± 2.13 and B was 0.215 ± 0.042 mM. Data represent n = 3 experiments and are reported as means ± SD. Data were normalized to control experiments at 6 mM K^+.

Similar experiments were performed to investigate if chrysoidine was competitive with Na^+ for ATPase. Specifically, we performed Na^+ activation experiments in the presence or absence of 0.5 mM chrysoidine and increasing Na^+ concentrations. Na^+ mediated ATPase activation occurs at the intracellular site as opposed to the K^+ activation experiments which occur at the extracellular site. Figure 6.5 shows results from these experiments. An initial curve fit of the data using equation 1 was used. Equation 1 is highly cooperative for Na^+ binding and requires only one Michaelis constant (B).

Equation 1: $Vo = Vmax \times Na \times Na / (C \times C + Na \times Na)$

Using this equation, in the absence of chrysoidine, Vmax was 103.0 ± 2.0 and Km was 14 ± 0.6 mM. In the presence of 0.5 mM chrysoidine Vmax was 77 ± 6.0 and Km was 23 ± 3.0 mM. For reasons outlined in the discussion section we also fit the data using equation 2. Equation 2 introduces an extra term (D x Na) and constrains Vmax to that of control (i.e. 103).

Equation 2: $Vo = 103 \times Na \times Na / (C \times C + D \times Na + Na \times Na)$

Using equation 2, the apparent binding affinities (C and D, respectively) were 13.6 ± 0.8 mM in the absence of chrysoidine and 16.0 ± 2.0 mM in the presence of chrysoidine.

K^+-dependent phosphatase experiments were performed in the presence of increasing concentrations of chrysoidine in order to investigate whether chrysoidine could inhibit this alternative mode of action. Figure 6.6 demonstrates that chrysoidine inhibited purified renal Na,K pNPPase activity in a dose dependent manner with an IC_{50} of 1.82 $^{\pm}$ 0.20 mM. Whether chrysoidine could compete with inside K^+ for pNPPase activity was also determined (Figure 6.7). A K+ dependence of pNPPase activity was performed in the presence and absence of 2 mM chrysoidine. Figure 6.7 demonstrates that in the absence of chrysoidine Vmax is 99.7 ± 2.8 mM and Km is 3.14 ± 0.39 mM. In the presence of 2 mM chrysoidine Vmax is 95.5 ± 2.7 mM and Km is 19.98 ± 1.7 mM.

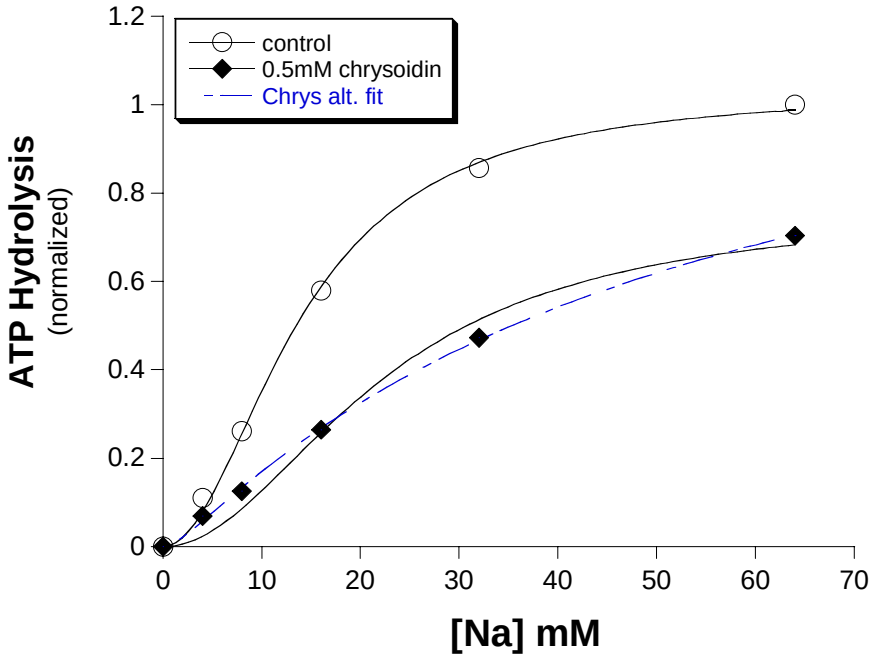


Figure 6.5: Na^+ activation ATPase in the presence and absence of chrysoidine. Ouabain-sensitive Na pump ATPase activity from purified sheep kidney preparations was measured in the presence of 3 mM ATP, 6 mM K, 20 mM Mg, increasing concentrations of sodium and the absence or presence of 0.5 mM chrysoidine. Both sets of data were fit to the equation v = Vmax × Na × Na / (C × C + Na × Na) where Na is the sodium concentration and C is, in the simplest case, the binding constants. Using this equation Vmax was 103.0 ± 2.0 and C was 14 ± 0.6 mM in the absence of chrysoidine. In the presence of 0.5 mM chrysoidine Vmax was 77 ± 6.0 and C was 23 ± 3.0 mM. We also fit the chrysoidine data to the equation Vo = 103 x Na x Na / (C x C + D x Na + Na x Na). Note that this equation constrains Vmax to that of control (103) and introduces the term D x Na. Using this equation the new binding constants are 13.6 ± 0.8 mM and 16 ± 2.0 mM in the absence and presence of chrysoidine respectively. Data represent n = 3 experiments and are reported as means ± SD. Data were normalized to control experiments at 64 mM Na^+.

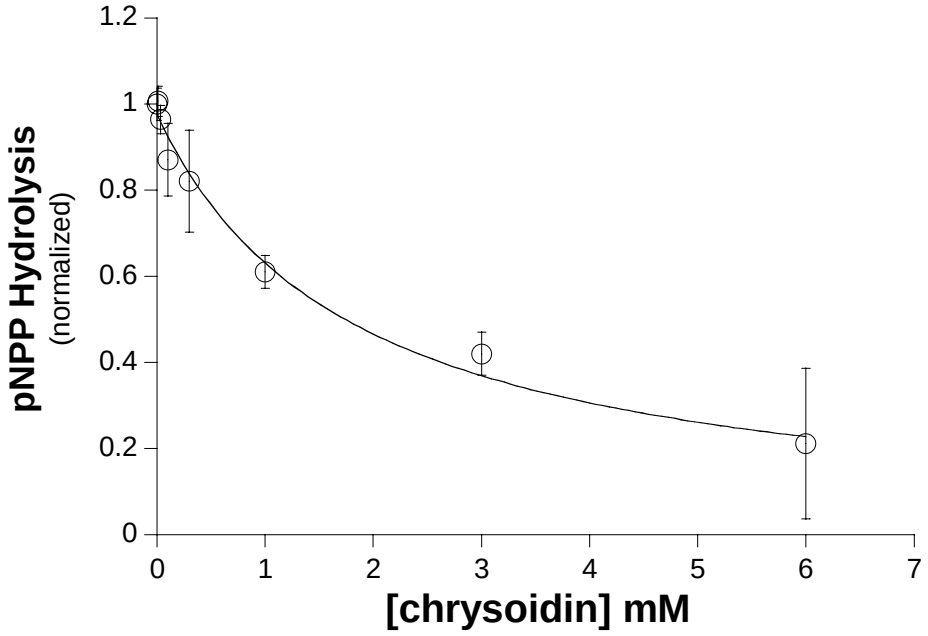


Figure 6.6: Chrysoidine inhibition of purified renal Na,K pNPPase. Ouabain-sensitive Na pump pNPPase activity from purified sheep kidney preparations was measured in the presence of 6 mM pNPP, 2.4 mM K, and 20 mM Mg and indicated concentrations of chrysoidine (0.01, 0.03, 0.1, 0.3, 1, 3 mM) at pH 7.4 The data were fit to the equation $v = Vo * IC_{50}/ (IC_{50} + I)$ where V is the maximal velocity in the absence of chrysoidine (i.e. I=0), and I is [chrysoidine]. The IC_{50} was 1.82 $\pm$ 0.20 mM. Data represent n = 3 experiments and are reported as means ± SD. Data were normalized to the activity in the absence of chrysoidine.

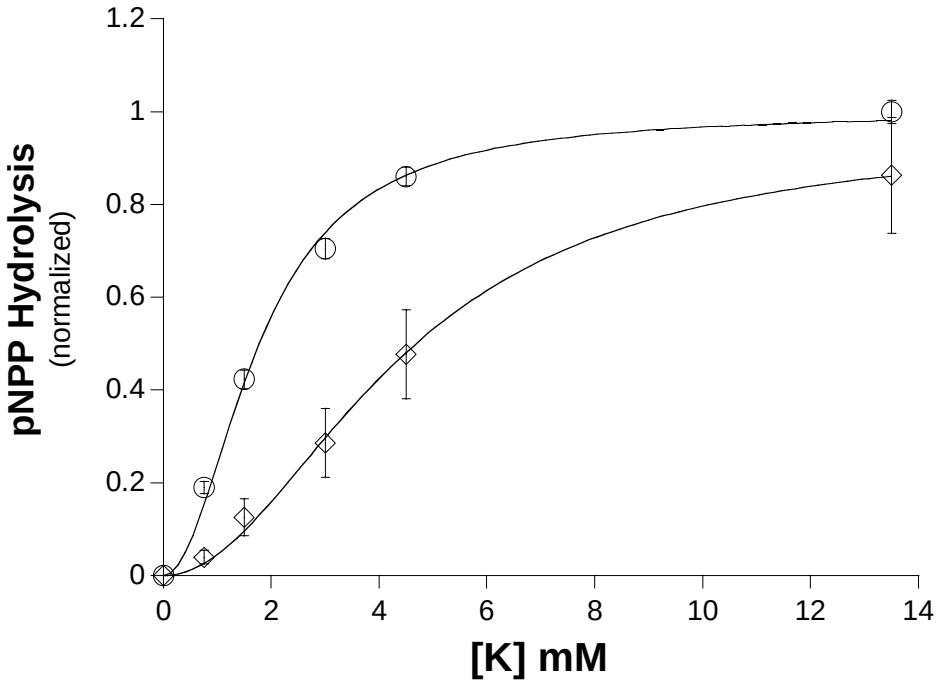


Figure 6.7: K^+ activation pNPPase in the presence and absence of chrysoidine. Ouabain-sensitive Na pump pNPPase activity from purified sheep kidney preparations was measured in the presence of 6 mM pNPP, 20 mM Mg, increasing concentrations of potassium and the absence or presence of 2 mM chrysoidine. The data were fit to v = Vmax × K × K / (B × B + K × K). In the absence of chrysoidine Vmax was 99.7 ± 2.8 and B was 3.14 ± 0.39 mM. In the presence of 2 mM chrysoidine Vmax was 95.5 ± 2.7 and B was 19.98 ± 1.7 mM. Data represent n = 3 experiments and are reported as means ± SD. Data were normalized to controls at 13.5 mM K^+.

DISCUSSION

In this paper we investigated 1) the binding selectivity of the outside transport site as well as 2) potential optical probes for the E_{in} and E_{out} transport sites. Our results suggest that the outside transport site is remarkably selective for monovalent cations over similarly sized divalent cations. In regards to potential optical probes for the transport sites we found that terbium is not competitive with $^{86}Rb^{+}$ influx suggesting Tb^{3+} is not binding to the outside transport site. This result eliminates Tb^{3+} as a potential probe for E_{out}. Further, we demonstrate that chrysoidine competes with K^{+} for pNPPase (an intracellular effect) but does not compete for ATPase (an extracellular effect). Finally, whether Na^{+} was able to compete with chrysoidine for ATPase depended upon the curve fit, however, we feel Na^{+} does compete with chrysoidine (see discussion below). These results suggest chrysoidine is able to bind to the E_{in} conformation.

Selectivity of outside transport site

Our data provides evidence that the outside transport site of the Na,K pump is highly selective for monovalent cations such as K^{+} and Rb^{+} over the divalent cations Ba^{2+} and Ca^{2+}. Many studies have shown that Ba^{2+} can inhibit K^{+} channels by binding to the pore but typically only concentrations of < 5 mM are used (Armstrong et al 1980; Vergara et al 1999; Vergara et al 1983). We show that even at exceedingly high concentrations neither Ca^{2+} nor Ba^{2+} were able to compete with $^{86}Rb^{+}$ to diminish the influx of Rb^{+}. These data suggest Ba^{2+} and Ca^{2+}, despite being similar in size to their monovalent counterparts, are unable to bind to E_{out}. This suggests that it is the divalent

nature of Ba^{2+} and Ca^{2+} , not size, that prevent their binding to the E_{out}. Intriguingly, the palytoxin treated pump is weakly permeant to extracellular Ca^{2+} or Ba^{2+} (Rouzaire-Dubois et al 1990; Artiga et al 2004).

Interestingly, divalent cations such as Ca^{2+} have been shown able to access E_{in} (Vasallo et al 1986; Forbush 1988). Vasallo and Post, examining the rate of phosphorylation of the Na pump, showed that Ca^{2+} mimicked K^+ in many respects (Vassallo et al 1986). Forbush showed that $^{45}Ca^{2+}$ directly bound to the pump and was occluded. However, Ca^{2+} was unable to be released into the extracellular space (Forbush et al 1988). In another study, Schneeberger and Apell, using the electrochromic fluorescent dye RH431, demonstrated the presence of a cytoplasmic binding site capable of binding cations including divalents independent of valence and size (Schneeberger et al 2001). Finally, we recently showed that both Ba^{2+} and Sr^{2+} competed with Na^+ for ATPase activity and with K^+ for pNPPase activity suggesting that these two divalent cations are able to bind to the intracellular transport site (Gatto et al).

It is interesting to note then that while the inside transport site is able to bind divalent cations the outside transport site is unable to bind divalent cations. A physiological rationale can be made to explain this difference in selectivity. In regards to the intracellular space, Ca^{2+}, especially under basal conditions, is kept at a much lower concentration (on the order of 0.1 μM) than Na^+ or K^+ (on the order of 10 or 100 mM, respectively). Even if the site bound Ca^{2+}, Na^+ and K^+ with equal affinity, under physiological conditions, Na^+ or K^+ would be much more likely to bind than Ca^{2+}. Therefore, there would have been very little, if any, pressure to select for Na pumps with intracellular selectivity of Na^+ over Ca^{2+}. In contrast, the divalent cation, Ca^{2+}, and the

monovalent cation K^+, are present in the extracellular fluid at similar concentrations. If the pump transported both Ca^{2+} and K^+, that would seem counterproductive as the cell works hard to pump Ca^{2+} out- why create another pathway for entry? In addition, consider what would happen with a putative Na^+ pump that bound Ca^{2+} or K^+ at the extracellular site, but transported only K^+. Then, under physiological conditions, this putative pump would be working less effectively at removing Na^+ than the existing pump. Therefore, there would be a selective advantage for pumps that could bind K^+ and not Ca^{2+}. In theory, this selection could have been based upon size, not charge (since Ca^{2+} is smaller than K^+). Our results show that the selection is actually based on charge, since Ba^{2+}, which is the same size as K^+, does not bind. As suggested by Post, a positively charged residue near the extracellular entrance to the transport site would account for this selectivity (Vasallo et al 1986). It would be interesting to try to create a pump that bound Ba^{2+} and K^+ outside by removing this charge.

Interpretation of Tb^{3+} results

Results from the Tb^{3+} experiments indicate that at both concentrations of terbium (15 and 25 μM) $^{86}Rb^+$ influx was significantly diminished. Further, increasing $^{86}Rb^+$ concentrations were unable to overcome the terbium induced inhibition suggesting that terbium is a non-competitive inhibitor of inward $^{86}Rb^+$ transport. Non-competitive inhibition suggests that the inhibitor, in this case terbium, is binding to a site unrelated to the substrate binding site. One possibility for the terbium binding site is the three adjacent glutamates in the first extracellular loop.

Interpretation of chrysoidine inhibition

As a potential optical probe for the inside transport sight we investigated chrysoidine. Chrysoidine (4-phenylazo-*m*-phenylenediamine) is a relatively large organic cationic dye that structurally resembles propyl diamine. We have previously shown that propyl diamine is a competitive inhibitor at the inside transport site (Gatto et al 2006). By establishing that the relatively large chrysoidine compound is able to gain access to E_{in} we are encouraged that a similarly bulky fluorescent compound based on propyl diamine may be a useful probe for Ein.

Potassium activates ATPase activity by binding to the extracellular transport site. We found that K^+ was not able to overcome the inhibition by chrysoidine of ATPase activity. This is consistent with chrysoidine not binding to E_{out}. Potassium also activates pNPPase activity but by binding to the intracellular transport site (Drapeau et al 1980). We found that K^+ was able to overcome the inhibition by chrysoidine of pNPPase activity. This is consistent with chrysoidine binding to E_{in}.

Sodium activates ATPase activity by binding to the intracellular transport site. Whether Na^+ overcomes the inhibition by chrysoidine of ATPase activity depends upon the curve fit. The simplest equation that fit the control activation curve is highly cooperative for Na and requires only one Michaelis constant. Using this equation for the chrysoidine curve gave a fit in which the Vmax value is significantly decreased. If Vmax is decreased, then chrysoidine and Na^+ do not compete. It is difficult, kinetically and structurally, to construct models in which intracellular K^+ and chrysoidine compete yet intracellular Na^+ and chrysoidine do not compete. Thus we also fit the data to a more general equation, which contains two Michaelis constants. This equation fits the

chrysoidine data better and is consistent with no change of Vmax for chrysoidine. Thus we feel the most likely explanation for our data is that chrysoidine competes with K^+ or Na^+ at the intracellular surface. The fact that the D term significantly increased suggests that there are concentrations of chrysoidine and Na^+ such that one of each is bound to the pump.

In summary, we show that the outside transport site is highly selective for the monovalent cation $^{86}Rb^+$ over the divalent cations Ca^{2+} and Ba^{2+}. We also show that Tb^{3+} is a non-competitive inhibitor of $^{86}Rb^+$ influx, which suggests that Tb^{3+} is not binding to the outside transport site and therefore is not a probable probe for the transport site. Lastly, we provide evidence that suggests chrysoidine competes with K^+ and Na^+ at the inside transport site. Chrysoidine will be used as a lead compound to develop a similarly structured fluorescent compound as a probe for the transport site.

CHAPTER 7

Discussion

7.1 Summary of Major Findings and Contributions

Chapter 2

In chapter 2 I investigated the effects of peroxynitrite on purified renal Na pump activity. Peroxynitrite is a reactive nitrogen species produced when nitric oxide and superoxide react. In vivo studies suggest that reactive oxygen species and perhaps peroxynitrite can influence Na,K-ATPase function (White et al 2008; Comellas et al 2006; Zhang et al 2002). However, the direct effects of peroxynitrite on Na,K-ATPase function remain unknown. We show that a single bolus addition of peroxynitrite inhibited purified renal Na, K – ATPase activity with an IC_{50} of 107 ± 9 μM. To mimic cellular/physiological production of peroxynitrite a syringe pump was used to slowly release (≈ 0.85 μM / second) peroxynitrite. This treatment was similarly effective at inhibiting Na,K-ATPase activity as a single bolus addition of equal cumulative concentration. Peroxynitrite treatment produced 3-nitrotyrosine residues on the α, β and FXYD subunits of the Na pump. Interestingly, the flavonoid epicatechin, which prevented tyrosine nitration was unable to blunt peroxynitrite induced ATPase inhibition suggesting that tyrosine nitration is not required for inhibition. Peroxynitrite lead to a decrease in iodoacetamidofluorescein labeling implying cysteine modifications were induced. Glutathione was unable to reverse ATPase inhibition. The presence of Na^+ and low Mg ATP during peroxynitrite treatment increased the IC_{50} to 145 ± 10 μM while the presence of K^+ and low Mg ATP increased the IC_{50} to 255 ± 13 μM. This result suggests that the E-PNa conformation of the pump is slightly more sensitive to peroxynitrite than

the E (K) conformation. Taken together these results show that peroxynitrite is a potent irreversible inhibitor of Na,K-ATPase activity and that peroxynitrite can induce nitration of α, β, and FXYD subunits and modification of cysteine residues. Glutathione did not reverse the ATPase inhibition. While results suggest that tyrosine nitration is not required for ATPase inhibition, nitration could alter trafficking and degradation of the Na pump (see below for more).

Chapter 3

In chapter 3 I attempted to address two questions; 1) can the Na pump be nitrated under cellular conditions and 2) is the Na pump nitrated in vivo. Detecting nitrated Na pumps would suggest that peroxynitrite is reacting with the Na pump in cellular conditions. To determine this, Na pumps were immunoprecipitated from either LLCPK1 cells, a porcine renal epithelial cell line, or from tissue homogenates from a sedentary pig fed a high fat diet. Immunoprecipitated Na pumps were then probed for the presence of 3-nitrotyrosine. Results presented in chapter 3 suggest that LLCPK1 cells treated with peroxynitrite contain nitrated Na pumps and that porcine tissue contains both nitrated proteins and Na pumps. However, more experiments are needed to determine if the Na pumps from the tissue are themselves nitrated.

Chapter 4

Given the potential for 3-nitrotyrosine to alter tyrosine phosphorylation and protein function, the presence of a denitrase enzyme capable of removing NO_2 groups could be an important signaling mechanism. It would further suggest that cellular 3-

nitrotyrosine levels are determined not only by peroxynitrite and other nitrating agents but also by the activity of the putative denitrase. In chapter 4 I presented some experiments attempting to detect a denitrase activity in red blood cells. Red blood cells were chosen because they circulate throughout the cardiovascular system and are exposed to a myriad of bioactive factors including nitric oxide and superoxide produced from endothelial cells, platelet cells, macrophages and perhaps red blood cells themselves (Sase et al 1995, Fialkow et al 2007; Kleinbongard et al 2006). Red blood cell exposure to peroxynitrite over 120 days could lead to accumulation of peroxynitrite induced protein modifications such as 3-nitrotyrosine which could disrupt red blood cell functioning. Further, because red blood cells contain no nucleus they are unable to synthesize new proteins and probably have a limited capacity to degrade damaged proteins. However, the presence of a denitrase activity might serve as a critical repair mechanism. In chapter 4, red blood cell membrane proteins were isolated and treated with peroxynitrte to induce nitration and then incubated with either red blood cell lysate or a control buffer. Experiments shown in chapter 4 hint at the possibility of a denitrase activity, however the totality of experiments neither clearly demonstrated an obvious denitrase activity nor ruled one out.

Chapter 5

Perivascular adipose tissue has been reported to blunt agonist induced arterial tone via a relaxing factor acting in a paracrine manner (Gollasch et al 2004). However, the exact chemical nature of this relaxing factor, termed adipocyte derived relaxing factor (ADRF), is unknown. The purpose of this study was to test the hypothesis that porcine

coronary artery perivascular adipose tissue blunts constriction and that the anti-contractile effect of perivascular adipose tissue is altered by diet and/or exercise training. Fourteen adult male pigs were fed a normal-fat diet and ten adult male pigs were fed a high-fat/cholesterol diet. Four weeks after the initiation of diet, pigs were exercise trained or remained sedentary for 16 weeks, yielding four groups:1) normal fat-sedentary, 2) normal-fat exercised, 3) high fat sedentary and 4) high fat exercise . Left circumflex coronary artery (LCX) rings were prepared with vascular adipose tissue left intact or removed. LCX reactivity to acetylcholine (ACh), endothelin (ET-1), bradykinin (BK) and sodium nitroprusside (SNP) was assessed using standard in vitro techniques. Results demonstrated that both ACh and ET-1 elicited dose dependent increases in tension from LCX rings from all groups. Removal of perivascular adipose had no significant effect on ACh-induced contractions in any group. In contrast, removal of perivascular adipose tissue increased ET-1-induced tension in LCX from normal fat-sedentary, high fat-sedentary and high fat - exercise but not normal fat-exercised animals. Perivascular adipose tissue had no significant effect on relaxation responses to BK except in high fat exercised animals where removal of perivascular adipose increased BK-induced relaxation. Removal of vascular adipose tissue decreased SNP-induced relaxation in LCX from high fat fed pigs, but not LCX from normal fat fed pigs, suggesting basal release of a relaxing factor LCX isolated from high fat fed pigs. In conclusion perivascular adipose tissue blunts contraction induced by ET-1 in LCX from normal fat and high fat fed pigs. While exercise abolished this effect of perivascular adipose tissue in normal fat fed pigs, exercise did not alter the anti-contractile effect in high fat fed pigs. While this study did

not address the mechanisms involved later in this chapter I will discuss the possibility that ADRF could be a reactive oxygen/nitrogen species (see below section 7.4).

Chapter 6

Optical probes have provided valuable insight into the structure/function of many proteins. With regard to the Na,K pump, for example, FITC has been shown to covalently label Lys 501 in the ATP binding site with a ratio of one fluorescein molecule per pump (Karlish et al 1980). The bound fluorescein acts as a convenient reporter group that has been used to monitor conformational changes (Karlish et al 1980). Another fluorescent probe used in Na,K studies is eosin, which, unlike FITC, binds non-covalently to the ATP site. Eosin has also been used to distinguish between conformations (Skou et al 1981). Despite these probes for the ATP site, there have been few studies investigating fluorescent probes at the Na pump transport site. Finding such a probe would allow studies that monitor the conformational changes at the transport site and would greatly contribute to our knowledge of pump structure/function.

Here we report on potential optical probes for both the inside and outside transport site. We investigated the effects of the trivalent, luminescent lanthanide terbium (Tb^{3+}), as a potential probe for the outside transport site (E_{out}) by measuring rubidium (Rb^{+}) influx into red blood cells. Eout is the conformation with the transport site facing extracellularly. We found that Tb^{3+} at 15 and 25 μM was a non-competitive inhibitor of $^{86}Rb^{+}$ influx suggesting Tb^{3+} is not binding to the transport site. This result reduces the usefulness of Tb^{3+} as a potential probe for the E_{out} conformation. We also investigated the specificity of E_{out} for monovalent cations by measuring Rb^{+} influx into red blood cells in

the presence of divalent cations. Ba^{2+}, Sr^{2+} and Ca^{2+}, at concentrations > 50 mM had minimal effects on Rb^{+} influx into red blood cells (1 mM Rb-out) suggesting that the outside transport site is very specific for monovalent cations over divalent cations. Chrysoidine was tested as a potential probe for $E_{in.}$ E_{in} is the conformation with the transport site facing intracellularly. Using a purified renal Na preparation we also found that chrysoidine (4-phenylazo-*m*-phenylenediamine) competes with Na^{+} for ATPase activity and K^{+} for pNPPase activity suggesting it is binding to the E_{in} conformation. Chrysoidine and similar compounds may be useful as optical probes of the E_{in} conformation.

7.2 Recurring themes from my work

At first glance it might appear that the topics presented here are somewhat unrelated to each other. However, there are several main themes that unify and weave throughout this body of work. The first theme, understanding and determining different aspects of Na pump function and regulation, can be found in chapters 2, 3 and 6. With regards to the first theme I have addressed specific gaps in knowledge regarding potential mechanism(s) that regulate Na pump activity. For example, in chapter 2 I presented original work focused on determining the effects of peroxynitrite on purified renal Na pump activity. Experiments presented in chapter 2 clearly demonstrate that peroxynitrite is a potent inhibitor of Na,K ATPase activity and that peroxynitrite nitrates alpha, gamma and FXYD tyrosine residues. Peroxynitrite also decreased IAF labeling demonstrating that thiol groups are modified. After determining the effects of peroxynitrite on purified Na pump I then attempted to determine if the Na pump could be a target of peroxynitrite

in vivo under cellular conditions. In chapter 3, I attempted to answer this question by immunoprecipitating Na pumps from peroxynitrite treated LLC PK1 cells and porcine tissue homogenates and then probing for the presence of 3-nitrotyrosine. The results presented in chapter 3 suggest that Na pumps in LLCPk1 cells treated with peroxynitrite contain 3-nitrotyrosine residues implying that it is possible to nitrate the Na pump under cellular conditions. Further, Western blots using tissue homogenates from high fat fed sedentary Yucatan pigs exhibited a positive 3-nitrotyrosine signal and a positive Na pump signal. Experiments aimed at immunoprecipitating Na pumps from these tissues and probing for 3-nitrotyrosine are ongoing.

I also have addressed some very basic aspects of the Na pump and potential tools that might lead to a better understanding of how the Na pump works. For example, in chapter 6, I presented a study providing evidence that the outside transport site of the Na pump is remarkably specific for monovalent cations over divalent cations. Potential probes for the transport site were also investigated. Specifically we investigated terbium, a luminescent lanthanide, and found that it is a non-competitive inhibitor of Rb^+ influx suggesting it did not bind to the outside transport site and thus reducing its potential as a fluorescent reporter. However, chrysoidine, a diamine, competed with Na^+ for ATPase activity suggesting it is binding to the inside transport site increasing the potential for it, or similar compounds, to used as tools to probe the inside transport site. Taken together, in many of the experiments from chapters 2,3 and 6, it was our goal to gain a better understanding of potential regulators of the Na pump, such as peroxynitrite and tyrosine nitration, and to address some very basic aspects of the Na pump such as cation selectivity.

The second major theme of my work is understanding how reactive oxygen and reactive nitrogen species (ROS/RNS), like peroxynitrite, influence protein function and signaling pathways. This theme can be found in chapters 2, 4 and 5. In chapter 2 I clearly show that the RNS species peroxynitrite is an irreversible inhibitor of ATPase activity. By incorporating both the Na pump and ROS chapter 2 clearly contains overlap between both themes. In chapter 4 I presented data from experiments attempting to detect a denitrase activity in red blood cell lysates. While the data did not conclusively demonstrate a consistent denitrase activity it also did not rule one out. If a denitrase activity is present it would represent a new mechanism regulating the influence of ROS/RNS on protein function. In chapter 5 I provided evidence that the presence of perivascular adipose tissue blunted agonist induced constriction induced by ET-1 in isolated coronary arteries from Yucatan pigs fed either a normal fat diet or a high fat diet. Interestingly, exercise seemed to abolish this effect in normal fed pigs but not in high fat fed pigs. Adipose tissue has been reported to release an adipose derived relaxing factor (ADRF) (see Gollasch et al 2004) and while we did not investigate the mechanism(s) responsible for the attenuation of agonist induced constriction it could have been due to ADRF. The exact chemical nature of ADRF is not known. It could be that ADRF is a ROS/RNS produced from perivascular adipose tissue that alters vascular function (see below in section 7.4).

One beneficial aspect of doing such varied work across the spectrum of physiology is that it allowed me to see first hand the pros and cons of using a myriad of technical approaches to answer biological questions. It seems there are often at least two different philosophical aspects with any given methodological approach. These are the

ability to generate physiologically relevant data vs. the ability, for lack of better term, to generate clean data. While not necessarily mutually exclusive it often seems that these two concepts are at odds with each other. That is, the more physiologically relevant data becomes the more difficult it becomes to include all the necessary controls to ensure a clean answer. For example, generating peroxynitrite ATPase dose response curves yielded a clean answer showing that peroxynitrite inhibited ATPase activity. However, this type of experiment does not address the cellular/physiological relevance of this reaction and to what extent it actually occurs in cells. On the other hand probing for the presence of nitrated Na pumps from tissue homogenates has a high degree of cellular/physiological relevance. However, based on experience interpreting Western blots, in all likelihood these experiments will not yield a clean result. For example, the presence of a denitrase activity and/or the potential for non-enzymatic reduction of nitrotyrosine to aminotyrosine can confound the results. In a similar manner the studies on isolated coronary blood vessels gave very physiologically relevant answers but did not address some of the mechanisms involved.

In many of my experiments kinetic analyses were used to infer conclusions on various aspects of Na pump function. While it is tremendously beneficial to know the protein structure, the power of kinetic data and kinetic analyses can not be underestimated. For example, proper kinetic analysis can provide a great deal of insight into mechanisms of enzymatic reactions and protein structure without necessarily knowing the exact structure of the protein of interest. Long before the crystal structure of the Na pump and SERCA were known a tremendous amount concerning the enzymatic cycle of the Na pump (see figure 1.2) was known. One advantage of using a kinetic

approach is that it provides "real time" information on a functioning enzyme. On the other hand a crystal structure provides only a snapshot of the protein in one conformation. In chapter 6 a kinetic approach was used to determine that terbium was not binding to the outside transport site which allowed us to conclude that terbium would not make a useful probe for the outside transport site. Specifically, figure 6.1 shows that the presence of 15 and 25 μM terbium significantly decreased Vmax but with no change in Km, consistent with terbium acting as a non-competitive inhibitor. In chapter 2 I presented data showing that the EP Na conformation had a lower IC50 compared to the IC50 of the E(K) conformation which allowed me to conclude that the EP Na conformation is more sensitive to peroxynitrite. Being exposed to these basic kinetic analyses provides a strong background for future experimental approaches.

7.3 Potential directions for future experiments/studies

Identifying which residues in the Na pump are nitrated

One issue that was not addressed was 1) which tyrosine residues within the Na pump are being modified and 2) where are those tyrosine residues located within the structure of the Na pump? The sheep alpha, beta, and FXYD subunits contain 25, 20 and 3 tyrosine residues respectively and clearly one or more tyrosine residue on each subunit is being nitrated by peroxynitrite. Identifying which residue(s) are nitrated is an interesting question and probably would have taken on a higher priority if the epicatechin data had clearly demonstrated that peroxynitrite induced inhibition was solely due to tyrosine nitration. In other words, if tyrosine nitration had exclusively been the cause of ATPase inhibition, as has been suggested for peroxynitrite induced SOD inhibition, then

determining which tyrosine(s) are nitrated and their location within the Na pump structure would have been particularly insightful. Because this doesn't appear to be the case knowing exactly which residues are modified and where they are located became less of a priority. However, knowing this information could still provide insight into how peroxynitrite induces inhibition. Further, it might shed light on the kind of local environmental conditions required for peroxynitrite induced tyrosine nitration to occur. For example, are tyrosine residues located in hydrophobic transmembrane spanning domains more or less likely to be nitrated than tyrosine residues located in the hydrophilic cytoplasmic domains. Evidence does suggest that hydrophobicity is important in tyrosine nitration (Bartesaghi et al 2007).

Bulk assay vs individual assay

Results presented in chapter 2 show that ~ 100 μM peroxynitrite reduced ATPase activity by half. These experiments and others reported in this dissertation were done using traditional bulk assay techniques which do not provide insight on what is occurring at the single molecule level. For example, when peroxynitrite treatment reduces bulk activity by half, is this because 1) each individual pump is working half as fast or 2) half of the pumps are completely inhibited but the other half are functioning at full capacity? Having an individual assay technique that could measure single molecule pump activity would shed light on this question and also provide insight into kinetics and distribution of single molecule pump activity rates. In essence such an assay would be analogous to patch clamping which allows for studies on single channels to be performed.

As an initial project I attempted to develop a novel technique for measuring single molecule Na pump activity. The basic premise was to place a single Na pump in a very small container (~ nanoliter to picoliter) and allow it hydrolyze 3-0-methylfluorescein phosphate (MFP), a non-fluorescent compound, into methylfluorescein (MF) which is fluorescent. The Na pump exhibits a potassium stimulated phosphatase activity which catalyzes the breakdown of MFP into MF. In a very small chamber the fluorescein concentration could reach as high as 100 nM in a relatively short time period (~ 10 minutes). Using a Zeiss 2 Photon Confocal System I attempted to measure an increase in fluorescence reflective of Na pump activity. The main obstacle encountered with this project was creating uniform nanoliter to picoliter sized chambers that we could fill with an aqueous solution. Several different techniques were used to generate the chambers including using polydimethylsiloxane (PDMS) to create a mold of an AFM calibration chip and using a spray bottle to generate very small drops of water that were then covered with a thin layer of oil to prevent evaporation. Ultimately this project was but on the back burner as other projects took hold. However, this is still a really interesting project and if executed could yield some very novel and interesting results.

Initially we were planning to use this single molecule technique to examine how superoxide anion and hydroxyl radical damaged the pump. However, these ROS species gave inconsistent inhibition results when used with our traditional bulk assay technique suggesting it would be difficult to use them with our single molecule assay. Perhaps this was because we were synthesizing superoxide from xanthine oxidase and hydroxyl radical from the Fenton reaction. In contrast to these ROS, peroxynitrite provided much more reliable, consistent inhibition. Further, because peroxynitrite is stable at alkaline

conditions it can be synthesized and stored until use. If the lab revisits the single molecule project it might be worth using peroxynitrite instead of superoxide and hydroxyl radical.

Alternative methods for addressing role of 3-nitrotyrosine in Na pump function

One issue that I attempted to address was whether 3-nitrotyrosine itself accounted for the loss of Na,K ATPase activity (see chapter 2). I attempted to address this issue by using the flavonoid epicatechin which selectively prevents nitration but reportedly not other peroxynitrite induced oxidation reactions (Schroeder et al 2001). If the presence of epicatechin would have returned ATPase activity to that of controls it would have suggested that tyrosine nitration accounted for the loss of peroxynitrite induced ATPase activity. However, this was not the case. In fact ATPase inhibition in the presence of epicatechin was actually enhanced. This result suggests that tyrosine nitration is not required for inhibition. However, we cannot conclude that solely nitrating Na pump tyrosines does not cause inhibition. At least two mechanisms might explain why the presence of epicatechin enhanced inhibition. It could be that peroxynitrite reacts with epicatechin to produc a substance that causes more inhibition than peroxynitrite alone. Alternatilvely, epicatechin itself could alter the conformation of the pump in turn exposing more ROS modifiable residues which enhances peroxynitrite induced inhibition. Another interpreatation is that 3-nitrtoyrinse induces a protective effect.

Another, and perhaps better, strategy for specifically addressing the role of tyrosine nitration on ATPase activity is to induce tyrosine nitration but avoid other confounding peroxynitrite induced side reactions/modifications such as those that occur

to cysteine residues. Tetranitromethane (TNM) is a compound that predominates as an oxidizing agent at pH 6.0. However, at pH 8.0 it reportedly acts exclusively as a tyrosine nitrating agent (Sokolovsky et al 1966). Using TNM at pH 8.0 I could have exclusively nitrated tyrosine residues and measured ATPase activity. However, given the dual nature of TNM it is likely that results obtained would have been difficult to interpret.

Another method to address this issue could be to engineer Na pumps that contain 3-nitrotyrosine residue(s), an unnatural amino acid. Incorporating unnatural amino acids into a protein is a method that has been used to address many aspects of protein function (Dougherty 2000). I recently learned about this technique while attending Dr. Horn's seminar at Dalton Cardiovascular Research Center. Dr. Horn was expressing fluorinated Phe449, an unnatural amino acid, in Shaker potassium channels as a way to probe how the channel blocker tetraethylammonium (TEA) worked (Ahern et al 2006). After hearing his seminar it occurred to me that in a similar manner it might be possible to express 3-nitrotyrosine residues in the Na pump or any protein for that matter. Expressing unnatural amino acids, such as 3-nitrotyrosine, is typically done by incorporating a TAG stop codon into mRNA of the protein to be studied (in this case Na pump) at the position of interest (i.e. sequence that codes for a tyrosine residue(s). A tRNA capable of recognizing the TAG sequence is then chemically altered such that it contains the unnatural amino acid (in this case 3-nitrotyrosine). Both the modified mRNA and tRNA are then incorporated into an appropriate expression system providing the protein of interest, now expressing unnatural amino acids, to be studied. Expressing the unnatural amino acid 3-nitrotyrosine in Na pumps would have the following advantages. First, there would be no confounding side reactions that occur when using chemicals such as peroxynitrite or

TNM. Secondly, this technique would allow incorporation of 3-nitrotyorisne into known locations. When using chemical compounds it is not necessarily known which tyrosine residues are nitrated without further experiments. Expressing 3-nitrotyrosine in known locations could be particularly beneficial when it comes to tyrosine 10 for example. Insulin induced phosphorylation of tyrosine 10 has been implicated in insulin induced Na pump trafficking. It would be interesting to see if nitrated tyrosine 10 mimics or inhibits this insulin response. One drawback to this technique is that the Na pump might not fold properly and as a result it might not make it out of the endoplasmic reticulum.

Confirming that the Na pump is nitrated in vivo

In chapter 3 I attempted to determine if the Na pump is a "target" of peroxynitrite under cellular condition and in vivo. To ascertain this I investigated if Na pumps from LLCPK1 cells and porcine tissue homogenates contained 3-nitrotyrosine residues. One possible concern confounding the interpretation of the Westernblot data from the porcine tissue is the specificity of the primary and secondary antibodies (see figure 3.2 and 3.3). For example, any putative 3-nitrotyrosine signal could be due to non-specific binding of the primary antibody (rabbit anti-3-nitrotyrosine) or the secondary antibody (goat anti rabbit HRP). I addressed non-specific binding of the secondary antibody by exposing one half of the membrane to only secondary antibody and the other half to both primary and secondary antibodies. Therefore, any signal that was the same between the two membranes was interpreted as a result of non-specific binding of the secondary antibody. Further, any signal present only in the membrane exposed to both primary and secondary antibodies was interpreted as being a true 3-nitrotyrosine signal.

Several additional approaches could be taken to further verify the binding specificify of the 3-nitrotyrosine primary antibody. First, prior to incubating the membrane blot with the primary antibody, the primary antibody could be reacted with a 10 fold excess of nitrated BSA (or some other nitrated protein). This should prevent the primary antibody from binding to membrane bound nitrated proteins and therefore should decrease the 3-nitrotyrosine signal. In this manner if serves as a useful method of verifying a true 3-nitrotyrosine signal. Another approach to verify an authentic 3-nitrotyrosine signal is to reduce 3-nitrotyrosine to aminotyrosine. Dithionite is a compound that can reportedly convert 3-nitrotyrosine to aminotyrosine (Xu et al 2006). In this manner tissue treated with dithionite should yield an attenuated 3-nitrotyrosine signal compared to non-treated tissue and provide another control verifying a true nitrotyrosine signal.

One issue that has not yet been discussed but is worth mentioning is whether the magnitude of peroxynitrite production and tyrosine nitration is large enough to have any substantial influence on protein function and cell behavior. One critique on research in this area, including my own, is that often exogenous peroxynitrite is used and while this has its advantages it does not address endogenous peroxynitrite production and 3-nitrotyrosine yield levels in cells. While a fair amount of immunohistochemical data showing the presence of 3-nitrotyrosine exists it doesn't specifically address the issue at hand. It has been reported that in stressed tissue 3-nitrotyrosine can occur between 10 and 100 pmol/mg tissue which is estimated to correspond to between 5 and 10 tyrosine residues over 10,000 tyrosine residues which is $\sim$ 100-500 umol/mol (Bartesaghi et al 2007). If correct this represents a fairly low nitration yield. However, one confounding

influence could be denitrase and ubiquitination activity which could keep global nitration levels low. Regardless, if these low nitration yield values are reality, the net effect on protein function and cell function could indeed be minimal. Certainly it seems that a substantially higher degree of nitration would be necessary for any significant loss of protein function to occur. For example, it is hard to believe that inhibiting 5-10 out of 1000 (~ 1.0%) pumps would have any significant impact on the cell. Curiously, in some instances there is evidence that peroxynitrite induced nitration of proteins occurs in large enough quantities to contribute to pathology. These include nitration of SOD in human renal allografts and actin in sickle cell disease (MacMillan-Crowet al 1996; Aslan et al 2003). Thus as of now it is difficult to say whether perxoynitrite/nitration reactions occur with enough frequency to influence protein function in vivo.

While nitrating a relatively small number of tyrosine residues might have minimal impact on the net function of a protein pool it could have a more substantial impact on cell signaling pathways. Amplification is a key feature of signal-transducing pathways and involves the amplification of an initially small signal into a much larger signal that can be several orders of magnitude larger than the originating signal. With this in mind nitrating a small number of tyrosine residues, which could mimic or inhibit tyrosine phosphorylation, could ultimately have a substantial impact on signaling cascades. Similarly Bartesaghi has suggested that tyrosine nitration could lead to a gain of function which could activate a previously absent signal. In this manner nitration of a relatively small tyrosine fraction/protein pool could have a substantial impact on signaling pathways.

Does tyrosine nitration lead to protein unfolding, ubiquitination and degradation?

Another area of interest that represents a potential future direction is whether peroxynitrite is capable of influencing protein degradation rates. In this sense peroxynitrite induced modifications, such as 3-nitrotyrosine, could alter protein structure/folding leading to ubiquitination and degradation. This could represent a repair mechanism to get rid of damaged proteins. Incidentally, an alternative repair mechanism could be due to a denitrase activity as described in chapter 4. Little is known about Na pump ubiquitination but ubiquitination and degradation of membranes transport proteins in general is thought to represent an important mechanism controlling the amount of protein localized to the membrane. Interestingly, the Na pump amino acid sequence between the 10^{th} and 30^{th} amino acids is **y**epaavehgd**kk**s**kk**a**kk**er. Lysine (k) residues are known ubiquitination sites and the multiple lysines in this section of the pump could be sites of ubiquitination. Further, nitration of tyrosine 10, located in this same region, either by itself or in conjunction with other oxidative modifications, could cause the Na pump to unfold and in turn expose previously hidden lysine residues that now could lead to protein ubiquitination and degradation. In this manner oxidatively damaged Na pumps, and other proteins, could be removed and thus not allowed to accumulate. One way to measure Na pump ubiquitination is to immunoprecipitate Na pumps and then probe for the presence of ubiquitination. Using this technique one could address under what conditions the Na pump is ubiquitinated including with peroxynitrite treatment. If nitration and other oxidative modifications induce ubiquitination then I would predict peroxynitrite treated LLC-PK1 cells would exhibit elevated levels of ubiquitination compared to non-treated controls.

Do diet and exercise alter levels of 3-nitrotyrosine?

Results from preliminary studies suggest that tissue from a sedentary Yucatan pig fed a high fat diet contained a 3-nitrotyrosine signal, particularly from cardiac tissue. If this preliminary result is repeatable it would be interesting to compare levels of 3-nitrotyrosine between differently treated groups of animals. For example, do exercise trained Yucatan pigs fed a normal diet contain similar, elevated or decreased 3-nitrotyrosine levels? Conventional wisdom would suggest that the exercised animals on a normal diet would be "healthier" from an oxidative/nitrosative stress perspective despite an increase in exercise induced oxygen utilization which promotes ROS formation. While this statement might seem paradoxical, evidence suggests that regular exercise training induces systemic adaptations that promote anti-oxidant and/or oxidative damage repair/eliminating pathways (Radak et al 2008). With this said, exercise trained animals fed a normal diet might contain less 3-nitrotyrosine compared to their sedentary high fat fed counterparts suggesting difference in nitrosative stress from peroxynitrite and/or possible alterations in denitrase activity. If sedentary high fat fed animals were found to contain elevated levels of 3-nitrotyrosine it would suggest that perhaps peroxynitrite and other nitrosative stressors might contribute to some of the pathologies seen in these animals. For example, because peroxynitrite is formed from nitric oxide reacting with superoxide, elevated formation of peroxynitrite could decrease the overall bioavailability of nitric oxide. Decreasing the bioavailability of nitric oxide could have a negative impact on many processes including endothelial dependent vasodilation. Interestingly, there are data suggesting that superoxide from perivascular fat alters vasoreactivity (Gao et al

2006). However, data presented in chapter 5 (figures 5.5 & 5.6) suggest the presence of perivascular adipose tissue did not effect bradykinin induced endothelium dependent vasodilaton. This result would seem to rule out the possibility that excess "spillover" superoxide from perivascular fat is reducing the bioavailability of nitric oxide. Another way that nitrosative stress could contribute to cell dysfunction is by damaging proteins like the Na pump, SERCA, SOD and others and thus preventing them from properly functioning.

7.4 ROS/RNS as signaling molecules

ROS and RNS acting as signaling molecules is an intriguing possibility that has been suggested but is still somewhat controversial (Rhee 2006, D'Autreaux et al 2007, Nathan 2003). A signaling role for ROS is counter-intuitive because implicit in signaling, which in essence is communication, is specificity. However, the reactive nature of ROS would seemingly lack the specificity required for effective signaling. As an analogy, a room full of people all shouting indiscriminately at each would represent ineffective communication in the same manner that ROS indiscriminately reacting with proteins would lead to ineffective cell signaling. However, anybody who has ever seen Wallstreet day traders know that it is possible to communicate during seemingly chaotic circumstances. So how then do ROS effectively signal given the constraints of their inherent reactivity?

To act as effective signaling molecules ROS must impart specificity onto their down stream targets. Exactly how specificity is obtained is unknown. However, one possible way in which ROS could impart specificity is if ROS react and modify only

specific kinds of amino acids. For example cysteine and tyrosine residues are particularly sensitive to ROS modifications. Further specificity could be obtained if only some fraction of total cysteine or tyrosine residues had the necessary local environment that allowed them to be modified by ROS. If protein behavior was altered in only a subset of all proteins that contained the ROS-modifiable amino acids further specificity could be obtained. For example it could be that ROS modifies cysteine and tyrosine residues in many different proteins but only in a few does this modification actually lead to functional and measurable changes in protein behavior. One emerging example of this comes from the Cohen group that has identified a thiol switch in SERCA (see chapter 1) (Adachi et al 2004).

Another possible way to gain specificity is to spatially organize ROS modifiable proteins around ROS production sites. This would, to a certain extent, negate the short half life of many ROS and would ensure the biggest effect. For example, if nitric oxide synthase (NOS) which produces nitric oxide, and NADPH oxidase which produces superoxide, co-localized in the same area as SERCA, the Na pump and/or other proteins they would in theory be exposed to more ROS/RNS. In effect this would create localized areas of elevated ROS, or "hotspots", which could impact the proteins in the immediate area. Interestingly, there is independent evidence supporting that the Na pump, NOS and NADPH are all localized to caveolin (Xie et al 2003; Liu et al 2003; Wang et al 2003; Shaul et al 1996; Garcia-Cardena et al 1996; Hilenski et al 2004). Further, a recent paper by Yang et al demonstrates that in BAECs, TNF-α activates caveolae -localized NADPH and eNOS which increased the presence of 3-nitrotyrosine in proteins localized to caveolae (Yang et al 2007). This group did not identify the specific proteins that were

nitrated but in theory the Na pump could be a target and if so I would predict, based on my own work on purified pump, that Na pumps in these caveloe would be damaged.

I suspect in the coming years more instances of ROS having a role in signaling will be identified. In some instances the end effect of these ROS pathways might already be described but the exact chemical nature (i.e which ROS) of the effector substance is unknown. This is not uncommon in science. For example, nitric oxide was originally described for its effect on vessel reactivity (i.e. endothelium derived relaxing factor (EDRF). It was only later that EDRF was identified as nitric oxide (Furchgott 1996). Likewise the existence of an adipocyte derived relaxing factor (ADRF) has recently been described (see Gallasch for review). Evidence of ADRF comes from vessels studies showing that the presence of perivascular adipose tissue blunts agonist induced constriction. However, the exact chemical nature of ADRF remains unknown. In chapter 5 I presented a paper investigating the effects of perivascular adipose on porcine coronary arteries. Consistent with the literature, in some of the conditions we observed that the presence of perivascular adipose tissue blunted agonist induced constriction. We did not investigate the mechanism but it is consistent with the ADRF theory. The chemical nature of ADRF is not known but the observed ADRF effect could be ROS induced. Spillover nitric oxide, superoxide, and/or peroxynitrite produced from the perivascular adipose tissue could promote vessel relaxation by influencing SERCA activity. As previously mentioned Adachi et al have shown that peroxynitrite induced s-glutathiolation of cys 674 can increase SERCA activity. Increasing SERCA activity would tend to decrease intracellular Ca^{2+} levels promoting a less constricted vessel. In this manner perivascular adipose tissue, acting through ROS/RNS, could impact smooth muscle SERCA activity.

Interestingly, we observed numerous macrophages in HF-SED perivascular adipose (see figure 5.1) and macrophages have the ability to produce a large amount of ROS/RNS in what is called a respiratory burst. One issue with this theory is that given the reactive nature of peroxynitrite and superoxide it would be difficult for them to diffuse from the sites of production in the perivascular fat to SERCA in smooth muscle cells.

7.5 Concluding Remarks

In conclusion I have presented a series of studies investigating the effects of peroxynitrite on Na pump activity, testing compounds as potential reporter probes for the Na pump transport site and investigating the effects of perivascular adipose tissue on porcine coronary artery reactivity. The findings from these studies which are contained in this dissertation report will certainly add in their small way to the larger scientific community by contributing to our understanding of peroxynitrite, pumps and perivascular adipose tissue.

Chapter 8

References

Ackermann U, Geering K. Mutual dependence of Na,K-ATPase alpha- and beta-subunits for correct posttranslational processing and intracellular transport. *FEBS Lett*. 269(1):105-8. 1990.

Adachi T, Weisbrod RM, Pimentel DR, Ying J, Sharov VS, Schöneich C, Cohen RA. S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med* 10(11):1200-7. 2004.

Ahern CA, Eastwood AL, Lester HA, Dougherty DA, Horn R. A cation-pi interaction between extracellular TEA and an aromatic residue in potassium channels. *J Gen Physiol.* 128(6):649-57. 2006.

Albers RW. Biochemical aspects of active transport. *Annu Rev Biochem* 36: 727-56, 1967.

Alvarez B, Radi R. Peroxynitrite reactivity with amino acids and proteins. *Amino Acids*. 25(3-4): 295-311, 2003.

Alvarez, M. N., Piacenza, L., Irigoin, F., Peluffo, G. & Radi, R. Macrophage-derived peroxynitrite diffusion and toxicity to Trypanosoma cruzi. *Arch. Biochem. Biophys.* 432, 222–232. 2004.

Ames, B. N., and M. K. Shigenaga. 1993. DNA and free radicals, p. 1-15. *In* B. Halliwell and O. I. Aruoma (ed.), Oxidants are a major contributor to cancer and aging. Ellis Horwood, New York, N.Y.

Andreassi, M. G. Coronary atherosclerosis and somatic mutations: an overview of the contributive factors for oxidative DNA damage. *Mutat. Res*. 543:67-86. 2003.

Arystarkhova E, Donnet C, Muñoz-Matta A, Specht SC, Sweadner KJ. Multiplicity of expression of FXYD proteins in mammalian cells: dynamic exchange of phospholemman and gamma-subunit in response to stress. *Am J Physiol Cell Physiol* 292(3): C1179-91, 2007.

Armstrong CM, Taylor SR. Interaction of barium ions with potassium channels in squid giant axons. *Biophys J*. 30: 473-88. 1980.

Artigas P, Gadsby DC. Large diameter of palytoxin-induced Na/K pump channels and modulation of palytoxin interaction by Na/K pump ligands. *J Gen Physiol*. 123: 357-76. 2004.

Aslan M, Ryan TM, Townes TM, Coward L, Kirk MC, Barnes S, Alexander CB, Rosenfeld SS, Freeman BA. Nitric oxide-dependent generation of reactive species in sickle cell disease. Actin tyrosine induces defective cytoskeletal polymerization. *J Biol Chem*. 278(6):4194-204. 2003.

Axelsen KB, Palmgren MG. Evolution of substrate specificities in the P-type ATPase superfamily *J Mol Evol* 46(1):84-101. 1998.

Balabanli B, Kamisaki Y, Martin E, Murad F. Requirements for heme and thiols for the nonenzymatic modification of nitrotyrosine. *Proc Natl Acad Sci U S A.* 96(23):13136-41. 1999.

Balasubramanian B, Pogozelski WK, Tullius TD. DNA strand breaking by the hydroxyl radical is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone. *Proc Natl Acad Sci U S A* 95(17):9738-43. 1998.

Bapat S, Verkleij A, Post JA. Peroxynitrite activates mitogen-activated protein kinase (MAPK) via a MEK-independent pathway: a role for protein kinase C. *FEBS Lett* 499(1-2):21-6. 2001.

Barry WH, Hasin Y, Smith TW. Sodium pump inhibition, enhanced calcium influx via sodium-calcium exchange, and positive inotropic response in cultured heart cells. *Circ Res* 56(2):231-41. 1985.

Bartesaghi S, Ferrer-Sueta G, Peluffo G, Valez V, Zhang H, Kalyanaraman B, Radi R. Protein tyrosine nitration in hydrophilic and hydrophobic environments. *Amino Acids* 32(4):501-15. 2007.

Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 271(5 Pt 1): C1424-37, 1996.

Béguin P, Crambert G, Guennoun S, Garty H, Horisberger JD, Geering K. CHIF, a member of the FXYD protein family, is a regulator of Na,K ATPase distinct from the gamma-subunit. *EMBO J* 20(15):3993-4002. 2001.

Béguin P, Crambert G, Monnet-Tschudi F, Uldry M, Horisberger JD, Garty H, Geering K. FXYD7 is a brain-specific regulator of Na,K-ATPase alpha 1-beta isozymes. *EMBO J* 21(13):3264-73. 2002.

Berberian G, Beauge L. Phosphatase activity and potassium transport in liposomes with Na+,K(+)-ATPase incorporated. *Biochim Biophys Acta* 1103: 85-93. 1992.

Bigelow DJ. Nitrotyrosine-modified SERCA2: a cellular sensor of reactive nitrogen species. *Pflugers Arch*. Jan 3, 2008.

Blaustein MP, Lederer WJ. Sodium/calcium exchange: its physiological implications. *Physiol Rev* 79(3): 763-854, 1999.

Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miwa S, Pakay JL, Parker N. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic Biol Med* 37(6):755-67. 2004.

Brotherus JR, Møller JV, Jørgensen PL. Soluble and active renal Na, K-ATPase with maximum protein molecular mass 170,000 +/- 9,000 daltons; formation of larger units by secondary aggregation. *Biochem Biophys Res Commun* 100(1): 146-54, 1981.

Butterfield, D. A., B. J. Howard, and M. A. LaFontaine. 2001. Brain oxidative stress in animal models of accelerated aging and the age-related neurodegenerative disorders, Alzheimer's disease and Huntington's disease. Curr. Med. Chem. **8:**815-828

Caverzasio J, Geering K, Martin PY, Favre H. Insulin-induced stimulation of Na+,K(+)-ATPase activity in kidney proximal tubule cells depends on phosphorylation of the alpha-subunit at Tyr-10. *Mol Biol Cell*. 10(9): 2847-59, 1999.

Chen TY, Hwang TC. CLC-0 and CFTR: chloride channels evolved from transporters. *Physiol Rev*. 88(2):351-87.2008.

Clausen T, Van Hardeveld C, Everts ME. Significance of cation transport in control of energy metabolism and thermogenesis. *Physiol Rev* 71(3):733-74. 1991.

Comellas AP, Dada LA, Lecuona E, Pesce LM, Chandel NS, Quesada N, Budinger GR, Strous GJ, Ciechanover A, Sznajder JI. Hypoxia-mediated degradation of Na,K-ATPase via mitochondrial reactive oxygen species and the ubiquitin-conjugating system. *Circ Res* 98(10): 1314-22, 2006.

Crambert G, Fuzesi M, Garty H, Karlish S, Geering K. Phospholemman (FXYD1) associates with Na,K-ATPase and regulates its transport properties. *Proc Natl Acad Sci U S A* 99(17):11476-81. 2002.

Crambert G, Li C, Claeys D, Geering K. FXYD3 (Mat-8), a new regulator of Na,K-ATPase. *Mol Biol Cell* 16(5):2363-71. 2005.

D'Autréaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* 8(10):813-24. 2007.

David P, Mayan H, Cragoe Jr EJ, Karlish SJ. Structure-activity relations of amiloride derivatives, acting as antagonists of cation binding on Na+/K(+)-ATPase. *Biochim Biophys Acta*. 1146: 59-64. 1993.

David P, Mayan H, Cohen H, Tal DM, Karlish SJ. Guanidinium derivatives act as high affinity antagonists of Na+ ions in occlusion sites of Na+,K(+)-ATPase. *J Biol Chem*. 267 (1992) 1141-9.

David P, Karlish SJ. Characterization of lanthanides as competitors of Na+ and K+ in occlusion sites of renal (Na+,K+)-ATPase. *J Biol Chem*. 266: 14896-902. 1991.

Denicola A, Souza JM, Radi R. Diffusion of peroxynitrite across erythrocyte membranes. *Proc Natl Acad Sci USA* 95: 3566–3571, 1998.

Dougherty DA. Unnatural amino acids as probes of protein structure and function. *Curr Opin Chem Biol* 4(6):645-52. 2000.

Drapeau P, Blostein R. Interactions of K^+ with (Na,K)-ATPase orientation of K^+-phosphatase sites studied with inside-out red cell membrane vesicles. *J Biol Chem* 255: 7827-7834. 1980.

Dubrovska, G., S. Verlohren, F. C. Luft, and M. Gollasch. Mechanisms of ADRF release from rat aortic adventitial adipose tissue. *Am J Physiol Heart Circ Physiol*. 286:H1107-1113, 2004.

Eakle KA, Lyu RM, Farley RA. The influence of beta subunit structure on the interaction of Na+/K(+)-ATPase complexes with Na+. A chimeric beta subunit reduces the Na+ dependence of phosphoenzyme formation from ATP. *J Biol Chem* 270(23):13937-47. 1995.

Eaton P. Protein thiol oxidation in health and disease: techniques for measuring disulfides and related modifications in complex protein mixtures. *Free Radic Biol Med* 40(11): 1889-99, 2006.

Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, van der Vliet A. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391(6665):393-7.1998.

Elsasser TH, Li CJ, Caperna TJ, Kahl S, Schmidt WF. Growth hormone (GH)-associated nitration of Janus kinase-2 at the 1007Y-1008Y epitope impedes phosphorylation at this site: mechanism for and impact of a GH, AKT, and nitric oxide synthase axis on GH signal transduction. *Endocrinology* 148(8): 3792-802, 2007.

Farley RA, Tran CM, Carilli CT, Hawke D, Shively JE. The amino acid sequence of a fluorescein-labeled peptide from the active site of (Na,K)-ATPase. *J Biol Chem* 259(15):9532-5, 1984.

Féraille E, Carranza ML, Gonin S, Béguin P, Pedemonte C, Rousselot M, Caverzasio J, Geering K, Martin PY, Favre H. Insulin-induced stimulation of

Na+,K(+)-ATPase activity in kidney proximal tubule cells depends on phosphorylation of the alpha-subunit at Tyr-10. *Mol Biol Cell* 10(9): 2847-59, 1999.

Ferdinandy P, Schulz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. *Br J Pharmacol* 138(4):532-43, 2003.

Feschenko MS, Sweadner KJ. Conformation-dependent phosphorylation of Na,K-ATPase by protein kinase A and protein kinase C. J Biol Chem 269(48): 30436-44, 1994.

Fialkow L, Wang Y, Downey GP. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free Radic Biol Med* 42(2):153-64. 2007.

Forbush B III. Rapid release of 45Ca from an occluded state of the Na,K-pump. *J Biol Chem.* 263: 7970-8. 1988.

Forbush III B. The interaction of amines with the occluded state of the Na, K-pump. *J Biol Chem* 263:7979-7988. 1988.

Forbush B 3rd, Kaplan JH, Hoffman JF. Characterization of a new photoaffinity derivative of ouabain: labeling of the large polypeptide and of a proteolipid component of the Na, K-ATPase. *Biochemistry* 17(17):3667-76. 1978.

Furchgott RF.The 1996 Albert Lasker Medical Research Awards. The discovery of endothelium-derived relaxing factor and its importance in the identification of nitric oxide. *JAMA* 276(14):1186-8. 1996.

Gao, Y.-J., K. Takemori, L.-Y. Su, W.-S. An, C. Lu, A. M. Sharma, and R. M. K. W. Lee. Perivascular adipose tissue promotes vasoconstriction: the role of superoxide anion. *Cardiovascular Research*. 71:363-373, 2006.

García-Cardeña G, Oh P, Liu J, Schnitzer JE, Sessa WC. Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. *Proc Natl Acad Sci U S A*. 93(13):6448-53. 1996.

Garty H, Karlish SJ. Role of FXYD proteins in ion transport. *Annu Rev Physiol* 68: 431-59, 2006.

Gatto C, Helms JB, Prasse MC, Arnett KL, Milanick MA. Kinetic characterization of tetrapropylammonium inhibition reveals how ATP and Pi alter access to the Na+-K+-ATPase transport site. *Am J Physiol Cell Physiol* 289(2):C302-11, 2005.

Gatto C, Lutsenko S, Kaplan JH. Chemical modification with dihydro-4,4'-diisothiocyanostilbene-2,2'-disulfonate reveals the distance between K480 and K501 in the ATP-binding domain of the Na,K-ATPase. *Arch Biochem Biophys* 340(1): 90-100, 1997.

Gatto C, Thornewell SJ, Holden JP, Kaplan JH. Cys(577) is a conformationally mobile residue in the ATP-binding domain of the Na,K-ATPase alpha-subunit. *J Biol Chem* 274(35): 24995-5003, 1999.

Gatto C, Arnett KL, Milanick MA. Divalent cation interactions with Na,K-ATPase cytoplasmic cation sites: implications for pNPPase reaction mechanism. Submitted.

Gatto C, Helms JB, Prasse MC, Huang SY, Zou X, Arnett KL, Milanick MA Similarities and differences between organic cation inhibition of the Na,K-ATPase and PMCA. *Biochemistry*. 45: 13331-45. 2006.

Gatto C, Helms JB, Prasse M, Arnett KL, Milanick MA. Tetrapropylammonium, an exclusive extracellular cation site probe in Na,K-ATPase, reveals how ATP and Pi alter access to the transport site. *Am. J. Physiol.* 289: C302-C311. 2005.

Gaut JP, Byun J, Tran HD, Lauber WM, Carroll JA, Hotchkiss RS, Belaaouaj A, Heinecke JW. Myeloperoxidase produces nitrating oxidants in vivo. *J Clin Invest* 109(10):1311-9, 2002.

Geering K. FXYD proteins: new regulators of Na-K-ATPase. *Am J Physiol Renal Physiol* 290(2): F241-50, 2006.

Geering K, Theulaz I, Verrey F, Häuptle MT, Rossier BC. A role for the beta-subunit in the expression of functional Na+-K+-ATPase in Xenopus oocytes. *Am J Physiol* 257(5 Pt 1):C851-8. 1989.

Geering K, Beggah A, Good P, Girardet S, Roy S, Schaer D, Jaunin P. Oligomerization and maturation of Na,K-ATPase: functional interaction of the cytoplasmic NH2 terminus of the beta subunit with the alpha subunit. *J Cell Biol*. 133(6):1193-204. 1996.

Geering K. Function of FXYD proteins, regulators of Na, K-ATPase. *J Bioenerg Biomembr* 37(6):387-92. 2005. 2005.

Geering K, Béguin P, Garty H, Karlish S, Füzesi M, Horisberger JD, Crambert G. FXYD proteins: new tissue- and isoform-specific regulators of Na,K-ATPase. *Ann N Y Acad Sci* 986:388-94. 2003.

Geiszt M, Kopp JB, Várnai P, Leto TL. Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U S A* 97(14): 8010-4, 2000.

Gheorghiade M, van Veldhuisen DJ, Colucci WS. Contemporary use of digoxin in the management of cardiovascular disorders. *Circulation* 113(21):2556-64. 2006.

Glynn IM. A hundred years of sodium pumping. *Annu Rev Physiol* 64:1-18. 2002.

Golden, T. R., D. A. Hinerfeld, and S. Melov. Oxidative stress and aging: beyond correlation. *Aging Cell* 1:117-1. 2002

Gollasch, M. and G. Dubrovska. Paracrine role for periadventitial adipose tissue in the regulation of arterial tone. *Trends Pharmacol Sci*. 25:647-653, 2004.

Gow AJ, Duran D, Malcolm S, Ischiropoulos H. Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. *FEBS Lett* 385(1-2): 63-6, 1996.

Grover AK, Kwan CY, Samson SE. Effects of peroxynitrite on sarco/endoplasmic reticulum Ca2+ pump isoforms SERCA2b and SERCA3a. *Am J Physiol Cell Physiol*. 285(6):C1537-43. 2003.

Grundy, S. M. Hypertriglyceridemia, insulin resistance, and the metabolic syndrome. *Am J Cardiol*. 83:25F-29F., 1999.

Gutiérrez-Martín Y, Martín-Romero FJ, Iñesta-Vaquera FA, Gutiérrez-Merino C, Henao F. Modulation of sarcoplasmic reticulum Ca(2+)-ATPase by chronic and acute exposure to peroxynitrite. *Eur J Biochem* 271(13): 2647-57, 2004.

Harrison ML, Rathinavelu P, Arese P, Geahlen RL, Low PS. Role of band 3 tyrosine phosphorylation in the regulation of erythrocyte glycolysis. *J Biol Chem* 266(7):4106-11, 1991.

Hasler U, Wang X, Crambert G, Béguin P, Jaisser F, Horisberger JD, Geering K. Role of beta-subunit domains in the assembly, stable expression, intracellular routing, and functional properties of Na,K-ATPase. *J Biol Chem* 273(46):30826-35. 1998.

Henrichot, E., C. E. Juge-Aubry, A. Pernin, J.-C. Pache, V. Velebit, J.-M. Dayer, P. Meda, C. Chizzolini, and C. A. Meier. Production of Chemokines by Perivascular Adipose Tissue: A Role in the Pathogenesis of Atherosclerosis? *Arterioscler Thromb Vasc Biol*. 25:2594-2599, 2005.

Herrera M, Garvin JL. Recent advances in the regulation of nitric oxide in the kidney. *Hypertension* 45(6): 1062-7, 2005.

Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 24(4):677-83. 2004.

Hoffman JF, Kaplan JH, Callahan TJ. The Na:K pump in red cells is electrogenic. *Fed Proc* 38(11):2440-1. 1979.

Horisberger JD. Recent insights into the structure and mechanism of the sodium pump. *Physiology (Bethesda)* 19: 377-87, 2004.

Hoving S, Bar-Shimon M, Tijmes JJ, Goldshleger R, Tal DN, Karlish SJ. Novel aromatic isothiouronium derivatives which act as high affinity competitive antagonists of alkali metal cations on Na/K-ATPase. *J Biol Chem*. 270: 29788-93.1995.

Hu YK, Kaplan JH. Site-directed chemical labeling of extracellular loops in a membrane protein. The topology of the Na,K-ATPase alpha-subunit. *J Biol Chem* 275(25):19185-91. 2000.

Huang WH, Wang Y, Askari A. (Na+ + K+)-ATPase: inactivation and degradation induced by oxygen radicals. *Int J Biochem* 24(4): 621-6, 1992.

Iacobellis, G., M. C. Ribaudo, F. Assael, E. Vecci, C. Tiberti, A. Zappaterreno, U. Di Mario, and F. Leonetti. Epicardial Adipose Tissue Is Related to Anthropometric and Clinical Parameters of Metabolic Syndrome: A New Indicator of Cardiovascular Risk. *J Clin Endocrinol Metab*. 88:5163-5168, 2003.

Imlay, J. A. Pathways of oxidative damage. *Annu. Rev. Microbiol*. **57:**395-418. 2003.

Jaisser F, Jaunin P, Geering K, Rossier BC, Horisberger JD. Modulation of the Na,K-pump function by beta subunit isoforms. *J Gen Physiol* 103(4):605-23.1994.

Ji Y, Neverova I, Van Eyk JE, Bennett BM. Nitration of tyrosine 92 mediates the activation of rat microsomal glutathione s-transferase by peroxynitrite. *J Biol Chem* 281(4):1986 -91. 2006.

Jorgensen PL. Purification and characterization of (Na+ plus K+)-ATPase. IV. Estimation of the purity and of the molecular weight and polypeptide content per enzyme unit in preparations from the outer medulla of rabbit kidney. *Biochim Biophys Acta* 356(1): 53 -67, 1974.

Jorgensen PL, Hakansson KO, Karlish SJ. Structure and mechanism of Na,K ATPase: functional sites and their interactions. *Annu Rev Physiol* 65: 817-49, 2003.

Kamisaki Y, Wada K, Bian K, Balabanli B, Davis K, Martin E, Behbod F, Lee YC, Murad F. An activity in rat tissues that modifies nitrotyrosine-containing proteins. *Proc Natl Acad Sci* U S A 95(20):11584-9, 1998.

Kang M, Ross GR, Akbarali HI. COOH-terminal association of human smooth muscle calcium channel Ca(v)1.2b with Src kinase protein binding domains: effect of nitrotyrosylation. *Am J Physiol Cell Physiol* 293(6): C1983-90, 2007.

Kaplan JH. Biochemistry of Na,K-ATPase. *Annu Rev Biochem* 71: 511-35, 2002.

Karlish SJD. Characterization of conformational changes in (Na,K) ATPase labeled with fluorescein at the active site. *J. Bioenerg. Biomembr* 12: 111-136. 1980.

Kelly JM, McBride BW. The sodium pump and other mechanisms of thermogenesis in selected tissues. *Proc Nutr Soc* 49(2):185-202. 1990.

Keyer K, Imlay JA. Superoxide accelerates DNA damage by elevating free-iron levels. *Proc Natl Acad Sci U S A* 93(24): 13635-40, 1996.

Kleinbongard P, Schulz R, Rassaf T, Lauer T, Dejam A, Jax T, Kumara I, Gharini P, Kabanova S, Ozüyaman B, Schnürch HG, Gödecke A, Weber AA, Robenek M, RobenekH, Bloch W, Rösen P, Kelm M. Red blood cells express a functional endothelial nitric oxide synthase. *Blood* 107(7):2943-51. 2006.

Klotz LO, Schroeder P, Sies H. Peroxynitrite signaling: receptor tyrosine kinases and activation of stress-responsive pathways. *Free Radic Biol Med* 33(6):737-43. 2002.

Koenig RJ, Peterson CM, Jones RL, Saudek C, Lehrman M, Cerami A. Correlation of glucose regulation and hemoglobin A1c in diabetes mellitus. *N Engl J Med* 295:417-420, 1976.

Kooy NW, Lewis SJ, Royall JA, Ye YZ, Kelly DR, Beckman JS. Extensive tyrosine nitration in human myocardial inflammation: evidence for the presence of peroxynitrite. *Crit Care Med*. 25(5):812-9. 1997.

Koppenol WH, Moreno JJ, Pryor WA, Ischiropoulos H, Beckman JS. Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem Res Toxicol* 5(6):834-42. 1992.

Laughlin, M. H., J. Cook, R. Tremble, D. Ingram, P. N. Colleran, and J. R. Turk. Exercise Training Produces Nonuniform Increases in Arteriolar Density of Rat Soleus and Gastrocnemius Muscle. *Microcirculation*. 13:175-186, 2006.

Laughlin, M. H. and J. M. Muller. Vasoconstrictor responses of coronary resistance arteries in exercise- trained pigs. *J Appl Physiol*. 84:884-889, 1998.

Laughlin, M. H., K. A. Overholser, and M. J. Bhatte. Exercise training increases coronary transport reserve in miniature swine. *Journal of Applied Physiology*. 67:1140-1149, 1989.

Laughlin, M. H., J. S. Pollock, J. F. Amann, M. L. Hollis, C. R. Woodman, and E. M. Price. Training induces nonuniform increases in eNOS content along the coronary arterial tree. *J Appl Physiol*. 90:501-510., 2001.

Lew, V.L., Glynn, I.M., and Ellory, J.C. Net synthesis of ATP by reversal of the sodium pump. *Nature* 225:865-866. 1970.

Li N, Yi FX, Spurrier JL, Bobrowitz CA, Zou AP. Production of superoxide through NADH oxidase in thick ascending limb of Henle's loop in rat kidney. *Am J Physiol Renal Physiol* 282(6): F1111-9, 2002.

Liu L, Mohammadi K, Aynafshar B, Wang H, Li D, Liu J, Ivanov AV, Xie Z, Askari A. Role of caveolae in signal-transducing function of cardiac Na+/K+-ATPase. Am J Physiol Cell Physiol. 284(6):C1550-60. 2003.

Low PS, Allen DP, Zioncheck TF, Chari P, Willardson BM, Geahlen RL, Harrison ML. Tyrosine phosphorylation of band 3 inhibits peripheral protein binding. *J Biol Chem* 262(10):4592-6, 1987

Lohn, M., G. Dubrovska, B. Lauterbach, F. C. Luft, M. Gollasch, and A. M. Sharma. Periadventitial fat releases a vascular relaxing factor. *FASEB J.* 16:1057-1063, 2002.

MacMillan-Crow LA, Crow JP, Thompson JA. Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. Biochemistry 37(6):1613-22. 1998.

MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS, Thompson JA. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc Natl Acad Sci U S A.* 93(21):11853-8. 1996.

Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 240 (4852):622-30.1988.

Matsudaira P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* 262(21): 10035-8, 1987.

Mazurek, T., L. Zhang, A. Zalewski, J. D. Mannion, J. T. Diehl, H. Arafat, L. Sarov-Blat, S. O'Brien, E. A. Keiper, A. G. Johnson, J. Martin, B. J. Goldstein, and Y. Shi. Human Epicardial Adipose Tissue Is a Source of Inflammatory Mediators. *Circulation*. 108:2460-2466, 2003.

Mercer RW, Biemesderfer D, Bliss DP Jr, Collins JH, Forbush B 3rd. Molecular cloning and immunological characterization of the gamma polypeptide, a small protein associated with the Na,K-ATPase. *J Cell Biol* 121(3):579-86. 1993.

Milanick MA, Arnett KL. Extracellular protons regulate the extracellular cation selectivity of the sodium pump. *J Gen Physiol*. 120: 497-508. 2002.

Moller J.V, Nissen P, Sorensen T.L, le Maire M. Transport mechanism of the sarcoplasmic reticulum Ca2+ -ATPase pump. *Curr Opin Struct Biol* 15:387-93. 2005.

Morth JP, Pedersen BP, Toustrup-Jensen MS, Sørensen TL, Petersen J, Andersen JP, Vilsen B, Nissen P. Crystal structure of the sodium-potassium pump. *Nature* 450(7172): 1043-9, 2007.

Muller, J. M., P. R. Myers, and M. H. Laughlin. Vasodilator responses of coronary resistance arteries of exercise- trained pigs. *Circulation*. 89:2308-2314, 1994.

Nathan, C. Points of control in inflammation. *Nature* 420**:**846-852. 2002.

Nathan C. Specificity of a third kind: reactive oxygen and nitrogen intermediates in cell signaling. *J Clin Invest* 111(6):769-78. 2003.

Okamura H, Denawa M, Ohniwa R, Takeyasu K. P-type ATPase superfamily: evidence for critical roles for kingdom evolution. *Ann N Y Acad Sci* 986:219-23. 2003.

Or E, David P, Shainskaya A, Tal DM, Karlish SJ. Effects of competitive sodium-like antagonists on Na,K-ATPase suggest that cation occlusion from the cytoplasmic surface occurs in two steps. *J Biol Chem*. 268 (1993) 16929-37. 1993.

Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 87(1): 315-424, 2007.

Parastatidis I, Thomson L, Fries DM, Moore RE, Tohyama J, Fu X, Hazen SL, Heijnen HF, Dennehy MK, Liebler DC, Rader DJ, **Ischiropoulos H**. Increased protein nitration burden in the atherosclerotic lesions and plasma of apolipoprotein A-I deficient mice. *Circ Res*. 101(4):368-76. 2007.

Peluffo RD, Hara Y, Berlin JR. Quaternary organic amines inhibit Na,K pump current in a voltage-dependent manner: direct evidence of an extracellular access channel in the Na,K-ATPase. *J Gen Physiol*. 123: 249-63. 2004.

Pierre SV, Xie Z. The Na,K-ATPase receptor complex: its organization and membership. *Cell Biochem Biophys* 46(3): 303-16, 2006.

Post RL, Kume S, Tobin T, Orcutt B and Sen AK. Flexibility of an active center in sodium-plus-potassium adenosine triphosphatase. *J. Gen. Physiol.* 54**:** 306–326, 1969.

Post RL, Hegyvary C, Kume S. Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. *J Biol Chem* 247(20):6530-40. 1972.

Quijano C, Castro L, Peluffo G, Valez V, Radi R. Enhanced mitochondrial superoxide in hyperglycemic endothelial cells: direct measurements and formation of hydrogen peroxide and peroxynitrite. *Am J Physiol Heart Circ Physiol* 293(6):H3404-14, 2007.

Radak Z, Chung HY, Goto S. Systemic adaptation to oxidative challenge induced by regular exercise. *Free Radic Biol Med*. 44(2):153-9. 2008.

Radi R. Nitric oxide, oxidants, and protein tyrosine nitration. *Proc Natl Acad Sci U S A* 101(12):4003-8, 2004.

Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 288(2): 481-7, 1991.

Radi R, Peluffo G, Alvarez MN, Naviliat M, Cayota A. Unraveling peroxynitrite formation in biological systems. *Free Radic Biol Med* 30(5): 463-88, 2001.

Rao A. V. and Balachandran B. 2002. Role of oxidative stress and antioxidants in neurodegenerative diseases. Nutr. Neurosci. **5:**291-309

Reuter H, Henderson SA, Han T, Ross RS, Goldhaber JI, Philipson KD. The Na+-Ca2+ exchanger is essential for the action of cardiac glycosides. *Circ Res* 90(3): 305-8, 2002.

Reynolds MR, Berry RW, Binder LI. Nitration in neurodegeneration: deciphering the "Hows" "nYs". *Biochemistry* 46(25):7325-36. 2007.

Rhee SG. Cell signaling. H2O2, a necessary evil for cell signaling. *Science* 312(5782):1882-3. 2006.

Robinson J.D. Modification of ligand binding to the Na+/K+-activated ATPase. *Biochim Biophys Acta*. 997: 41-8. 1989.

Rouzaire-Dubois B and Dubois JM. Characterization of palytoxin-induced channels in mouse neuroblastoma cells. *Toxicon*. 28: 1147-58. 1990.

Sase K, Michel T. Expression of constitutive endothelial nitric oxide synthase in human blood platelets. *Life Sci* 57: 2049–2055, 1995.

Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. *J Nutr* 130(8S Suppl):2073S-85S, 2000.

Schieke SM, Briviba K, Klotz LO, Sies H. Activation pattern of mitogen-activated protein kinases elicited by peroxynitrite: attenuation by selenite supplementation. *FEBS Lett* 448(2-3):301-3. 1999.

Schneeberger A, Apell HJ. Ion selectivity of the cytoplasmic binding sites of the Na,K ATPase: II. Competition of various cations, *J Membr. Biol*. 179: 263-273. 2001.

Schoner W, Scheiner-Bobis G. Endogenous and exogenous cardiac glycosides and their mechanisms of action. *Am J Cardiovasc Drugs* 7(3): 173-89, 2007.

Schroeder P, Klotz LO, Buchczyk DP, Sadik CD, Schewe T, Sies H. Epicatechin selectively prevents nitration but not oxidation reactions of peroxynitrite. *Biochem Biophys Res Commun* 285(3): 782-7, 2001.

Schuurmans Stekhoven FM, Swarts HG, Lam GK, Zou YS and De Pont JJ. Phosphorylation of (Na^+ + K^+)-ATPase; stimulation and inhibition by substituted and unsubstituted amines. *Biochim Biophys Acta* 937: 161-76. 1988.

Schuurmans Stekhoven FM, Zou YS, Swarts HG, Leunissen J, De Pont JJ. Ethylenediamine as active site probe for Na^+/K^+-ATPase, Biochim Biophys Acta 982:103-114. 1989.

Shaul PW, Smart EJ, Robinson LJ, German Z, Yuhanna IS, Ying Y, Anderson RG, Michel T. Acylation targets emdothelial nitric-oxide synthase to plasmalemmal caveolae. *J Biol Chem*. 271(11):6518-22. 1996.

Skou JC. The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim Biophys Acta* 23(2):394-401. 1957.

Skou JC, Esmann M. The Na,K-ATPase. *J Bioenerg Biomembr* 24(3): 249-61, 1992.

Skou JC, Esmann M. Eosin, a fluorescent probe of ATP binding to the (Na^+ +K^+)-ATPase. *Biochim. Biophys. Acta* 647: 232-240. 1981.

Smallwood HS, Lourette NM, Boschek CB, Bigelow DJ, Smith RD, Pasa-Tolić L, Squier TC. Identification of a denitrase activity against calmodulin in activated macrophages using high-field liquid chromatography--FTICR mass spectrometry. *Biochemistry* 46(37):10498-505. 2007.

Smith TW. Digitalis. Mechanisms of action and clinical use. *N Engl J Med* 318(6): 358-65, 1988.

Sokolovsky M, Riordan JF, Vallee BL. Tetranitromethane. A reagent for the nitration of tyrosyl residues in proteins. *Biochemistry* 5(11):3582-9.1966.

Sonveaux P, Lobysheva II, Feron O, McMahon TJ. Transport and peripheral bioactivities of nitrogen oxides carried by red blood cell hemoglobin: role in oxygen delivery. *Physiology (Bethesda)* 22:97-112, 2007.

Srere, P. A. Citrate synthase. *Methods in Enzymology*. 13:3-5, 1969.

Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. *J Biol Chem*. 276(18):14533-6. 2001.

Sweadner KJ, Donnet C. Structural similarities of Na,K-ATPase and SERCA, the Ca(2+)-ATPase of the sarcoplasmic reticulum. *Biochem J* 356(Pt 3): 685-704, 2001.

Szabó C, Ischiropoulos H, Radi R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nat Rev Drug Discov* 6(8): 662-80, 2007.

Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension* 42(6):1075-81. 2003.

Thomas, T. R., J. Pellechia, R. S. Rector, G. Y. Sun, M. S. Sturek, and M. H. Laughlin. Exercise training does not reduce hyperlipidemia in pigs fed a high-fat diet. *Metabolism*. 51:1587-1595., 2002.

Thompson, M. A., K. K. Henderson, C. R. Woodman, J. R. Turk, J. W. E. Rush, E. Price, and M. H. Laughlin. Exercise preserves endothelium-dependent relaxation in coronary arteries of hypercholesterolemic male pigs. *J. Appl. Physiol.* 96:1114-1126, 2004.

Tosteson DC, Hoffman JF. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. *J Gen Physiol*. 44:169-94. 1960.

Toyoshima C, Inesi G. Structural basis of ion pumping by Ca2+-ATPase of the sarcoplasmic reticulum. *Annu Rev Biochem* 73: 269-92. 2004.

Toyoshima C, Nakasako M, Nomura H, Ogawa H. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. *Nature* 405(6787):647-55. 2000

Traylor LA, Mayeux PR. Superoxide generation by renal proximal tubule nitric oxide synthase. *Nitric Oxide* 1(5): 432-8, 1997.

Turk, J. R., K. K. Henderson, G. D. Vanvickle, J. Watkins, and M. H. Laughlin. Arterial endothelial function in a porcine model of early stage atherosclerotic vascular disease. *International Journal of Experimental Pathology*. 86:335-345, 2005.

Upmacis RK, Crabtree MJ, Deeb RS, Shen H, Lane PB, Benguigui LE, Maeda N, Hajjar DP, Gross SS. Profound biopterin oxidation and protein tyrosine nitration in tissues of ApoE-null mice on an atherogenic diet: contribution of inducible nitric oxide synthase. *Am J Physiol Heart Circ Physiol* 293(5):H2878-87. 2007.

Van der Hijden HT, F.M. Schuurmans Stekhoven FM, and De Pont JJ. Sidedness of the effect of amines on the steady-state phosphorylation level of reconstituted $Na^{+/}K^{+}$-ATPase. *Biochim Biophys Acta* 987: 75-82. 1989.

van der Vliet A, Eiserich JP, Halliwell B, Cross CE. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite: A potential additional mechanism of nitric oxide-dependent toxicity. *J Biol Chem* 272(12): 7617-25, 1997.

Vasallo PM, Post RL. Calcium ion as a probe of the monovalent cation center of sodium, potassium ATPase. *J Biol Chem.* 261: 16957-62. 1986.

Vergara C, Alvarez O, Latorre R. Localization of the K+ lock-In and the Ba2+ binding sites in a voltage-gated calcium-modulated channel. Implications for survival of K+ permeability. *J Gen Physiol.* 114: 365-76. 1999.

Vergara C, Latorre R. Kinetics of Ca2+-activated K+ channels from rabbit muscle incorporated into planar bilayers. Evidence for a Ca2+ and and Ba2+ blockade. *J Gen Physiol*. 82: 543-68. 1983.

Verlohren, S., G. Dubrovska, S.-Y. Tsang, K. Essin, F. C. Luft, Y. Huang, and M. Gollasch. Visceral Periadventitial Adipose Tissue Regulates Arterial Tone of Mesenteric Arteries. *Hypertension*. 44:271-276, 2004.

Viappiani S, Schulz R. Detection of specific nitrotyrosine-modified proteins as a marker of oxidative stress in cardiovascular disease. *Am J Physiol Heart Circ Physiol* 290(6): H2167-8, 2006.

Walker LM, Walker PD, Imam SZ, Ali SF, Mayeux PR. Evidence for peroxynitrite formation in renal ischemia-reperfusion injury: studies with the inducible nitric oxide synthase inhibitor L-N(6)-(1-Iminoethyl)lysine. *J Pharmacol Exp Ther* 295(1): 417-22, 2000.

Wang, H., Haas, M., Cai, T., and Xie, Z. Sodium pump as a signal transducer: A role of caveolae. *Biophysical J.* 83: 268a. 2003.

Wellen, K. E. and G. S. Hotamisligil. Obesity-induced inflammatory changes in adipose tissue. *J Clin Invest*. 112:1785-1788, 2003.

White CN, Hamilton EJ, Garcia A, Wang D, Chia KK, Figtree GA, Rasmussen HH. Opposing effects of coupled and uncoupled NOS activity on the Na+-K+ pump in cardiac myocytes. *Am J Physiol Cell Physiol* 294(2): C572-8, 2008.

Whiteman M, Halliwell B. Protection against peroxynitrite-dependent tyrosine nitration and alpha 1-antiproteinase inactivation by ascorbic acid. A comparison with other biological antioxidants *Free Radic Res* 25(3): 275-83, 1996.

Williams JA, Withrow CD, **Woodbury DM**. Effects of ouabain and diphenylhydantoin on transmembrane potentials, intracellular electrolytes, and cell pH of rat muscle and liver in vivo. *J Physiol* 212(1):101-15. 1971.

Wolin MS, Ahmad M, Gupte SA.Oxidant and redox signaling in vascular oxygen sensing mechanisms: basic concepts, current controversies, and potential importance of cytosolic NADPH. *Am J Physiol Lung Cell Mol Physiol*. 289(2):L159-73.2005.

Xie Z. Molecular mechanisms of Na/K-ATPase-mediated signal transduction. *Ann N Y Acad Sci* 986: 497-503, 2003.

Xie Z, Cai T. Na+-K+--ATPase-mediated signal transduction: from protein interaction to cellular function. *Mol Interv* 3(3): 157-68, 2003.

Xu S, Ying J, Jiang B, Guo W, Adachi T, Sharov V, Lazar H, Menzoian J, Knyushko TV, Bigelow D, Schöneich C, Cohen RA. Detection of sequence-specific tyrosine nitration of manganese SOD and SERCA in cardiovascular disease and aging. *Am J Physiol Heart Circ Physiol* 290(6): H2220-7, 2006.

Yamakura F, Taka H, Fujimura T, Murayama K. Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J Biol Chem*. 273(23): 14085-9, 1998.

Yang B, Rizzo V. TNF-alpha potentiates protein-tyrosine nitration through activation of NADPH oxidase and eNOS localized in membrane rafts and caveolae of bovine aortic endothelial cells. *Am J Physiol Heart Circ Physiol*. 292(2):H954-62. 2007.

Zhang C, Imam SZ, Ali SF, Mayeux PR. Peroxynitrite and the regulation of Na(+),K(+)-ATPase activity by angiotensin II in the rat proximal tubule. *Nitric Oxide* 7(1): 30-5, 2002.

Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 258: p468–471, 1992.

Zolotarjova N, Ho C, Mellgren RL, Askari A, Huang WH. Different sensitivities of native and oxidized forms of Na+/K(+)-ATPase to intracellular proteinases. *Biochim Biophys Acta* 1192(1): 125-31, 1994.

Zouki C, Zhang SL, Chan JS, Filep JG. Peroxynitrite induces integrin-dependent adhesion of human neutrophils to endothelial cells via activation of the Raf-1/MEK/Erk pathway. *FASEB J* 15(1):25-27. 2001.

VITA

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