The Role of Sulfenic Acid Modification in the Regulation of the Cardiovascular Voltage-Gated Potassium Channel, Kv1.5

by

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To my family

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4AP	4-Aminopyridine
CE	Cigarette Extract
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CSE	Cigarette Smoke Extract
Cys-SO_2H	Cysteine Sulfinic Acid
Cys-SO_3H	Cysteine Sulfonic Acid
Cys-SOH	Cysteine Sulfenic Acid
DTT	Dithiothreitol
EEA1	Early Endosomal Antigen-1
ERK	Extracellular Signal-Related Kinase
GFP	Green Fluorescent Protein
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione (Glutathione Disulfide)
HERG	human Ether-à-go-go
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
IK	Delayed rectifier potassium current
IKss	Steady-state delayed rectifier potassium current
Ito	Transient outward potassium current
JNK	c-Jun N-terminal Kinase
KCNH2	HERG1 Channel Gene
Kv Channel	Voltage-Gated Potassium Channel
MG132 inhibitor	N-(benzyloxycarbonyl)leucinylleucinylleucinal protease
MI	Myocardial Infarction
NAC	*N*-Acetylcysteine
NEM	N-ethylmaleimide
NO	Nitric Oxide
NO2	Nitrogen Dioxide
ONOO-	Peroxinitrite
PAH	Pulmonary Arterial Hypertension
PDZ	Post synaptic density proten, Drosophila disc large tumor suppressor, and zonula occludens-1 protein
ROS	Reactive Oxygen Species
S^-	Thiolate Anion.
S-Acylation	Thioacylation
SAE1/SAE2	Sumo-Activating Enzyme
SERCA	Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} ATPase

-SH	Thiol
SNO	S-Nitrosothiol
SOD	Superoxide Dismutase
SUMO	Small Ubiquitin-Like Modifier
*t*BOOH	*tertiary* Butyl Hydroperoxide
TEA	Tetraethylammonium

CHAPTER I: INTRODUCTION

Overview

Potassium channels play an essential role in the complex electrical responses of the cardiovascular and nervous systems, where they establish the resting membrane potential, facilitate cellular repolarization, and control neuronal excitability [1]. These channels open and close in response to diverse stimuli, including changes in membrane voltage, fluctuations in intracellular calcium, or binding of ligands such as ATP [2]. Voltage-gated potassium (Kv) channels are abundantly expressed in the cardiovascular system, where they play a pivotal role in normal physiology. In the heart, the generation of myocardial action potentials is governed by a balance between inward, depolarizing (Na^+ and Ca^{2+}) and outward, repolarizing (K^+) ion channel currents. Kv channels underlie the repolarizing currents initiated in early and late phases of the action potential, which result in a return to resting membrane potential (Figure 1.1) [3]. In the vasculature, Kv channels are ubiquitously expressed, where they open in response to membrane depolarization to promote vasodilation, as well as establish vessel tone and resting membrane potential [2]. Alterations in Kv channel function are linked to several disease states, including diabetes mellitus [4, 5], pulmonary arterial hypertension [6], atrial fibrillation [7, 8], heart failure [9,

10] , ischemia-reperfusion injury [11-13], atherosclerosis [14], and post-myocardial infarction electrical remodeling [7, 15-17]. The focus of this thesis is on the rapidly activating, essentially non-inactivating, delayed rectifier Kv channel, Kv1.5. This channel underlies the ultrarapid repolarizing current, IK_{ur}, in the atrium of the heart [3]. Kv1.5 is also expressed in the vasculature, where it functions in regulation of vessel contractility in response to changes in membrane potential [18, 19], and in the pulmonary vasculature, promotes vasodilation in response to increased oxygen tension [6].

Diversity and Structure of Kv Channels

Voltage-gated (Kv) channels comprise the largest and most diverse class of potassium channels. Twelve families of Kv channels have been identified, displaying distinct electrophysiological characteristics and tissue distributions [20]. The Kv1.x family, the first family of Kv channels to be cloned from *Drosophila*, is designated the Shaker family of Kv channels [20]. Additional homologous alpha subunit families were subsequently cloned from *Drosophila*, and designated Shab (Kv2.x), Shaw (Kv3.x), and Shal (Kv4.x) [20]. Families of Kv alpha subunits were discovered, designated Kv5.x-6.x and Kv8.x-Kv9.x, which are electrically silent and unable to form functional homotetramers [20]. Instead, these subunits form heterotetramers with Kv2 family channels and modifiy their gating properties and/or expression levels [20]. Further cloning studies in *Drosophila* led to the identification of the eag family of Kv alpha subunits, the human homologues of which were designated HERG [20]. These channels exhibit lower amino acid sequence homology to Shaker channels, but possess similar membrane topology [20]. Positional cloning techniques led to

the discovery of the KvLQT subfamily [20]. Mutations in the KvLQT gene are implicated in an inherited form of long QT syndrome, a lethal ventricular arrhythmia [20]. Additional electrically silent modulatory subunits have been discovered, including minK, which coassembles with KvLQT1, and Kv beta subunits [20]. Three Kv beta subunit homologues, designated Kv ß1, ß2 and ß3, are cytosolic proteins which couple to Kv1 family channels to modulate channel function, surface expression, and stability [20]. Although alternative splicing of Kv channel transcripts occurs in some mammalian Kv alpha subunits, considerable diversity in Kv channel currents occurs, because each subfamily has multiple members [20]. Still further diversity is generated through hetero-tetramerization of Kv channel alpha subunits with members of the same subfamily. The extent of heteromultimerization and distinct composition of heteromultimers *in vivo* is the subject of intense research.

In the cardiovascular system, several repolarizing Kv currents have been identified based on their time- and voltage-dependent properties and sensitivity to pharmacological agents, and can be broadly classified into two categories: rapidly activating/inactivating transient outward currents, and delayed rectifier currents [3]. In the heart, Kv channel currents encoded by multiple Kv channel genes function in repolarization of cardiac myocytes and pacemaker cells after initiation of an action potential [3]. The timely activation/inactivation and proper expression of these channels are critical for maintenance of healthy rhythm, automaticity, and contractility in the heart. Delayed rectifier channels, which display slow kinetics of activation and inactivation, function in repolarization of

the atria and ventricles [1]. Several delayed rectifier currents, designated I_K, have been identified in the heart, and differ based on their time- and voltage-dependence and sensitivity to pharmacological agents [3]. Rapid (I_{Kr},), slow (I_{Ks}), steady-state (I_{Kss}), and ultrarapid (I_{Kur}) components of I_K have been identified, with differences in their expression depending on the region of the heart and the species studied [3]. Many of the Kv channel genes encoding these currents have been identified. The slow component of I_K is likely encoded by KvLQT (KCNQ1) channels, as well as Kv1.2, Kv1.5, and Kv2.1, while the rapid component of I_K is encoded by the HERG (KCNH2) channel [3]. The ultrarapid component of I_K is encoded by Kv1.5, and Kv2.1 underlies I_{Kss} in the mouse atrium [3, 21]. A-type Kv channels exhibit rapid kinetics of inactivation, and encode transient outward (I_{to}) currents in the heart [3]. These channels are primarily responsible for the initial, early phase repolarization of the atrial and ventricular action potentials [1]. Similar to I_K currents, slow (I_{tos}, encoded by Kv1.4) and fast (I_{tof}, encoded by Kv4.2 and 4.3) components of I_{to} have been identified [3]. Accessory subunits (Kv ß, minK, and KChIP) may also associate with Kv channel alpha subunits in the heart to modify channel current properties, although the details of these associations are still primarily unclear [3]. The subunit minK appears to associate with KvLQT (KCNQ1) in generating I_{Ks} currents [3]. Kv ß and KChIP subunits are also expressed in the heart, and an interaction between Kv ß and HERG channels has been reported [3, 22]. The precise role for Kv accessory subunits in shaping repolarizing cardiac currents requires further

experimentation. Appendix A lists the Kv channel subfamilies, their current properties and tissue distributions.

As illustrated in Figure 1.2, Kv channel alpha subunits assemble to form a tetrameric structure with a central ion conducting pore [23]. Each alpha subunit possesses six transmembrane alpha helices, designated S1-S6 [24]. The S1-S4 transmembrane helices function in voltage sensing, and the S4 helix possesses conserved basic arginine residues, which respond to electrostatic forces [25]. The S5, S6 and pore helices comprise the ion conducting pore, with the highly conserved selectivity filter GYG amino acid sequence located in the pore helix [25]. Figure 1.3 illustrates the topology of Kv alpha subunits in the plasma membrane.

Regulation of Kv Channels by Oxidative Stress in Cardiovascular Disease

Cardiovascular Kv channels are regulated by numerous factors, including cyclic AMP-dependent pathways activated by the autonomic nervous system [26-28], antiarrhythmic drugs and other pharmaceuticals [29-31], and hormones [32]. An important cellular mechanism by which the expression and function of Kv channels are regulated is via changes in cellular reduction-oxidation (redox) state. A pro-oxidant shift in cellular redox state leads to oxidative stress, defined as excessive production of reactive oxygen species (ROS), and/or diminished antioxidant capacity. Oxidative stress has been implicated in aging, inflammation, environmental exposures, as well as in numerous disease states [33, 34]. Both endogenous and exogenous sources of ROS regulate the cellular redox environment [35]. In the mitochondria, hydrogen peroxide and superoxide generated during electron transport are an important source of ROS [35]. ROS

are also generated by endogenous enzyme systems, including NADPH oxidase, as a result of signal transduction via receptor tyrosine kinase pathways, or via cytochrome P450- mediated metabolism of xenobiotics and drugs [35]. In the innate immune response, myeloperoxidase generates significant quantities of hydrogen peroxide and hypochlorous acid, generating cytotoxic radicals in defense against pathogens [35]. Additionally, nitric oxide synthase, under oxidative stress or when depleted of necessary cofactors, may produce toxic radicals such as peroxinitrite and superoxide anion [35]. Environmental exposures such as cigarette smoke and UV light are significant, socially relevant sources of oxidative stress [36, 37]. In the cardiovascular system, several pathophysiological states are characterized by increased oxidative stress. Rapid pacing of the atrium leads to increased ROS and a decrease in tissue levels of antioxidants [38, 39]. This observation has important implications for atrial fibrillation, a cardiac arrhythmia associated with oxidative stress that becomes self-perpetuating once initiated [7, 40]. Additionally, diabetes and hypertension are associated with oxidative stress; hyperglycemia and signaling via angiotensin-II result in an increase in superoxide levels and impaired vasodilation [41, 42]. Generation of superoxide via the angiotensin-II pathway likely occurs through induction of NADPH oxidase [43], while the source of superoxide in hyperglycemia may be the cyclooxygenase pathway [44]. Pathological alterations in cellular glucose utilization, which diminish stores of NADPH, can deplete reduced glutathione (GSH), an endogenous antioxidant [17, 45]. This is an important contributing factor in the pathogenic electrical remodeling that

occurs after myocardial infarction [17]. Thus, oxidative stress is an unavoidable consequence of normal physiological functions, as well as an unintended result of environmental exposures and disease states, including cardiovascular disease.

Several studies support a role for oxidative stress in regulation of Kv channels in the cardiovascular system, with important physiological implications [4, 6, 13, 46-51]. Several pathophysiological states are characterized by altered Kv channel function and expression as well as altered cellular redox state, including diabetes mellitus [4, 5], pulmonary arterial hypertension [6], atrial fibrillation [7, 8], heart failure [9, 10] , ischemia-reperfusion injury [11-13], atherosclerosis [14], and post-myocardial infarction electrical remodeling [7, 15-17]. Redox sensitivity of Kv channels has been reported in both native cells and in heterologous expression systems. Depending on the oxidant used and the tissue and type of Kv channel studied, both decreases and increases in Kv channel currents have been reported in response to altered cellular redox state.

There is a compelling body of evidence demonstrating redox regulation of Kv channels expressed in the heart. In a heterologous expression system, oxidation of a cysteine in the N-terminus of A-type Kv channels abolishes rapid inactivation, which is restored by application of the thiol reducing agents, glutathione or dithiothreitol [52]. Similarly, the oxidizing agent rose bengal significantly reduces inactivation of Kv1.4 channels, which underlie I_{to}, and inhibits Kv1.5 currents, which contribute primarily to I_{Kur} [49]. In post myocardial infarction (MI) ventricular myocytes, there is a significant decrease in the total

cellular ratio of reduced to oxidized glutathione (GSH:GSSG), an indicator of oxidative stress [17]. The increase in oxidative stress is accompanied by a significant reduction in the rapidly inactivating transient outward Kv channel current (I_{to}). Importantly, the I_{to} current in post MI myocytes is restored to control levels by exogenous glutathione or *N*-acetylcysteine (NAC), a pharmacological precursor to glutathione [17]. In a similar study, administering exogenous pyruvate, which bolsters production of NADPH, restores I_{to} currents in post-MI myocytes, and this restoration in currents is blocked by inhibitors of thioredoxin [53]. Levels of mRNA and protein for Kv4.2, 4.3, and KChIP2 are significantly reduced in post-MI myocytes, and expression is significantly restored after incubation of the cells with pyruvate [53]. These results suggest that myocardial infarction induces cellular oxidative stress, which likely serves an important role in the mechanism of electrical remodeling. The results also implicate endogenous antioxidant systems, for which NADPH provides reducing equivalents, in regulation of Kv channel currents in the heart. Exogenous oxidants have similar effects on Kv channel currents in cardiac myocytes. Treatment of ventricular myocytes with diamide, which oxidizes intracellular reduced glutathione and thereby indirectly induces oxidative stress, significantly reduces both I_{to} and the slowly inactivating I_{Kss} Kv channel currents [13]. Dichloroacetate, which stimulates glucose metabolism and increases cellular NADPH, reverses these effects. The ability of dichloroacetate to restore I_{to} and I_{Kss} channel current after oxidative stress is sensitive to inhibitors of the thioredoxin and glutaredoxin systems, respectively [13]. In contrast with these

results, others have demonstrated that hypoxia causes a reduction in I_{Ks} currents, effects which are mimicked by thiol reducing agents [54]. Discrepancies in the data may reflect differences in experimental models. In contrast with the studies conducted in post-MI myocytes, hypoxia treatments were conducted in *ex vivo*, isolated cardiac myocytes. Recent evidence suggests that ROS significantly reduce HERG1 (KCNH2) channel currents, effects which are partially dependent on a single COOH-terminal cysteine [55]. Hydrogen peroxide can also modulate the gating properties and current density of HERG [56, 57]. Similarly, hyperglycemia-induced oxidative stress reduces HERG current density [5]. Although these HERG channel studies were conducted in heterologous expression systems, they suggest that redox modulation of HERG currents in the heart may play a role in arrhythmias observed in patients with diseases such as diabetes [5]. Thus, Kv channel currents in the heart are redox-sensitive and regulated by endogenous antioxidant systems, and this may have important implications for cardiovascular disease.

In addition to the heart, significant evidence demonstrates that Kv channels in the vasculature are also redox sensitive. Vasoconstriction is mediated by the depolarizing influence of voltage-gated Ca^{2+} channels, store-operated Ca^{2+} channels, and stretch-activated cation channels [2]. Delayed rectifier Kv channels in the vasculature provide an important counter-balance to these depolarizing, vasoconstrictive effects, controlling myogenic tone and regulating vessel contractility [1]. Consequently, down-regulation of Kv channels,

observed in disease states such as pulmonary arterial hypertension [6] and diabetes [4], promotes excessive vasoconstriction. Significant evidence supports a role for redox regulation of Kv channels in maintaining vessel tone in healthy blood vessels, as well as in disease states characterized by excessive vasoconstriction, such as diabetes and systemic and pulmonary hypertension [6, 42, 58]. Hydrogen peroxide (H_2O_2) induces relaxation of coronary arteries, which is sensitive to the Kv channel inhibitors, tetraethylammonium (TEA) and 4-aminopyridine (4-AP) [58]. In a subsequent study, the authors demonstrate that H_2O_2 causes a 4-AP sensitive increase in Kv channel current in dissociated smooth muscle cells from these arteries [59]. The effects of H_2O_2 are reversed upon administration of the thiol reductant, dithiothreitol (DTT) [58]. The authors hypothesize that cysteine oxidation of Kv channels may promote vasodilation. However, high doses of TEA and 4-AP [58, 59] are used in these experiments, making it difficult to determine the specific molecular targets affected. Furthermore, modification of accessory proteins may also account for their findings. Contrary to these results, in disease states such as diabetes, oxidative stress appears to have an inhibitory effect on Kv channel function. Incubation of rat small coronary arteries in high glucose causes a dramatic increase in the level of superoxide radical, which is sensitive to scavengers of superoxide and hydrogen peroxide, and a significant reduction in whole cell Kv channel current in dissociated smooth muscle cells from these arteries [46]. Additional studies *in vivo* suggest that Kv channel mediated vasodilation is impaired in small coronary arteries of diabetic rats, and is partially restored by free radical scavengers [4,

46]. These results implicate high glucose in the diabetic state as a source of oxidative stress, which inhibits function of Kv channels and may contribute to the impaired vessel contractility observed in this disease. Further evidence supports a role for redox regulation of Kv channel currents in pulmonary arteries, with conflicting results among research groups. It is well-established that pulmonary hypertension is characterized by enhanced oxidative stress and chronic down regulation of Kv channel subunits in pulmonary arterial smooth muscle [6]. Oxygen-sensitive current in resistance pulmonary arteries is carried by Kv1.5 and Kv2.1 subunits, and Kv1.5 currents are inhibited by hypoxia [6]. Interestingly, restoring normoxic conditions and mitochondrial oxidative phosphorylation leads to an increase in Kv1.5 currents [6]. The authors therefore conclude that ROS increase Kv channel currents, and that ROS are decreased under hypoxic conditions, leading to inhibition of channel function [6]. However, whether hypoxia causes an increase or decrease in ROS is the subject of significant debate [6, 60, 61]. Recent work using a fluorescent, redox-sensitive, ratiometric sensor indicates that although generation of ROS decreases in the mitochondrial matrix during hypoxia, it is increased in the cystosol [62]. This finding highlights the possibility that redox status may vary based on the subcellular compartment(s) studied. It is therefore possible that ROS modulate Kv1.5 currents during both hypoxia and oxidative stress, through different mechanisms. In contrast with these findings, ROS cause a reduction in Kv channel current in pulmonary arterial smooth muscle cells, and enhanced contractility of isolated pulmonary arteries, effects which are sensitive to a scavenger of H_2O_2 or

inhibition of NADPH oxidase [48]. Reduced Kv channel function, mediated by oxidative stress and sensitive to treatment with the antioxidant, tiron, are also observed in a fetal lamb model of persistent pulmonary hypertension of the newborn [63]. In line with these findings, hydrogen peroxide causes constriction of, and inhibits Kv channel currents in, the ductus arteriosus [64]. Although the definitive mechanism for regulation of Kv channels in the pulmonary vasculature requires further investigation, it is clear that Kv channel currents in the pulmonary vasculature, particularly Kv1.5, are sensitive to ROS.

Additional studies in heterologous expression systems provide evidence for the redox sensitivity of modulatory Kv beta subunits. Kv beta subunits have an aldo keto reductase fold, and are capable of reducing aldehyde substrates using bound NADPH as a cofactor [65]. When co-expressed with Kv1.4, oxidation of bound NADPH on the Kv beta subunit increases Kv1.4 current [65]. Similarly, Kv beta 1.3 confers A-type inactivation on Kv1.5, and the oxidized cofactors NAD+ and NADP+ abolish this effect [66].

As noted above, the effects of oxidative stress on Kv channel function have differed among various research groups. Several important factors may have contributed to discrepancies in the findings. Channel redox sensitivity is likely affected by the nature of the redox-sensitive modification, the interaction with other, potentially redox-sensitive, accessory subunits [67], the species of oxidant [49], and the tissue type. Other factors, including the presence of free iron or other metal ions [68], pH [69, 70], or expression of endogenous oxidant or antioxidant enzymes likely affect cellular redox reactions and the sensitivity of Kv

channels to these reactions. Furthermore, patch clamping studies frequently use calcium chelating agents in the intracellular solution. We have recently discovered that drug-induced channel internalization is calcium-dependent [29]. Thus, in voltage clamp experiments studying the acute effects of oxidative stress on channel current, the contribution of oxidant-induced channel internalization may be missed. In the pulmonary vasculature, differences in distribution and quantity of voltage-dependent, store-operated, and receptor-operated calcium channels relative to other tissues may explain the discrepant vasoconstrictive responses to hypoxia, ischemia, and oxidative stress when compared to other tissues [71]. Indeed, differences in calcium channel expression and function appear to contribute to discrepancies in agonist-mediated contraction of vessels within the right and left branches of the pulmonary artery itself [71]. Expression of factors mediating vasodilation that are present in the endothelial layer of blood vessels may further alter the cellular response to oxidative stress. For example, in pulmonary arterial rings pre-constricted with high extracellular potassium, relaxation in response to treatment with diamide is attenuated, although not inhibited, upon removal of the endothelium [71]. Lastly, very different mechanisms may govern the redox modulation of channel function in the context of basal physiological signaling, compared to modulation by oxidative stress. Thus, although there is significant evidence in support of potassium channel redox sensitivity, the cellular mechanisms governing this sensitivity are, and will continue to be, the subject of intense research. Furthermore, the mechanistic

role for redox regulation of potassium channels in the development and perpetuation of disease remains poorly understood.

A Role for Kv1.5 and Oxidative Stress in Cardiovascular Disease

Among redox-sensitive cardiovascular Kv channels, the redox sensitivity of the Kv1.5 channel is an important factor in human diseases, including atrial fibrillation and pulmonary arterial hypertension [6, 8]. Both of these conditions are associated with oxidative stress, as well as with reduced expression of Kv1.5 [6-8, 40, 48, 63, 72]. Atrial fibrillation, the most common cardiac arrhythmia in the US, is a significant concern to public health, and represents a substantial financial burden on the US health care system [73]. The condition is independently associated with an increased risk of stroke, congestive heart failure, and death [40, 73]. Several risk factors for atrial fibrillation have been identified, including age, pre-existing heart disease, high blood pressure, hyperthyroidism and other chronic conditions, and environmental exposures such as smoking and binge drinking [74, 75]. Atrial fibrillation is also a common complication after open-heart surgery [76]. In a small number of cases, inherited gene mutations can also increase the risk of this arrhythmia [77]. Although the pathogenesis of atrial fibrillation is largely unknown, the condition is strongly associated with oxidative stress and inflammation [78]. Electrophysiologically, atrial fibrillation is characterized by significant shortening of the atrial effective refractory period, and reduced duration of the atrial action potential [8]. On a molecular level, in patients with chronic atrial fibrillation, there is down-regulation of Kv1.5 protein expression, with no change in levels of mRNA [8]. This finding, combined with the atrium-specific expression of the channel human heart, make

Kv1.5 an important therapeutic target for atrial fibrillation [79]. Kv1.5 is the focus of a significant amount of research into the mechanisms governing the onset and perpetuation of this condition [29]. However, in spite of significant evidence linking atrial fibrillation to oxidative stress and to reduced Kv1.5 expression, the mechanistic link between these observations remains unclear. Pulmonary arterial hypertension (PAH), a significant cause of morbidity and mortality, is characterized by obstruction of the pulmonary vasculature and failure of the right ventricle of the heart [20]. The risk factors that lead to PAH are poorly understood, and include female gender, a family history of the disease, anorexigen (appetite suppressant drug) use, and HIV infection [80-83]. On a molecular level, several factors, including enhanced serotonin signaling, and expression of survivin, an anti-apoptotic protein, appear to play a role in the disease [6]. Like chronic atrial fibrillation, PAH is characterized by reduced expression of Kv1.5, as well as oxidative stress, and dysregulation of Kv1.5 by reactive oxygen species appears to play an important role in the pathogenesis of this disease [6, 48, 63]. In spite of this, the molecular means for this regulation is unclear. Given the significant public health implications of cardiovascular diseases, and a potential role for Kv1.5 in the pathogenesis of several diseases, it is of interest to determine the molecular mechanisms governing the regulation of channel expression under normal circumstances and in pathophysiological states.

The focus of the work in this thesis is to understand, on a molecular level, how oxidative stress regulates Kv1.5 expression, and the implications that this

has for cardiovascular disease, including atrial fibrillation and pulmonary arterial hypertension. A link between oxidative stress and down-regulation of Kv channels is currently the subject of intense research [4, 6, 46]. However, studies of the redox sensitivity of Kv channels thus far have not explored redox-sensitive changes in channel surface levels. Surface levels of several Kv channels, including HERG1 and Kv1.5, are sensitive to other factors, such as intracellular signaling molecules [84] and pharmacological agents [29]. Indeed, recent work in our laboratory has demonstrated that Kv1.5 undergoes a dynamic trafficking process, in which the channel undergoes endocytosis from the cell surface, is internalized to EEA1- positive early endosomal compartments, and recycled back to the cell surface (Figure 1.4) [85]. Importantly, we have further demonstrated that this constitutive trafficking process can also be regulated by pharmacological interventions, such as antiarrhythmic drugs [29]. Therefore, in addition to altering the biophysical properties of Kv channels, drugs, toxicants, and changes in metabolic state may regulate cellular excitability through modulation of channel trafficking. In this study (currently under review for publication), we demonstrate that oxidative stress, via oxidative posttranslational modification of the channel itself, reduces cell surface levels of channel, interferes with channel trafficking, and leads to degradation. This may represent an important mechanism governing dysregulation of channel expression in cardiovascular disease.

Posttranslational Modifications to Kv Channels: A Molecular Mechanism for Channel Redox Sensitivity

As noted above, the redox sensitivity of Kv channels in the cardiovascular system is well documented. Several potential mechanisms exist for modulation

of Kv channel current, including blocking the pore of the channel itself, altering its trafficking and cell surface localization, altering protein stability, and affecting channel transcription or translation [47, 86]. For example transcription of the KCNA5 gene, which encodes Kv1.5, is sensitive to oxidative stress [87]. Oxidative stress may regulate channel expression through any of these mechanisms, either through direct or indirect effects on the channel itself. One important direct mechanism for Kv channel regulation is via redox-sensitive posttranslational modifications to the channel itself. As outlined below, several different posttranslational modifications to Kv channels are redox sensitive, with different consequences, promoting specificity in the cellular response to oxidative stress.

Methionine Oxidation

With a sulfur atom that is amenable to oxidation, methionine residues are common cellular targets for ROS. Addition of a single oxygen to the sulfur moiety on methionine leads to formation of methionine sulfoxide, which is reversible via the action of methionine sulfoxide reductases [88]. Methionine sulfoxide can proceed via further oxidation to methionine sulfone, an irreversible modification that may be relevant only in an experimental setting [88]. Although methionine sulfoxide can lead to protein inactivation with insufficient antioxidant capacity, it also provides a means for reversible regulation of protein function in response to changes in cellular redox state [88]. While methionine is relatively nonpolar, oxidation leads to an increase in polarity, which influences protein stability and function [88]. In a heterologous expression system, reversible oxidation of a methionine residue in the N-terminus of a *Drosophila* Kv channel

abolishes N-type inactivation of the channel [89]. In HEK 293 cells, methionine oxidation also causes a significant reduction in HERG channel current [90]. The effects of methionine oxidation in these studies were attenuated in cells over-expressing methionine sulfoxide reductase [89, 90]. These findings collectively suggest that reversible oxidation of methionine may represent an acute means of regulating cellular excitability in response to changes in cellular redox state.

Phosphorylation

Phosphorylation is the reversible, enzymatic addition of a phosphate (PO_4) group to serine, threonine, or tyrosine residues on proteins [91]. Addition of the polar PO_4 group to proteins induces a conformational change to the protein, which can result in either activation or inactivation [92]. Regulation of proteins via phosphorylation occurs in the context of normal physiological function, where it is a pivotal mechanism for signal transduction [93]. In addition, phosphorylation is an important means of protein regulation in response to cellular stressors, including oxidative stress [93, 94]. In the cardiovascular system, several signaling pathways lead to redox-sensitive activation of protein kinases, including activation of growth factor receptors [95] and angiotensin-II [96]. Several kinases that modulate phosphorylation are redox-sensitive, including big mitogen-activated kinase-1 (BMK-1) [97], ERK MAP kinase [97], c-jun N-terminal kinase (JNK) [98], Src [99], and p38 MAP kinase [98]. Additionally, protein tyrosine phosphatases are inactivated upon oxidation of a redox sensitive cysteine in the active site, leading to an accumulation of phosphorylated proteins [100]. Changes in the phosphorylation state of Kv channels are implicated in ischemic preconditioning [11], heart failure, and diabetes [101]. Ischemic preconditioning

in neurons results in dephosphorylation of Kv2.1, an increase in channel conductance, and a hyperpolarizing shift in the voltage dependence of activation, effects which are sensitive to an inhibitor of the phosphatase, calcineurin [11]. In cardiac myocytes, expression of the kinase p90RSK is dramatically increased in disease states such as heart failure, ischemia, and diabetes [101]. Transgenic mice with increased activity of p90RSK display prolongation of the QT interval and increased action potential duration in the ventricle, with corresponding decreases in I_{tof}, IK_s, and IK_{ss} [101]. In this same study, the authors demonstrate that p90RSK expression inhibits both Kv1.5 and Kv4.3 current in HEK 293 cells, and that Kv4.3 is a substrate for phosphorylation by p90RSK [101]. In human ventricular tissue, Kv1.5 associates with Src, a kinase which is reported to be redox-sensitive [102]. In this same study, phosphorylation of Kv1.5 by Src in HEK 293 cells reduces channel current [102]. The alpha subunit of the rapidly inactivating Kv4.2 channel, which underlies I_{to} in the heart, is a substrate for ERK MAP kinase phosphorylation at 3 amino acid residues [103], suggesting that this channel may be redox-sensitive through phosphorylation. Phosphorylation of a residue on the N-terminus of Kv1.4, which also underlies I_{to} in the heart, slows channel inactivation and accelerates its recovery from inactivation, a process regulated by the calcineurin/inhibitor-1 protein phosphatase [104]. HERG channel possesses several putative phosphorylation sites, and it is a substrate for phosphorylation by protein kinase C, Src, and protein kinase B [105-107]. Thus, phosphorylation plays an important role in regulation of cardiovascular potassium channels under normal and pathophysiological conditions, and through regulation

of the enzymes controlling phosphorylation and de-phosphorylation, this process can be sensitive to changes in cellular redox state.

Tyrosine Nitration

Tyrosine nitration is the covalent addition of nitrating agents to protein tyrosine residues [108]. Nitrating agents include peroxynitrite ($ONOO^-$) and nitrogen dioxide ($^{\cdot}NO2$), which form from reaction with nitric oxide ($^{\cdot}NO$) under conditions of oxidative stress [108]. Although tyrosine nitration was once thought to be an indiscriminate biomarker of oxidative insult, new evidence suggests that it occurs with a surprising level of specificity. The susceptibility of tyrosine residues in proteins to nitration is influenced by intrinsic factors, such as neighboring amino acid residues in the secondary structure of the target protein, as well as extrinsic factors, such as proximity of the target protein to nitric oxide synthases [108]. Nitration of a single tyrosine is sufficient for inactivation of Mn superoxide dismutase, suggesting that this modification may be a means of functional protein regulation [108]. New evidence suggests that Kv channels may be regulated by tyrosine nitration in disease states such as diabetes [4]. In rats administered streptozotocin to induce diabetes, there is an increase in tyrosine nitration on Kv1.2 in small coronary arteries [4]. This increase in tyrosine nitration is accompanied by a decrease in channel protein expression and current, and correolide-sensitive vasodilation [4]. Importantly, treatment with ebselen, a glutathione peroxidase mimetic and scavenger of $ONOO^-$, reduces tyrosine nitration of Kv1.2, restores channel expression and partially restores correolide-sensitive vasodilation [4]. Interestingly, tyrosine nitration and protein expression of Kv1.5, also expressed in small coronary arteries, remained

unchanged in the diabetic rats, suggesting differential sensitivity of specific Kv channel isoforms to oxidative and nitrosative stress [4]. Although the authors do not definitively prove that tyrosine nitration alone modulates Kv1.2 expression in diabetes, they provide an intriguing hypothesis that merits further studies. Thus, tyrosine nitration may play an important role in regulation of potassium channel function in cardiovascular disease, and this remains a burgeoning area for mechanistic studies.

S-Acylation

Thioacylation (S-acylation) is the reversible, posttranslational, covalent addition of long chain fatty acids to cysteine residues of proteins via a thioester linkage [109]. This modification is critical in regulation of trafficking, membrane association, intracellular signaling, and protein-protein interactions [109, 110]. Although S-palmitoylation is the most commonly studied form of S-acylation, protein modification with other saturated and unsaturated fatty acids has also been reported [111, 112]. The enzymatic pathways regulating protein fatty acylation are still poorly understood, although DHHC protein S-acyltransferases may play an important role in regulating thioacylation *in vivo* [110]. Palmitoylation of KChIP, the calcium sensitive auxillary subunit for Kv4 channels, promotes KChIP surface expression, and is necessary for KChIP enhancement of Kv4 channel surface expression [113]. Kv1.1 channels also undergo palmitoylation, which affects voltage sensing through modulation of channel interaction with the surrounding lipid environment [114]. We and others have previously reported that Kv1.5 undergoes S-acylation on intracellular cysteines, and that this modification regulates steady-state channel expression on the cell

surface [109, 115]. Fatty acylation occurs early in the biosynthesis of Kv1.5, and is important for permitting trafficking of mature, glycosylated channel to the cell surface [109]. Several reports suggest that fatty acylation is sensitive to oxidative stress, providing another dimension for redox regulation of protein expression and membrane targeting [116, 117]. Although we found no acute effects of oxidative stress on Kv1.5 fatty acylation, it remains to be determined whether chronic oxidative stress disrupts fatty acylation in the biogenesis and trafficking of new channel.

SUMOylation

SUMOylation is the covalent modification of proteins with the SUMO (small ubiquitin-like modifier) protein [118]. Modification of proteins with SUMO plays an important role in numerous physiological processes, including transcription, regulation of the cell cycle, DNA repair, and chromatin remodeling, and is sensitive to changes in cellular redox state [118, 119]. SUMOylation occurs via a sequence of enzymatic steps, in which SUMO is activated via the E1 (SAE1/SAE2) ligase, followed by transfer to the E2 ligase (Ubc9) [118]. Ubc9 catalyzes the addition of the COOH-terminus of SUMO to the amino group of lysine on the target protein, a process which is facilitated by E3 SUMO ligases [118]. The consensus sequence for SUMOylation consists of a hydrophobic reside preceding the target lysine, and a negatively charged amino acid two residues downstream [118]. SUMOylation is reversible, via the activity of mammalian SENP isopeptidase enzymes [118]. In addition to the well-established role for SUMOylation in regulation of transcription, it is emerging as a means of posttranslational regulation of ion channels. Reversible SUMOylation

of a single lysine on the two pore potassium channel K2P1 silences channel current, which is restored upon de-sumoylation [120]. This finding, however, is the subject of controversy [121]. SUMOylation of Kv1.5 occurs on two lysines of the channel, and disruption of this modification by mutation of the target lysines or overexpression of SENP2 causes a hyperpolarizing shift in the voltage dependence of inactivation [122]. SUMOylation has also been demonstrated to regulate Kv2.1 current. Kv2.1 is expressed in the pulmonary vasculature, and mRNA for this channel is also present in the heart [1]. SUMOylation attenuates Kv2.1 current in HEK cells and alters excitability of native pancreatic beta cells, through modulation of channel inactivation [123] . However, it is currently unclear whether SUMOylation occurs directly on the channel, or on an accessory protein. Interestingly, a role for SUMOylation has been demonstrated in disease states associated with oxidative stress, including ischemia and diabetes [124, 125]. Periods of ischemia in the brain are associated with a dramatic, global increase in SUMOylated proteins, commensurate with increased oxidative stress [125]. Whether redox-sensitive SUMOylation, through modulation of potassium channel function, plays a role in the pathogenesis of cardiovascular diseases remains to be determined, and represents a fertile area for continued research.

Cysteine Oxidation: A Principal Posttranslational Mechanism for Regulating Protein Functionality

Although other amino acids are subject to oxidation, the nucleophilic nature and relatively low pKa of the free thiol (-SH) moiety of cysteine make it an important target for ROS [68]. Even though the pKa of cysteine residues in peptides is approximately 9, the pKa of protein thiols can be as low as 3.5 [68],

facilitating ionization at physiological pH to their corresponding thiolate anion (S^-). As the thiolate anion is more nucleophilic than the parent sulfhydryl, these cysteine residues are more reactive toward formation of posttranslational modifications [45]. As shown in Figure 1.5, several different oxidative cysteine modifications (oxoforms) have been identified *in vivo*, and they perform a variety of functions, including catalysis, regulation of protein turnover, regulation of gene expression, and signal transduction [68]. Oxidative cysteine modifications are regulated by several endogenous antioxidant systems. Multiple antioxidant enzymes indirectly maintain cysteines in a reduced state by scavenging cellular free radicals [45]. These include: catalase, which scavenges hydrogen peroxide, superoxide dismutase, which catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide, and peroxiredoxins, which catalyze reduction of hydrogen peroxide, organic hydroperoxides, and peroxinitrite [45]. Additional enzyme systems function to directly reduce oxidized sulfhydryls in cells. The thioredoxin/thioredoxin reductase system functions to reduce oxidized sulfhydryls using NADPH as a cofactor [45]. Other antioxidant functions of thioredoxin include reduction and reactivation of peroxiredoxin and methionine sulfoxide reductase [45]. Similarly, the glutathione/glutathione reductase/glutaredoxin system promotes reduction of oxidized cysteines using NADPH as a cofactor [45].

Formation of Disulfide Bonds

Perhaps the most heavily studied cysteine oxidation reaction involves reversible formation of inter- or intra-molecular disulfide bonds, which are essential for maintaining protein structure and function. For example, inter-chain

disulfide bonds between heavy and light chains of immunoglobulins stabilize their structure [126]. Formation of an intra-molecular disulfide bond in the bacterial transcription factor OxyR permits DNA binding and transcription of genes in response to oxidative stress [127]. Disulfide bond formation in cardiovascular Kv channels has been reported in response to oxidative stress, although the functional effects of this modification appear to be channel-dependent. Nitric oxide inhibits the rapidly inactivating Kv4.1peak current through formation of an inter-subunit disulfide bond at the T1-T1 interface of adjacent alpha subunits [128]. The Shaker Kv channel can also form inter-molecular disulfide bonds between N- and COOH-terminal cysteines in response to oxidant exposure, although this modification has no effect on current amplitude or the biophysical properties of the channel [129]. Interestingly, we demonstrate that Kv1.5, a member of the Shaker family of Kv channels, does not undergo formation of inter-molecular disulfide bonds with acute oxidative stress, although oxidant treatment significantly reduces the amplitude of channel current. This discrepancy may reflect differences in the number and position of intracellular cysteines on Shaker channels, and suggests that there is a high degree of specificity among Kv channels in their response to oxidative stress. A specific disulfide bond may also occur in response to oxidative stress, through formation of adducts with the endogenous antioxidant, glutathione. Reversible glutathionylation represents a mechanism for protection of cysteines from irreversible over-oxidation during oxidative stress, and also functions in regulation of protein function [130]. Although oxidoreductase systems, including

the glutathione/glutathione reductase system, clearly regulate Kv channel function in the heart [13], glutationylation of Kv channels has not been reported. Consistent with this, our experiments show that Kv1.5 does not form adducts with glutathione under acute oxidative stress. The molecular mechanism by which the glutathione/glutathione reductase system regulates Kv channel function requires further experimentation.

S-Nitrosylation

Cysteine residues are capable of undergoing modification with nitric oxide. Nitric oxide plays a vital role in numerous cardiovascular functions, including vasodilation, angiogenesis, regulation of blood clotting in the vasculature, excitation-contraction coupling, mitochondrial respiration, and ischemic pre-conditioning in the heart [131, 132]. Regulation of proteins by nitric oxide can occur primarily via guanyl cyclase-dependent signal transduction, or direct posttranslational modification of proteins [131, 133]. S-nitrosylation is the covalent, posttranslational attachment of nitric oxide (NO) to cysteine thiols on proteins to form S-nitrosothiol (SNO) [133]. Although no distinct consensus sequence exists for formation of SNO, proximal acidic (Glu, Asp) and basic (Lys, Arg, His) residues increase the likelihood of SNO formation [133]. Furthermore, nitric oxide has a much smaller diffusion radius than other oxidants in cells, such as hydrogen peroxide [35]; thus, proximity to nitric oxide synthases likely influences the protein targets for nitric oxide signaling [108]. Coordination of cysteine thiols with Zn^{2+} ions lowers the pKa of cysteine and further facilitates SNO formation [133]. This modification permits NO to directly regulate proteins independent of guanyl cyclase. In guinea pig cardiac myocytes, S-nitrosylation

increases the amplitude of I_{Ks}, resulting in shortening of the action potential duration [134]. Further studies by the same research group demonstrate that in HEK293 cells, a nitric oxide donor induces S-nitrosylation of KCNQ1(KvLQT1) on a single, membrane-proximal cysteine and increases current amplitude in a calmodulin-dependent fashion [135]. Nitric oxide donors induce S-nitrosylation of, and inhibit currents from, Kv4.3 channels, which underlie the cardiac I_{to} current [136]. Kv1.5 is also reported to be modified via S-nitrosylation, resulting in a decrease in current amplitude which is independent of guanyl cyclase [51]. Lastly, nitric oxide inhibits the HERG channel when expressed in *Xenopus* oocytes, although S-nitrosylation of the channel itself was not directly demonstrated [137]. Thus, reversible, covalent modification of cysteines on potassium channels by nitric oxide may represent a mechanism for regulating cardiac cellular excitability in response to changes in cellular redox state. Interestingly, ischemic pre-conditioning results in a significant increase in S-nitrosylated proteins in the heart [132], suggesting that the metabolic conditions in brief periods of ischemia favor formation of S-nitrosothiols, and that they may protect proteins from irreversible over-oxidation and degradation.

Sulfenic Acid Modification

Of the currently known cysteine oxoforms (Figure 1.5), reversible formation of cysteine sulfenic acid (Cys-SOH) is currently the focus of intense research, and is emerging as a critical mechanism for signal transduction and protein regulation in response to changes in cellular redox state [68]. Sulfenic acid can form during conditions of cellular oxidative stress and has multiple cellular fates, making it a versatile, unique pivot point in signaling [68].

Depending on the protein microenvironment, sulfenic acid may exist as a metastable modification or as a transient species, leading to more stable disulfide, sulfinic acid, or sulfenyl-amide forms [68]. Sulfenic acids were identified in proteins several decades ago [138]; however, the transient and labile nature of the modification, combined with lack of specificity in inhibitors and pharmacological tools, have precluded identification of sulfenic acid-modified proteins *in vivo*. Accordingly, little is known about the role for sulfenic acid modification in the pathophysiology of disease states. Early studies of sulfenic acid-modified proteins were typically conducted *in vitro*, and often in bacteria [139]. Several recent proteomic analyses have begun to reveal the targets for sulfenic acid modification in cells and tissues. Using a cell-permeant, biotinylated dimedone analogue, analysis of rat hearts exposed to hydrogen peroxide revealed approximately 22 sulfenic acid-modified proteins [140]. Most proteins identified were expressed at relatively high levels, including myosin heavy chain and alpha actin [140]. Thus, the presence of low-abundance sulfenic acid-modified proteins is difficult to discern. Furthermore, the analysis revealed two proteins which do not contain cysteines, indicating that false positives are a potential disadvantage to proteomic studies. A similar proteomic study conducted in spontaneously hypertensive rats (SHR) revealed a global increase in sulfenic acid-modified proteins in the kidney medulla of SHR compared to normotensive rats [141]. Although this study identified primarily high-abundance proteins and encountered false positives, the results indicate an important association between sulfenic acid modification of proteins and cardiovascular disease. Using

the sulfenic acid-specific chemical probe, DAz-2, analysis of sulfenic acid-modified proteins in HeLa cells revealed 175 candidate proteins, although in a subset of these proteins, sulfenic acid is likely a precursor to other oxidative modifications [142]. Recently, the level of sulfenic acid modification in tumors was analyzed using protein microarray analysis of tumor tissues [143]. Interestingly, the level of sulfenic acid-modified proteins in breast tumors is significantly higher for grade II, relative to grades I and III. These data suggest that breast cancer in mid-stage is characterized by higher levels of oxidative stress, potentially implicating thiol oxidation in tumorigenesis [143]. It is clear from these data that the list of mammalian sulfenic acid-modified proteins is still relatively small. Furthermore, among the known sulfenic acid-modified proteins, the regulatory role for this modification is poorly understood. As novel chemical probes continue to be developed for detection and study of sulfenic acids *in vivo*, the functional consequences of this modification on protein function continue to emerge. Examples of regulation of protein function by sulfenic acid *in vivo* include regulation of enzymatic activity of protein tyrosine phosphatases [100], peroxiredoxins [127], methionine sulfoxide reductase [127], activation of T cells in the immune system [144], and regulation of transcription factors, including fos, jun, and the p50 subunit of NF-kB [127]. Recently, work in our laboratory identified a functional role for sulfenic acid modification in regulation of Kv1.5. Under acute oxidative stress, sulfenic acid modification on a single COOH-terminal cysteine triggers internalization of Kv1.5, and inhibits recycling of the channel back to the cell surface. Importantly, this modification does not proceed

to an inter-molecular disulfide bond or adduct with glutathione, suggesting that sulfenic acid is not merely an unstable intermediate *en route* to a more stable modification. This is the first example demonstrating sulfenic acid modification to a transmembrane protein, and that this modification can acutely regulate cell surface protein levels. Furthermore, we find that sustained oxidative stress promotes channel degradation. Our results indicate that sulfenic acid modification represents a fate switch, triggering internalization of channel and diverting it from a recycling pathway to a degradation pathway. As shown in Figure 1.6, the single cysteine undergoing sulfenic acid modification is conserved across multiple mammalian species. This observation suggests a conserved, functional role for this cysteine as a molecular sensor for changes in cellular redox state. Oxidation of this cysteine to sulfenic acid enables cells to translate acute changes in redox state into altered cellular excitability. Furthermore, during persistent oxidative stress, which is characterized by more prolonged changes in redox state, further oxidation of this cysteine may precipitate protein degradation, as work in our lab has demonstrated. The conservation of this cysteine may thus provide insight into the unique role for Kv1.5 in oxygen sensing in the pulmonary vasculature, and possibly in the heart. Recent functional site profiling has revealed that sulfenic acid-modified proteins are flanked by polar amino acid residues capable of forming hydrogen bonds [145]. It is interesting that the modified cysteine (red box) is preceded by a polar serine residue, which is also conserved. Thus, the protein microenvironment flanking

reactive cysteines may explain the propensity for particular cysteines to form sulfenic acid.

Sulfonic/Sulfinic Acid Modification

Under conditions of prolonged oxidative stress or insufficient antioxidant capacity, sulfenic acid may be further oxidized to sulfinic (SO_2H) or sulfonic (SO_3H) acid [68]. The Parkinson's disease protein, DJ-1 [146], copper-zinc superoxide dismutase (SOD1) [147], sarcoplasmic reticulum Ca(2+) ATPase [148], and the antioxidant enzyme peroxiredoxin [149] are among the few proteins that have been reported in the literature to be hyper-oxidized to sulfinic/sulfonic acid. Although sulfinic acid modification of DJ-1 is necessary for proper protein function and localization, cysteine hyper-oxidation is largely thought to lead to protein inactivation and degradation [127, 148]. Both sulfinic and sulfonic acid modification were thought to be irreversible; however, it was recently discovered that sulfinic acid modification of peroxiredoxin is enzymatically reversible via the enzyme sulfiredoxin [149]. To date, there have been no reports of sulfinic/sulfonic acid modification of Kv channels in the cardiovascular system. Interestingly, however, reduced expression of Kv1.2 is observed in the coronary arteries of diabetic rats, which is reversible upon oral treatment with ebselen, a scavenger of peroxides and peroxinitrite [4]. Free radical scavengers may prevent cysteine hyper-oxidation and protein degradation. Our recent experiments in HL-1 atrial myocytes demonstrate that prolonged exposure to oxidative stress causes degradation of Kv1.5, and we hypothesize that this degradation is due to hyper-oxidation of intracellular cysteines. Consistent with our hypothesis, Kv1.5 degradation is partially blocked

by concurrent treatment with dimedone, an agent that prevents cysteine hyper-oxidation by binding specifically to sulfenic acid-modified proteins, trapping them in a sulfenic acid-modified state. Collectively, these results suggest that chronic oxidative stress occurring in disease states may play a mechanistic role in the observed pathological reduction in Kv channel expression and altered cellular excitability. Much work is needed to understand the role for cysteine hyper-oxidation in Kv channel degradation. A mechanistic understanding of this process is particularly desirable, given that several disease states, including pulmonary arterial hypertension[6], atrial fibrillation [8], and diabetes mellitus [4] are associated with both oxidative stress and Kv channel degradation. Cysteine hyper-oxidation is therefore an exciting and fruitful area for future work.

Summary

In summary, the existence of multiple Kv channel alpha subunits capable of forming homo- and heterotetramers, and their association with modulatory subunits, generate significant diversity in Kv channel currents. This is particularly apparent in the heart, where multiple, complex Kv channel currents synergize to facilitate repolarization. These repolarizing currents are widely reported to be redox-sensitive, with important consequences for heart and blood vessel function. Not surprisingly, alterations in Kv channel currents are associated with several cardiovascular diseases that are correlated with oxidative stress, such as cardiac arrhythmias and hypertension. Although there is significant evidence in support of the redox sensitivity of Kv channels, the molecular nature of this sensitivity is the focus of intense study.

Multiple mechanisms for regulation of Kv channel currents exist, including alterations in the biophysical properties of the channel, effects on protein stability, and disruption of channel trafficking. On a molecular level, each of these mechanisms for regulation may be achieved through redox-sensitive posttranslational modifications to the channel itself. As summarized in the reviewed examples of posttranslational modifications, the redox-sensitive modification is dependent upon the type of oxidizing agent, the dose and duration of the oxidative stimulus, and the properties and location of the target protein. Additionally, the propensity to form specific posttranslational modifications, such as S-nitrosothiols, cysteine sulfenic acids and sumoylation, and their subsequent stability, is dependent on the amino acid sequence of the protein [108, 122, 145]. All of these factors, along with cross-talk between multiple pathways, promote significant diversity in cellular responses to oxidative stress.

In the remaining chapters of this thesis, we demonstrate that redox-sensitive modification of Kv1.5 with sulfenic acid has significant effects on channel expression and function in atrial myocytes. Redox-sensitive posttranslational modifications therefore represent a key mechanism by which ROS modulate cellular protein function and homeostasis, and provide a critical molecular link between oxidative stress and disease.

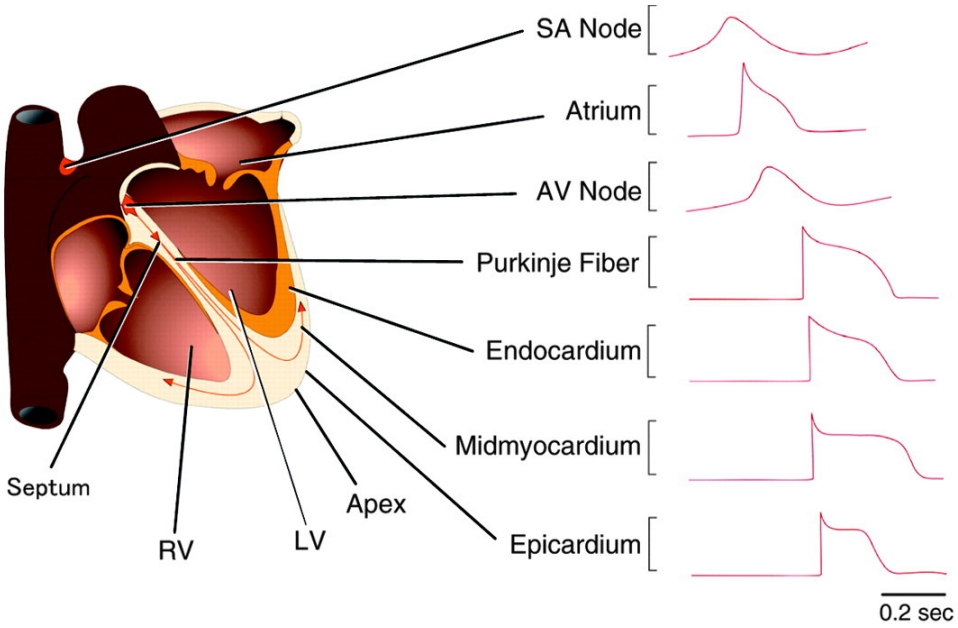

Figure 1.1: Electrical Activity in Different Regions of the Heart
Different regions of the heart display distinct action potential waveforms. The differences in action potential duration are governed in part by expression of distinct, repolarizing Kv channel currents. Repolarization of the action potential is represented by a downward deflection on the trace. Adapted from [3].

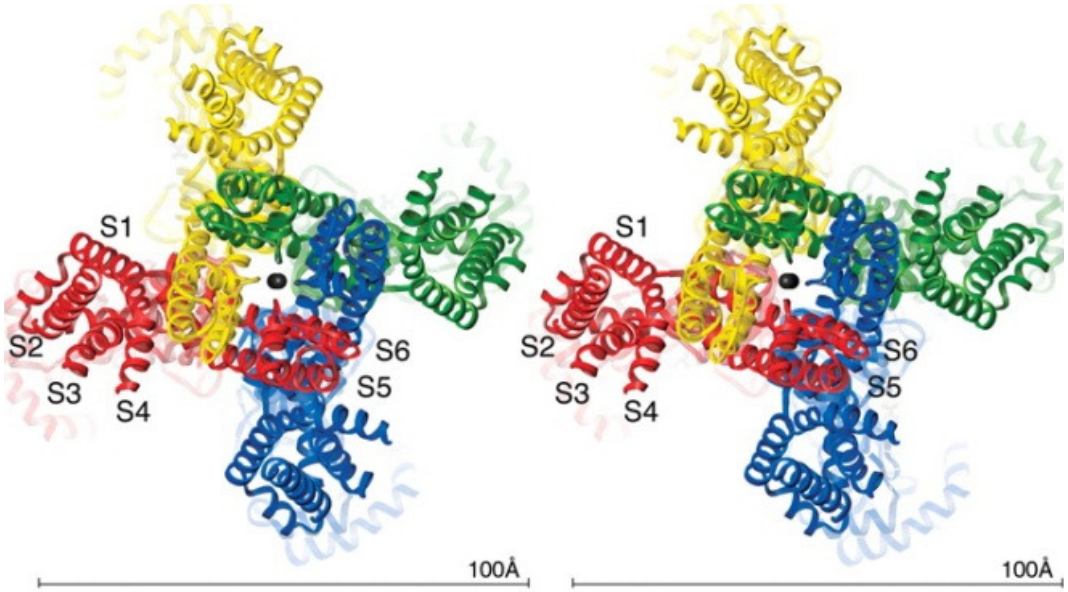

Figure 1.2: Kv Channel Alpha Subunits Arrange to Form Tetramers in vivo
Stereoview of a Shaker Kv channel, represented as a ribbon diagram, viewed from the extracellular side of the pore. Each alpa subunit is colored individually, and potassium ion is shown in black. Adapted from [23].

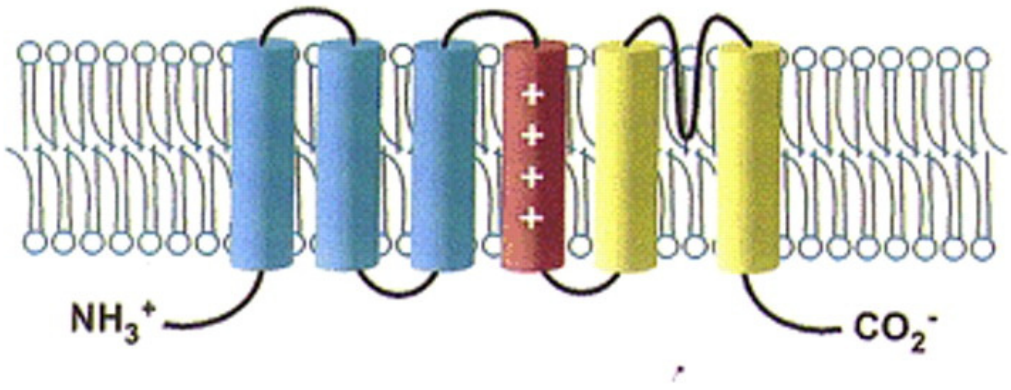

Figure 1.3: Topology of a Kv Alpha Subunit in the Cell Membrane
Illustration of a single Kv channel alpha subunit. S1-S3 colored blue, positively charged S4 region colored red, and S5-S6 colored yellow. Adapted from [24].

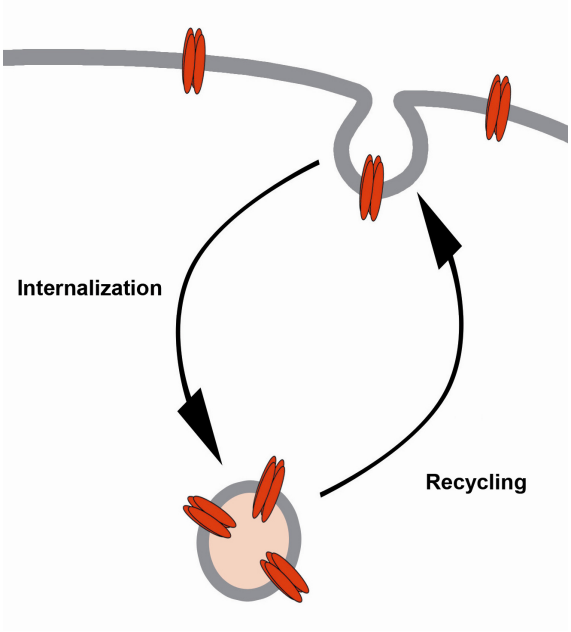

Figure 1.4: Kv1.5 Trafficking in Cardiac Myocytes
Kv1.5 undergoes a dynamic process of constitutive internalization to EEA1-positive compartments, and recycling back to the cell surface via a Rab-dependent mechanism [85]. Pharmacological interventions [29] and oxidative stress can alter this trafficking process.

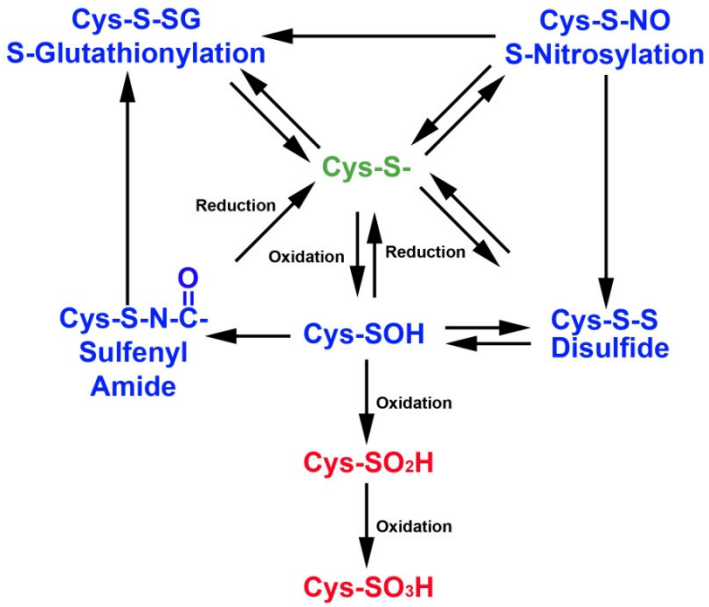

Figure 1.5: Multiple Fates of a Cysteine Thiol in Cells
Cysteines with sufficiently low pKa exist as a thiolate anion at physiological pH (green). Several cysteine modifications have been reported. All reversible modifications are shown in blue, and their potential fates are designated by the arrows. With the exception of cysteine sulfinic acid modification to peroxiredoxin [150], cysteine sulfinic and sulfonic acid (red) are irreversible. Adapted from [151].

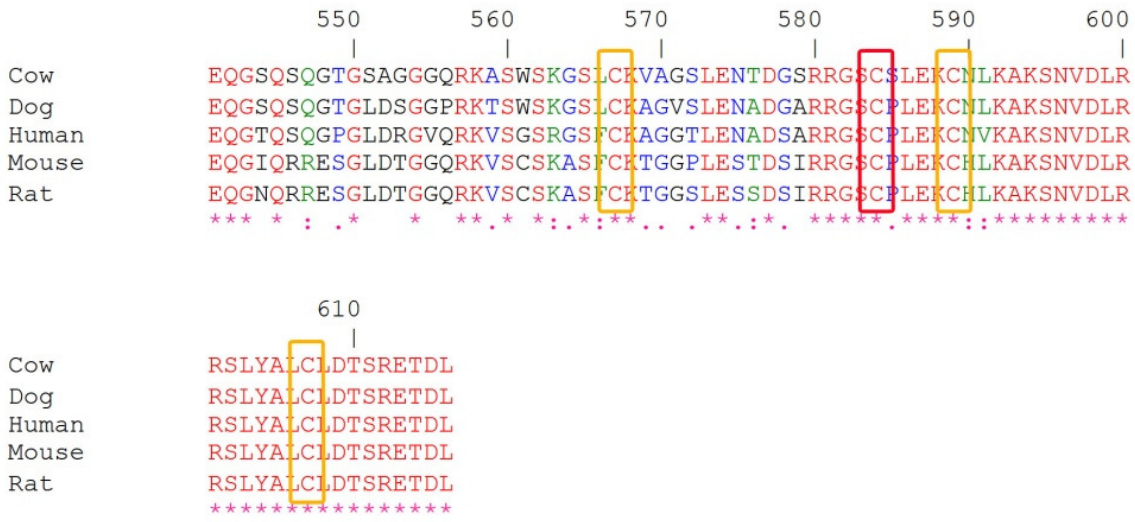

Figure 1.6: The COOH-Terminal Cysteines on Kv1.5 are Conserved Across Species.
Sequence alignment of the COOH-terminal segment of Kv1.5 containing the four cysteines (outlined in boxes), including sulfenic acid-modified C581 (red box). Residues shown in red are identical (also denoted by asterisk), green residues are strongly similar (denoted by colon), and blue residues are weakly similar (denoted by period). Data generated using the ClustalW multiple alignment function in Network Protein Sequence Analysis. http://npsa-pbil.ibcp.fr

CHAPTER II: KV1.5 UNDERGOES REDOX-SENSITIVE MODIFICATION WITH SULFENIC ACID

Introduction

The voltage-gated potassium (Kv) channel, Kv1.5 underlies IKur, the major repolarizing current in the human atrium. Kv1.5 is widely reported to be sensitive to changes in cellular redox balance [6, 47, 152, 153]. Disease states such as atrial fibrillation and hypoxic pulmonary hypertension are strongly associated with down-regulation of Kv1.5 expression as well as with oxidative stress [6-8, 40, 78]. Although both oxidative stress and dysregulation of Kv1.5 have been reported in the context of cardiovascular disease, the molecular mechanism(s) linking these observations remains unclear. The thiol (-SH) group of the amino acid cysteine is a principal target for ROS in many proteins, including enzymes, signaling proteins, and transcription factors [127]. Oxidation of key cysteine residues is an important mechanism whereby changes in cellular redox balance can lead to modification of protein function. Several different oxidative cysteine modifications (oxiforms) have been identified *in vivo*, and they play a crucial role in protein stability and function [68]. Of these cysteine oxoforms, reversible formation of cysteine sulfenic acid (Cys-SOH) is emerging as an important mechanism for dynamic regulation of protein function in response to changes in cellular redox state [68, 127]. Cys-SOH can form during

conditions of cellular oxidative stress and, depending on the protein microenvironment, afford a metastable modification or represent a transient species leading to more stable disulfide or sulfinic acid form [68]. Kv1.5 possesses six intracellular cysteines on both the NH_2 and COOH termini. Therefore, given the reported redox sensitivity of channel current, we hypothesized that Kv1.5 is modified with sulfenic acid on intracellular cysteines.

Using a novel chemical probe in LTK cells, a mouse fibroblast cell line, we demonstrate herein that Kv1.5 is modified with sulfenic acid on a single COOH-terminal cysteine. Furthermore, this modification is redox-sensitive, and occurs after treatment with multiple oxidants. Under conditions of acute oxidative stress, sulfenic acid modification of Kv1.5 is not a precursor to formation of inter-molecular disulfide bonds or adducts with glutathione. Although Kv1.5 undergoes thioacylation, which has been reported by others to be redox-sensitive [116, 117], formation of sulfenic acid under acute oxidative stress has no significant effect on Kv1.5 thioacylation. Importantly, we confirmed our findings in cells exposed to cigarette smoke extract, a socially relevant source of oxidative stress and a significant cause of cardiovascular disease [36, 75, 154]. Cigarette smoke extract induces a robust increase in sulfenic acid modification to Kv1.5. Together, these results reveal a novel, redox-sensitive posttranslational modification to an ion channel, and this finding will likely have important implications in the study of oxidative stress, dysregulation of ion channels, and cardiovascular disease.

Experimental Procedures

Materials

Mouse anti-V5 antibody (used at 1:5000) was purchased from Invitrogen (Carlsbad, CA). Rabbit anti-alpha tubulin (used at 1:1000) was purchased from Abcam (Cambridge, MA). HRP-streptavidin conjugate (used at 1:10,000) was purchased from Pierce (Rockford, IL). Anti-glutathione monoclonal antibody (used at 1:500) was purchased from ViroGen (Watertown, MA). Rabbit antiserum against the 112-amino acid NH2-terminal region of Kv1.5 was provided by the Tamkun laboratory [155]. Complete protease inhibitor cocktail tablets were obtained from Roche Applied Science (Indianapolis, IN). EZ-Link Biotin-BMCC was from Pierce (Rockford, IL). Protein G- and protein A-agarose beads, *N*-ethylmaleimide, tertiary-butyl hydroperoxide (*t*BOOH), diamide, hydrogen peroxide (H_2O_2) and dimedone were purchased from Sigma (St. Louis, MO). HL-1 cells were a generous gift from Dr. William Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA).

Transient Transfection

HL-1 (mouse cardiomyocyte) cells in 35-mm culture dishes were transfected with human Kv1.5 [156] at 50-60% confluence with 0.65µg of DNA combined with 1.5µL of lipofectamine 2000 reagent (Invitrogen) in Opti-Mem (Gibco) for 3–5 h, changed to normal medium, and allowed 48 hours for protein expression.

Stable Cell Lines

Stable cell lines expressing Kv1.5 wild type and cysteine mutant constructs were created in LTK cells (a mouse fibroblast cell line) using the Retro-X Universal Packaging System from Clontech (Mountain View, CA), according to manufacturer's instructions. Stable cell lines expressing the following mutant constructs were generated: Kv1.5 wild type (WT), Kv1.5-6CS, in which the six NH_2-and COOH-terminal cysteines were mutated to serine, and Kv1.5-4CS, in which the COOH terminal cysteines were mutated to serine. Four COOH-terminal point mutant constructs were created by mutating each individual cysteine to serine: Kv1.5C564S, Kv1.5C581S, Kv1.5 C586S, and Kv1.5C604S. Four additional Kv1.5 constructs were created by re-introducing individual cysteines on the COOH terminus into the null background of Kv1.5 6CS: Kv1.5S604C, Kv1.5S586C, Kv1.5S581C and Kv1.5S564C.

Western Blot

LTK or HL-1 cells were harvested in denaturing lysis buffer containing 50 mM Tris-Cl, 10% glycerol, and 2% SDS containing complete protease inhibitors (Roche Applied Science). Membranes were isolated and separated by SDS-PAGE on a NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen). Proteins were transferred to nitrocellulose and probed with the indicated primary antibody for 1 h at room temperature. Blots were then incubated with secondary antibody conjugated to horseradish peroxidase (1:5000), and visualized using Western Lightning enhanced chemiluminescent reagent according to the manufacturer's protocol (PerkinElmer Life Sciences). Images were captured using the EpiChemi3 darkroom (UVP, Inc., Upland, CA).

DAz-1 Labeling of Sulfenic Acid, Staudinger Ligation, and Immunoprecipitation of Kv1.5

LTK or HL-1 cells were harvested in non-denaturing lysis buffer containing 150 mM NaCl, 50 mM Tris-Cl, 5 mM EDTA, 1% Triton X-100, and 5 mM DAz-1, pH 7.5 with protease inhibitors (Roche), followed by gentle rocking at 4℃ for 20 min. Lysates were then dounce homogenized, followed by centrifugation at 16,000 g for 4 min at 4℃ to remove cell debris. Supernatant was then incubated at 37℃ for 2-2.5h with gentle rocking to allow labeling of sulfenic acid modified proteins with DAz-1. Protein (250µg) was immunoprecipitated overnight at 4℃ with 1.5 µL of anti-V5 antibody conjugated to 60 µL of Protein G-agarose beads. The following day, the beads were washed twice with wash buffer containing protease inhibitors: 150 mM NaCl, 50 mM Tris-Cl, 5 mM EDTA, and .1% Triton X-100. For the ligation reaction, beads were resuspended in wash buffer with 250 µM p-biotin and incubated 2h at 37℃ with gentle rocking, prior to electrophoresis and streptavidin-HRP Western blotting. For labeling of whole cell lysates with DAz, cells were harvested in non-denaturing lysis buffer with 1 mM DAz, as in the immunoprecipitation experiments. Lysates were then nutated at 37℃ for 2.5h, followed by a 4 minute spin at 16,000 g and 4℃. To remove excess DAz, supernatant was transferred to Amicon Microcon YM-3 spin columns and spun at room temperature, 10,000 g for 90 min. Retentate was resuspended in fresh lysis buffer and spun for another 60 min. For Staudinger ligation, lysate (at a concentration of 1mg/mL) was incubated in 100µM p-biotin and 5 mM DTT for 2 hours, nutating at 37℃. Samples were then resuspended in SDS sample buffer and frozen at -20℃ prior to streptavidin western blotting.

Detection of Fatty Acylation on Kv1.5

Fatty acylation of Kv1.5 was assessed using fatty acyl exchange labeling, as outlined previously [157]. Briefly, cells were lysed in non-denaturing lysis buffer containing 150 M NaCl, 50 mM Tris-Cl, 10 mM EDTA, 1% Triton X-100, and 250 mM N-ethylmaleimide (NEM). The supernatant was collected and precleared with 75 µL of protein G-agarose beads for 1h at 4℃ with gentle rocking. Precleared protein (250µg) was immunoprecipitated overnight at 4℃ with 1 µg of anti-V5 antibody conjugated to Protein G-agarose beads. The following day, the beads were washed twice for 5 min at room temperature with wash buffer containing protease inhibitors: 150 mM NaCl, 50 mM Tris-Cl, 10 mM EDTA, and .1% Triton X-100, then resuspended in 1 M hydroxylamine in 1 M Tris, pH 7.4, and nutated for 1 h at room temperature. After two 5 min washes, the beads were resuspended in 1 ml of 2µM EZ Link Biotin-BMCC and nutated for 2 h at room temperature, followed by electrophoresis and streptavidin-HRP Western blotting.

Detection of Glutathionylated Kv1.5

Glutathionylation of Kv1.5 was assessed by immunoprecipitation and Western blotting with anti-glutathione antibody. Briefly, cells were lysed in non-denaturing lysis buffer containing 150 M NaCl, 50 mM Tris-Cl, 10 mM EDTA, 1% Triton X-100, and 100 mM NEM. The supernatant was collected and precleared with 50 µL of protein A-sepharose beads for 1h at 4℃ with gentle rocking. Precleared protein (250µg) was incubated overnight at 4℃ with 1 µL of anti-Kv1.5 antibody and 75 µL of Protein A-sepharose beads. The next day, the beads were washed twice for 5 min at room temperature with wash buffer containing protease

inhibitors: 150 mM NaCl, 50 mM Tris-Cl, 10 mM EDTA, and .1% Triton X-100 and 100 mM NEM. Beads were then resuspended in non-reducing SDS sample buffer, boiled 2 min. and separated via electrophoresis under non-reducing conditions.

Production of Cigarette Extract and Cigarette Smoke Extract

To prepare cigarette smoke extract, each cigarette was burned for approximately 3-4 minutes using a vacuum line connected to a flask of water, as previously described [158]. Cigarette smoke (one cigarette per mL of water) was bubbled into 20 mL of water. Cigarette extract was prepared from crushed, unsmoked cigarettes, as previously described [158]. The cigarette extract and cigarette smoke extract were stored at -80°C.

N-Glycosidase Treatment of Kv1.5

Vehicle-treated cells were lysed in the presence of DAz-1, and Kv1.5 was immunoprecipitated and labeled with p-biotin as outlined in the method. After washing, sample on the beads was incubated in 1X glycoprotein denaturing buffer for 10 min at 100°C, followed by incubation overnight in 1 µl of 10x G7 reaction buffer, 1 µl of 10% NP-40, and 1 µl of *N*-glycosidase F at 37°C. Supernatant from beads was analyzed via western blot.

Statistics

Statistics were performed using Prism software Version 5 from Graphpad Prism Software (San Diego, CA). All data are expressed as mean +/- SEM. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test, or unpaired, two-tailed t-test. A p value of <0.05 was considered significant.

Results

Kv1.5 possesses six intracellular cysteines on both the NH_2 and COOH termini which may be candidates for oxidation to sulfenic acid (Figure 2.1). Therefore, we hypothesized that sulfenic acid modification of Kv1.5 may account for its reported redox sensitivity. To test this hypothesis, we utilized the novel chemical probe, DAz-1. When coupled to a biotinylated secondary agent, DAz-1 enables detection of sulfenic acid-modified Kv1.5 in cells [33] (Figure 2.2). Kv1.5 appears as a doublet, with a nascent, non-glycosylated form and a mature, glycosylated form [109]. In LTK cells stably expressing Kv1.5 WT, we found that Kv1.5 is modified with sulfenic acid, as indicated by a doublet appearing at approximately 80 kDa on the HRP-streptavidin Western blot (Fig. 2.3a, lane 3). To confirm that this modification occurs on intracellular cysteines, we created a second stable cell line in LTK cells expressing Kv1.5 with all six intracellular cysteines mutated to serine (Kv1.5-6CS). Mutation of the six intracellular cysteines on Kv1.5 abolished this labeling, despite efficient immunoprecipitation (Figure 2.3a, lane 4), indicating that sulfenic acid modification occurs on intracellular cysteines. Omitting DAz-1 or p-biotin resulted in few nonspecific bands, indicating that DAz-1 is specific for sulfenic acid-modified proteins, and that p-biotin is specific for proteins labeled with DAz-1(Figure 2.3a, lanes 1-2).

As an additional control to confirm that the observed bands were indeed Kv1.5, we performed DAz-1 labeling and immunoprecipitation, followed by deglycosylation of the channel with PNGase. Consistent with literature evidence indicating that Kv1.5 undergoes glycosylation [109], PNGase treatment eliminated the upper, glycosylated band in both the HRP-streptavidin Western

blot and the anti-V5 Western blots, further indicating that the sulfenic acid-modified protein is Kv1.5 (Figure 2.3b). To test the specificity of this labeling, we utilized the parent compound and competitive inhibitor of DAz-1, 5,5-dimethyl-1,3-cyclohexanedione (dimedone) [33]. Including dimedone with DAz-1 in the lysis buffer blocked labeling of sulfenic acid-modified Kv1.5 (Figure 2.3c), confirming the specificity of DAz-1 for sulfenic acid-modified Kv1.5.

Kv1.5 possesses two intracellular cysteines on the N-terminus, and four intracellular cysteines on the COOH terminus (Figure 2.1). To determine which cysteines on Kv1.5 were modified with sulfenic acid, we utilized LTK cells stably expressing Kv1.5-WT or 6CS, as well as an additional cysteine mutant (4CS) in which all four COOH-terminal cysteines were mutated to serine. Importantly, all cysteine mutant channels undergo proper folding, are able to traffic to the cell surface, and are fully functional. Using DAz-1 labeling and immunoprecipitation of Kv1.5, we found that mutation of either the four COOH-terminal cysteines, or all six intracellular cysteines, substantially reduced sulfenic acid modification of Kv1.5, as indicated by HRP-streptavidin Western blotting (Figure 2.4a-b). These results were quantified using densitometry and normalized to total Kv1.5 protein, assessed by Western blotting for the V5 epitope. Mutation of the four COOH-terminal cysteines caused a significant reduction in sulfenic acid modification to Kv1.5 ($p<.001$). This change was similar in magnitude to the reduction achieved by mutation of all six cysteines, indicating that sulfenic acid modification occurs primarily on the COOH-terminus of the channel. To determine the individual cysteine(s) on the COOH terminus that are important for this modification, we

developed four stable cell lines in which each individual cysteine on the COOH-terminus of Kv1.5 WT was mutated to serine (Kv1.5 C564S, C581S, C586S, and C604S). Mutation of each individual cysteine in Kv1.5 WT had little effect on the extent of sulfenic acid modification (data not shown). To improve sensitivity and allow for positive detection of labeling signal as opposed to a decrease in signal, we developed four additional stable cell lines in LTK cells, this time expressing Kv1.5 with each COOH-terminal cysteine re-introduced individually into the null background of the 6-cysteine mutant (Kv1.5 S564C, S581C, S586C, and S604C). As outlined in Fig. 2.4c-d, re-introducing C581 restored sulfenic acid modification of Kv1.5 to a level that was similar to wild type. These results indicate that C581 is the principal site where sulfenic acid modification occurs on Kv1.5.

To explore the redox sensitivity of sulfenic acid modification, we induced oxidative stress in LTK cells stably expressing WT Kv1.5 using the organic hydroperoxide, tertiary butyl hydroperoxide (*t*BOOH). Using DAz-1 labeling, immunoprecipitation of Kv1.5 and HRP-streptavidin Western blotting, we found that 30 min treatment with 200µM *t*BOOH caused a significant increase in sulfenic acid modified-Kv1.5 ($p<.01$, Fig. 2.5a). Importantly, concurrent treatment with the thiol-specific antioxidant, *N*-acetylcysteine (NAC), abolished the *t*BOOH-induced increase in sulfenic acid, while having no significant effect alone at this time point. Consistent with its published role as a precursor to *de novo* glutathione synthesis [159], prolonged treatment with NAC reduced the level of basal sulfenic acid modification to Kv1.5 (Figure 2.5b). This increase in sulfenic

acid was observed after treatment with the more physiologically relevant oxidizing agent, hydrogen peroxide, or by depletion of reduced intracellular glutathione with diamide [160] (Figure 2.5 c-d), indicating that the modification is not specific to a particular oxidant. Furthermore, this modification was not cell type-specific, because we observed a similar increase in sulfenic acid modification to Kv1.5 in HL-1 atrial myocytes after treatment with *t*BOOH or diamide (Figure 2.6 a-b). Together, these results show that sulfenic acid modification of Kv1.5 reflects changes in cellular redox state via multiple means.

We have previously demonstrated that Kv1.5 undergoes fatty acylation on intracellular cysteines [109]. Furthermore, others have reported that fatty acylation is sensitive to oxidative stress [116, 117]. Because fatty acylation and sulfenic acid modification are both posttranslational and reversible, we sought to determine the effects of oxidative stress on fatty acylation of Kv1.5. Using fatty acyl exchange labeling [157], immunoprecipitation and HRP-streptavidin Western blotting, we found that 60 min treatment with 200μM *t*BOOH had no significant effect on channel fatty acylation, $p>0.05$ (Figure 2.7a), suggesting that acute oxidative stress modulates sulfenic acid modification, but not fatty acylation, of Kv1.5 (Figure 2.7b).

Formation of mixed disulfide bonds between adjacent cysteine residues in proteins, a common cellular response to oxidative stress, is frequently preceded by sulfenic acid [127]. Previous evidence suggests that Kv channels can form inter-molecular disulfide bonds under oxidizing conditions [129]. Furthermore, disulfide bond formation has profound effects on protein conformation,

localization and function. Therefore, we sought to determine whether sulfenic acid modification of Kv1.5 is a precursor to mixed disulfide bond formation under oxidizing conditions. We treated HL-1 cells stably expressing Kv1.5 for 1h with *t*BOOH and lysed cells in the presence or absence of 100 mM NEM, which blocks free sulfhydryl groups. Consistent with a previous report [129], under non-reducing conditions, cysteines on Kv1.5 are in close enough proximity to form inter-subunit disulfide bonds, as indicated by high molecular weight oligomers (Figure 2.8a, left). However, when free thiols are blocked with NEM, the majority of Kv1.5 exists in monomeric form on a non-reducing gel, and there is no increase in disulfide bond formation after oxidant treatment (Figure 2.8a, right). We observed a similar effect in LTK cells stably expressing WT Kv1.5, indicating that this is not a cell type-specific phenomenon (Figure 2.8b).

Sulfenic acid modification on proteins may also be a precursor to formation of adducts with cellular thiol antioxidants such as glutathione. Reversible glutathione conjugation (S-glutathionylation) of proteins represents an important means by which proteins are protected from over-oxidation, inactivation, and degradation [130, 161]. To explore whether oxidative stress leads to glutathionylation of Kv1.5 via a sulfenic acid intermediate, we performed immunoprecipitation experiments in HL-1 cells stably expressing wild-type channel, followed by Western blotting with anti-glutathione antibody. In spite of efficient immunoprecipitation of Kv1.5, and a robust increase in the level of glutathionlyated proteins in HL-1 cells, we found no Kv1.5 immunoreactivity in vehicle- or *t*BOOH-treated cells (Figure 2.8c). The results of these experiments

indicate that sulfenic acid modification of Kv1.5 is not a precursor to S-glutathionylation under acute oxidative stress.

Environmental exposures such as cigarette smoke are strongly associated with oxidative stress, cardiovascular disease, and reduced expression of Kv1.5 [36, 75, 154, 162]. Therefore, we hypothesized that exposure to cigarette smoke would induce sulfenic acid modification to Kv1.5. LTK cells exposed to an aqueous extract of cigarette smoke (CSE) exhibited a substantial increase in sulfenic acid-modified proteins, compared to cells treated with extract derived from unsmoked cigarettes (CE) (Figure 2.9a). Importantly, CSE, but not CE, also induced a robust increase in sulfenic acid modification to Kv1.5 in LTK cells (Figure 2.9b), and in cardiac myocytes (Figure 2.9c). Concurrent treatment of cells with the chemical antioxidant, NAC abolished the CSE-dependent increase in channel oxidation (Figure 2.9b). Moreover CSE did not induce labeling of Kv1.5-6CS, implicating oxidation of intracellular cysteines on the channel. Thus, cigarette smoke, a socially relevant environmental toxicant, also induces sulfenic acid modification to Kv1.5.

Discussion

The voltage-dependent potassium (Kv) channel, Kv1.5 plays a vital role in controlling resting membrane potential and shaping action potentials in numerous excitable cells [1]. The activity of multiple ion channel proteins, including Kv1.5, is reported to be sensitive to the redox state of cells, linking oxidative stress to cellular excitability [6, 34, 47, 152, 163]. However, direct evidence for channel protein as the principal biological sensor of oxidative stress has not been established. Here, we show that a single cysteine in Kv1.5 undergoes oxidation

to sulfenic acid. To our knowledge, this is the first report demonstrating sulfenic acid modification to an ion channel, or to any transmembrane protein. Furthermore, this modification is redox-sensitive, and is not oxidant- or cell type-specific.

The finding that sulfenic acid modification occurs on a single COOH-terminal cysteine emphasizes the fact that, although formation of sulfenic acid occurs via a non-enzymatic mechanism, it is not a random occurrence. Indeed, the propensity for a cysteine to form a sulfenic acid is largely dependent on its pKa; the deprotonated thiolate anion is more nucleophilic than the protonated thiol, and is substantially more likely to form sulfenic acid [45]. Several factors influence the pKa of cysteines, including the presence of neighboring amino acid residues that participate in hydrogen bonding, such as threonine, serine, and histidine [145]. Although neighboring positively charged amino acid residues and solvent accessibility were thought to influence formation of the thiolate anion and affect cysteine reactivity, additional evidence suggests that this may not be the case. Further study will be required to elucidate the factors contributing to formation of sulfenic acid specifically on C581 of Kv1.5. Although an analysis of the amino acid sequence flanking C581 may lend insight into the factors contributing to modification of this cysteine, it is important to note that the tertiary structure of the channel COOH-terminus is currently unknown. Thus, formation of sulfenic acid may be influenced by amino acid residues outside of the immediate vicinity of C581. Interestingly, the presence of proximal cysteines adjacent to sulfenic acid is reported to increase the likelihood of disulfide bond

formation, and hence decrease the stability of sulfenic acid [127]. As outlined in Figure 2.1a, Kv1.5 possesses another cysteine at position 586. However, our results indicate that this cysteine does not participate in formation of sulfenic acid (Figure 2.4c). Furthermore, if this cysteine quenched sulfenic acid modification of C581, then mutation of C586 to serine should have produced a significant increase in the level of sulfenic acid modification to Kv1.5. Our initial experiments studying the effects of knocking out each individual COOH-terminal cysteine (Kv1.5 C564S, C581S, C586S, and C604S, data not shown) demonstrated that this was not the case.

Although we detect sulfenic acid modification specifically to C581, the stoichiometry of this modification remains an unanswered question. Alpha subunits of Kv1.5 assemble into a tetramer *in vivo*, and the number of subunits which are modified with sulfenic acid at any given time is unclear. A key strength of the DAz-1 labeling technique is that it enables facile detection of fold-changes in modified Kv1.5 under oxidative stress conditions. Precise quantification of the absolute percentage of sulfenic acid-modified channel in cells was not in line with the focus of this study, and given the tetrameric structure of Kv1.5, is unlikely to lend additional insight into the means by which sulfenic acid modulates Kv1.5 expression in a physiological setting.

We and others have previously reported that Kv1.5 undergoes thioacylation on intracellular cysteines [109, 115]. Thioacylation occurs during the biogenesis of nascent channel, and regulates expression of mature, glycosylated protein [109]. Additional evidence suggests that thioacylation may

be sensitive to oxidative stress [116, 117]. Given that sulfenic acid modification and thioacylation both occur on intracellular cysteines, we evaluated whether acute oxidative stress affects thioacylation of Kv1.5 (Figure 2.7). Our data indicate that channel thioacylation is not sensitive to acute oxidative stress. These results suggest that there may be disparate roles for fatty acylation and sulfenic acid modification in regulating Kv1.5 function. Indeed, thioacylation of Kv1.5 occurs primarily on the N-terminal C26 and COOH-terminal C604 [109], which are not prominent sites of sulfenic acid modification. Our finding is consistent with our previous work showing that thioacylation modulates biogenesis and trafficking of nascent channel subunits, and is not a mechanism for acute regulation of channel expression and function [109]. We did not explore the effects of prolonged oxidative stress on channel thioacylation in this study. Given that the fate of intracellular cysteines on Kv1.5 under prolonged oxidative stress is unknown, this represents an interesting area for future experiments.

The electrophilic sulfur atom of sulfenic acid can undergo nucleophilic attack by a proximal thiolate, leading to disulfide bonds, a common cellular response to oxidative stress [127]. Others have demonstrated that oxidant exposure can drive formation of disulfide bonds between adjacent alpha subunits of Shaker Kv channels [129]. We therefore investigated whether cysteines within the four pore-forming subunits of Kv1.5 form disulfide bond(s) under acute oxidative stress. Despite the presence of multiple cysteine residues in the COOH-terminal domain, labeling studies with the thiol-reactive reagent, *N*-ethylmaleimide (NEM) provided no evidence for disulfide bond formation in

response to *t*BOOH exposure (Figure 2.8). These results indicate that sulfenic acid is not a precursor for inter-molecular disulfide bond formation between Kv1.5 subunits under acute oxidative stress. Although Kv1.5 alpha subunits do not form disulfide bonds under our conditions, we cannot rule out the possibility that alpha subunits of the channel form disulfide bonds with other cellular proteins. Indeed, oxidative stress drives formation of disulfide bonds in a vast number of cytoplasmic proteins, suggesting that it is a common mechanism for oxidative protein regulation [164]. Identification of proteins that interact with sulfenic acid modified Kv1.5 under oxidative stress is outside the scope of the current study. Future proteomic experiments using immunoprecipitation and mass spectrometry, or immunohistochemical approaches could be utilized to address this question.

Reversible glutathione conjugation (*S*-glutathionylation) of cysteine residues represents a mechanism by which proteins can be protected from over-oxidation, inactivation, and degradation [130, 161]. S-glutathionylation has also been reported to modulate activity of several ion channels, including the ryanodine receptor type I, the CFTR channel, and the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) [130]. Although glutathionylation of potassium channels has not been explicitly reported, the thioredoxin/glutaredoxin systems may protect myocardial Kv channels from irreversible oxidative damage [16]. Moreover, oxidative stress inhibits myocardial Kv channel currents *in vivo* through modulation of oxidoreductase systems, including the glutaredoxin system [13]. Using an antibody that detects the formation of protein adducts with

glutathione, we tested whether oxidative stress leads to glutathionylation of Kv1.5. No immunoreactivity of Kv1.5 was observed in vehicle- or oxidant-treated cells, despite efficient immunoprecipitation of the channel and robust, peroxide-dependent detection of other *S*-glutathionylated proteins (Figure 2.8).

Collectively, these studies indicate that sulfenic acid modification of Kv1.5 is not accompanied by detectable increases in disulfide bond formation or *S*-glutathionylation (Figure 2.8). This is an interesting finding, because it suggests that sulfenic acid may be a more stable modification, rather than a transient intermediate en route to further oxidation. Stable sulfenic acids have been reported, and their stability may be governed by hydrogen bonding between adjacent amino acid residues [127, 165]. Additional experiments will be required to explore the stability of sulfenic acid modification to Kv1.5. Our data indicate that there is likely a basal tone of sulfenic acid modification, even in unstimulated cells (Figure 2.3a). The functional effects of this modification under basal and oxidant-stimulated conditions are summarized in Chapter 3.

In summary, the important point of this study is that the extent of channel oxidation reflects alterations in cellular redox balance. This may be an important mechanism for ROS signaling disease in states such as atrial fibrillation and pulmonary arterial hypertension, where sulfenic acid modification may be linked to pathophysiological outcomes.

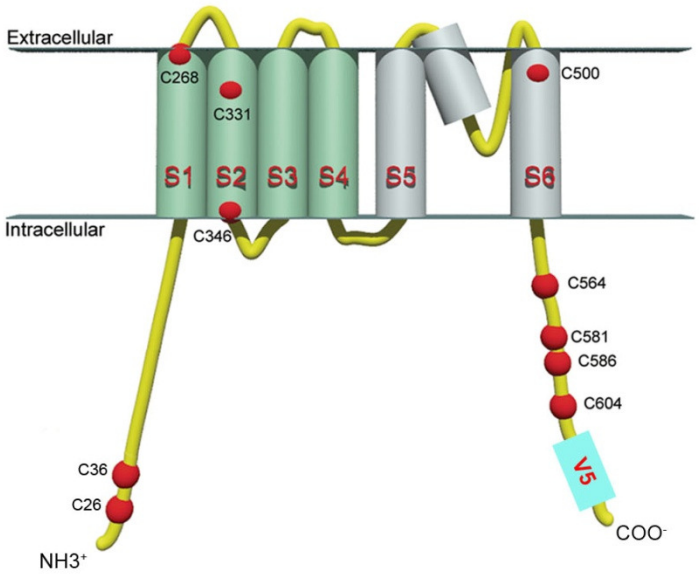

Figure 2.1: Kv1.5 possesses six, potentially redox-sensitive intracellular cysteines.
Topology of a single alpha subunit of wild type, human Kv1.5, showing all ten cysteine residues. The channel also possesses a COOH-terminal V5 tag for immunodetection.

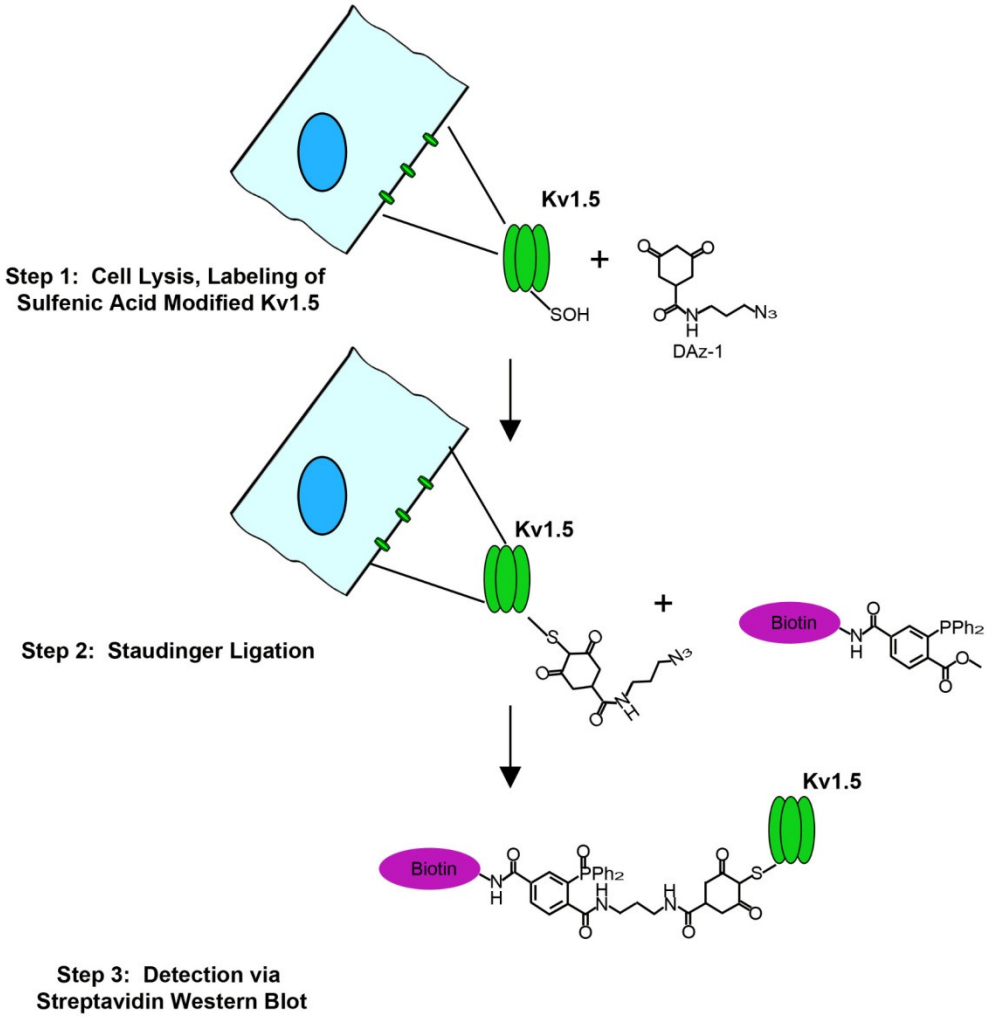

Figure 2.2: Strategy for detecting sulfenic acid-modified Kv1.5 using the novel chemical probe, DAz-1.
Cells are lysed in the presence of DAz-1, which covalently binds to sulfenic acid-modified proteins. DAz-1 labeled proteins are then conjugated to a secondary, biotinylated agent, enabling detection of the sulfenic acid modification via streptavidin western blot.

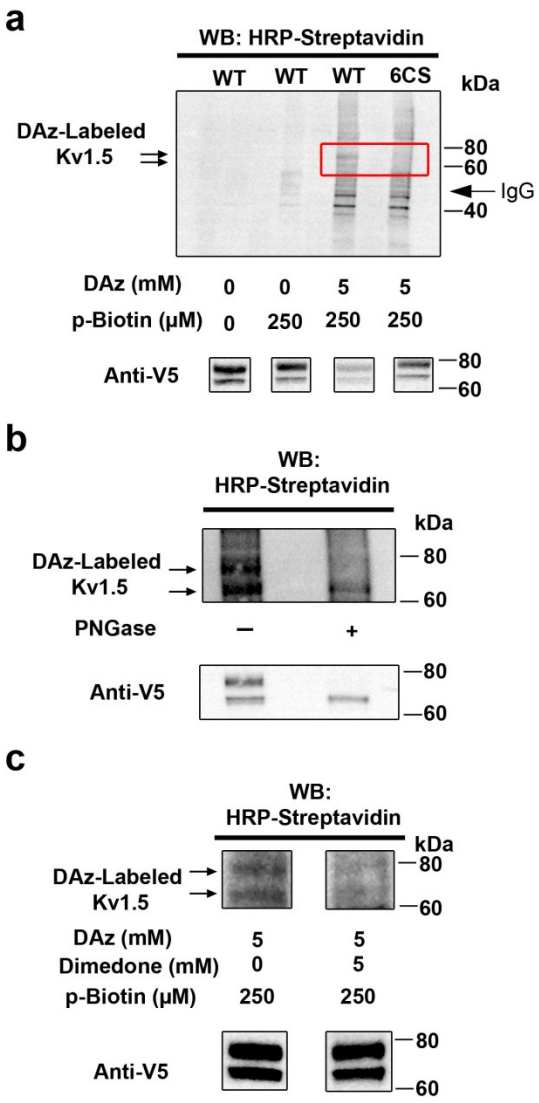

Figure 2.3: Kv1.5 is modified with sulfenic acid on intracellular cysteines. a: LTK cells stably expressing V5-tagged wild-type (WT) Kv1.5 or Kv1.5-6CS were labeled with DAz-1 and conjugated to p-biotin (lanes 3 and 4). Sulfenic acid modification was detected by HRP-streptavidin western blot. Immunoprecipitation efficiency did not differ among samples.
b: LTK cells stably expressing Kv1.5-WT were labeled with DAz-1. Lane 2 sample was then treated overnight at 37°C with PNGase to deglycosylate Kv1.5, which abolishes the doublet, leaving a single, non-glycosylated band. The blot was then stripped and re-probed with anti-V5 antibody to verify efficient immunoprecipitation.
c: LTK cells stably expressing Kv1.5-WT were labeled with DAz-1, alone or concurrently with dimedone, the parent compound of DAz-1. Dimedone blocks DAz-1 labeling of sulfenic acid-modified Kv1.5.

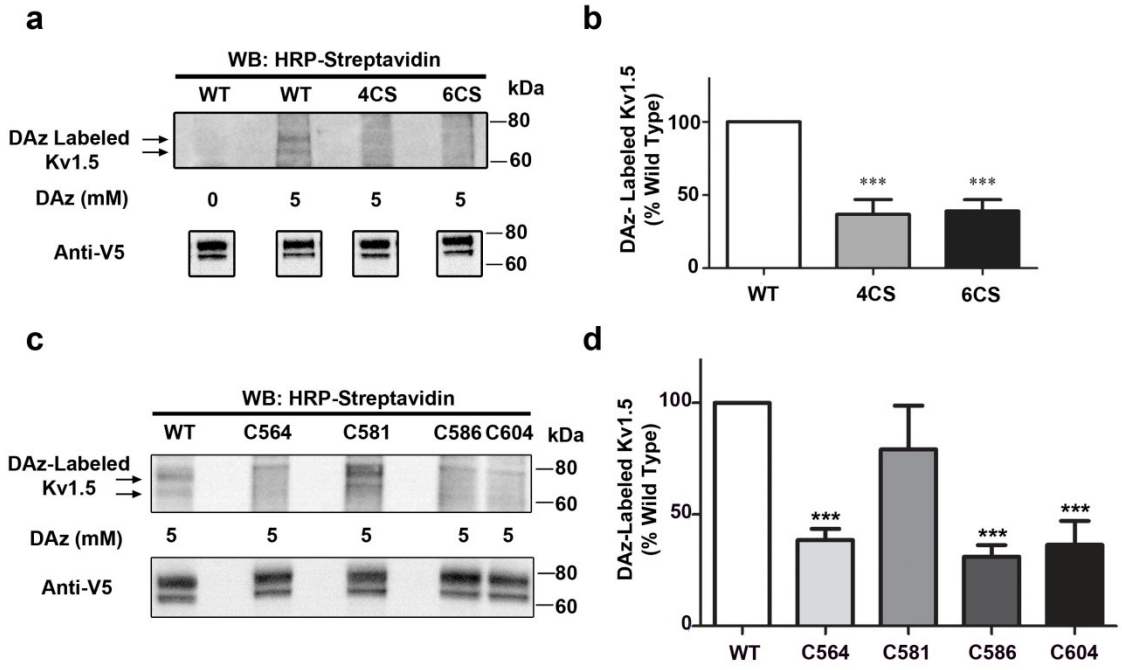

Figure 2.4: Kv1.5 is modified with sulfenic acid on a single, COOH-terminal cysteine.
a: Representative HRP-streptavidin western blot, showing constitutive sulfenic acid modification of Kv1.5-WT. This modification is substantially reduced upon mutation of the 4 COOH-terminal cysteine residues (4CS), or the 6 NH2- and COOH-terminal cysteine residues (6CS), to serine. LTK cells stably expressing Kv1.5-WT, 4CS, or 6CS were lysed in the presence of 5 mM DAz-1. Kv1.5 was immunoprecipitated overnight using anti-V5 antibody, followed by Staudinger ligation with 250 µM p-biotin. Sulfenic acid modification was detected by streptavidin-HRP western blot. Blots were stripped and re-probed with anti-V5 antibody to verify immunoprecipitation.
b: HRP-streptavidin signal for Kv1.5 (n=3 for 4CS, n=6 for 6CS) was quantified using densitometry and normalized to the V5 signal to factor out differences in immunoprecipitation efficiency. Data were converted to ratios, relative to WT channel and analyzed using 1-way ANOVA, followed by Tukey's post-hoc comparison. *** p<.0001 relative to wild type.
c: COOH-terminal cysteines were individually re-introduced into the null background of Kv1.5-6CS. Sulfenic acid modifications were detected by labeling with DAz-1, quantified via densitometry, and compared to WT Kv1.5.
d: Summary of at least three experiments quantified via densitometry as in (b), normalized to WT Kv1.5, and analyzed using 1-way ANOVA, followed by Tukey's post-hoc comparison. *** indicates p<0.0001 relative to WT.

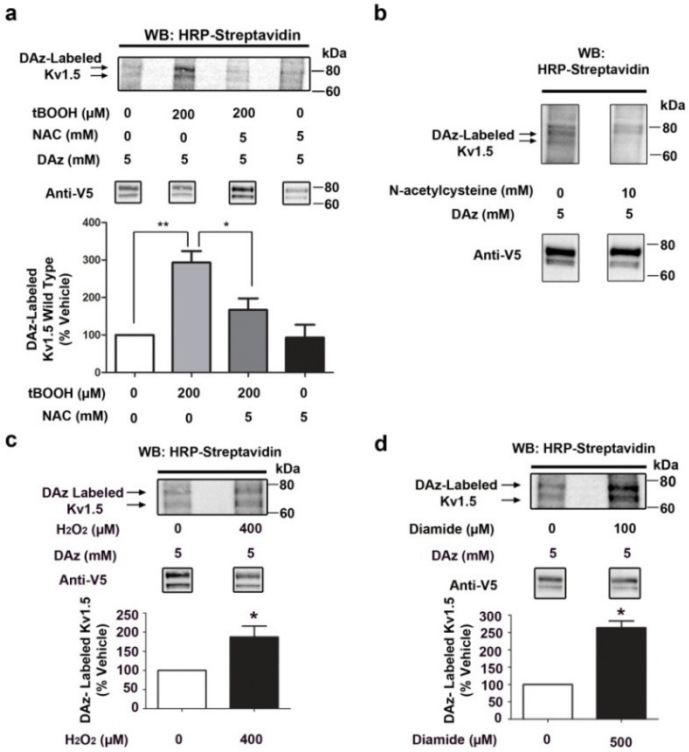

Figure 2.5: Sulfenic acid modification is redox-sensitive and is not oxidant-specific.
a: Top: HRP-streptavidin western blot depicting LTK cells stably expressing Kv1.5-WT, treated with *t*BOOH or vehicle 30 min. in the presence or absence of *N*-acetylcysteine (NAC), followed by labeling with DAz-1. Bottom: Summary of three separate experiments outlined above, analyzed using 1-way ANOVA, followed by Tukey's post-hoc comparison. ** indicates $p<0.01$, * indicates $p<0.05$.
b: LTK cells stably expressing V5-tagged Kv1.5-WT were incubated 16 hours with *N*-acetylcysteine, followed by labeling with DAz-1 and p-biotin. Sulfenic acid modification was detected by HRP-streptavidin western blot. *N*-acetylcysteine abrogates constitutive sulfenic acid modification to Kv1.5.
c: Top: LTK cells stably expressing Kv1.5-WT were treated with H_2O_2 or vehicle 30 min. followed by labeling with DAz-1 and HRP-streptavidin western blot. Bottom: Data from three separate experiments were quantified via densitometry. HRP-streptavidin signal was normalized to V5 (Kv1.5) signal, converted to ratios (relative to vehicle) and analyzed via unpaired t-test with Welch's correction. *, $p<0.05$.
d: Top: LTK cells stably expressing wild type Kv1.5 were treated with diamide or vehicle 30 min. followed by labeling with DAz-1 and HRP-streptavidin western blot. Bottom: Data from three separate experiments were quantified via densitometry. HRP-streptavidin signal was normalized to V5 (Kv1.5) signal, converted to ratios (relative to vehicle) and analyzed via unpaired t-test with Welch's correction. *, $p<.05$.

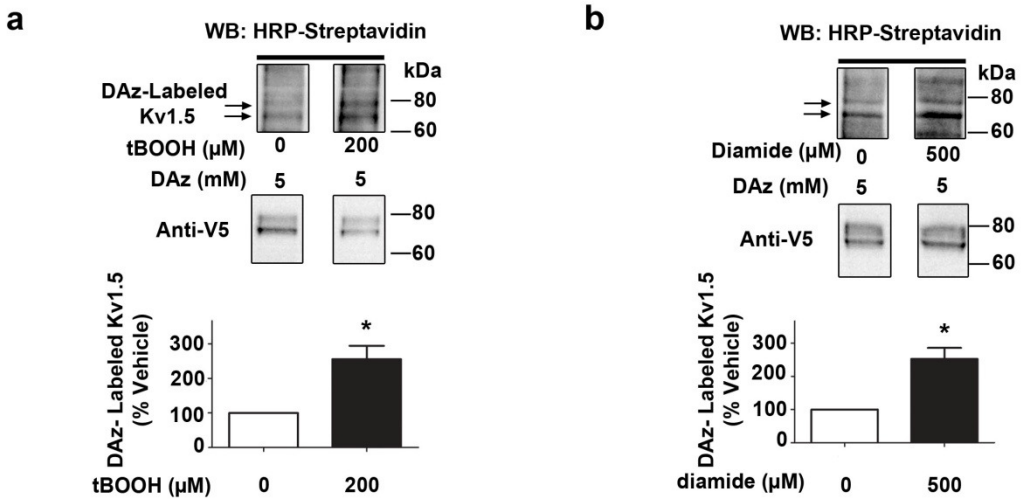

Figure2.6: Sulfenic acid modification of Kv1.5 is not cell-type specific.
a: HL-1 cells stably expressing Kv1.5-WT were treated for 60 min. with *t*BOOH, followed by labeling with DAz-1. Results were quantified via densitometry analyzed via unpaired t-test. *indicates $p<0.05$ relative to control.
b: HL-1 cells stably expressing Kv1.5-WT were treated for 60 min. with *t*BOOH, followed by labeling with DAz-1. Results were quantified via densitometry analyzed via unpaired t-test. *indicates $p<0.05$ relative to control.

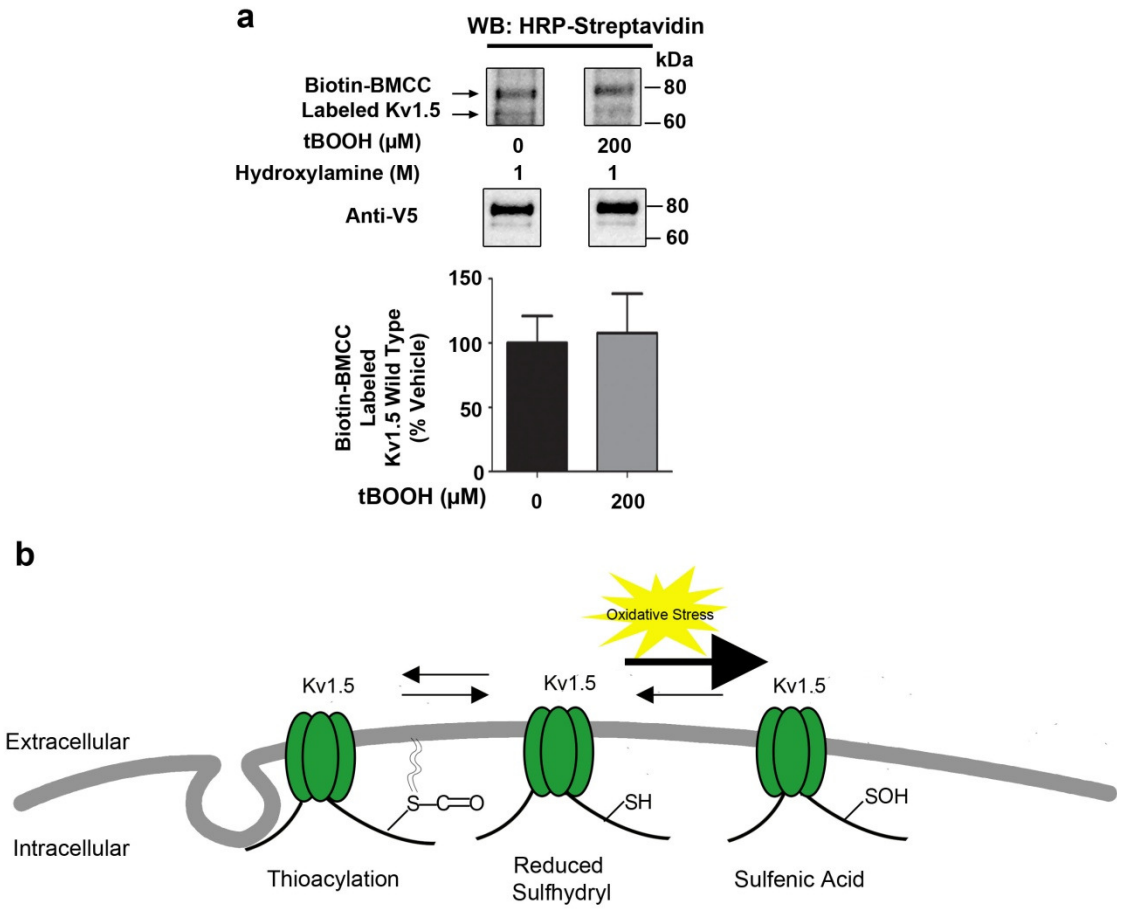

Figure 2.7: Acute oxidative stress does not perturb fatty acylation of Kv1.5.
a: LTK cells stably expressing Kv1.5-WT were treated with *t*BOOH or vehicle 30 min., followed by fatty acyl exchange labeling [157]. Data from three experiments were quantified via densitometry and analyzed using unpaired t-test ($p>0.05$).
b: Cartoon illustrating the potential cysteine modifications on Kv1.5 in cells.

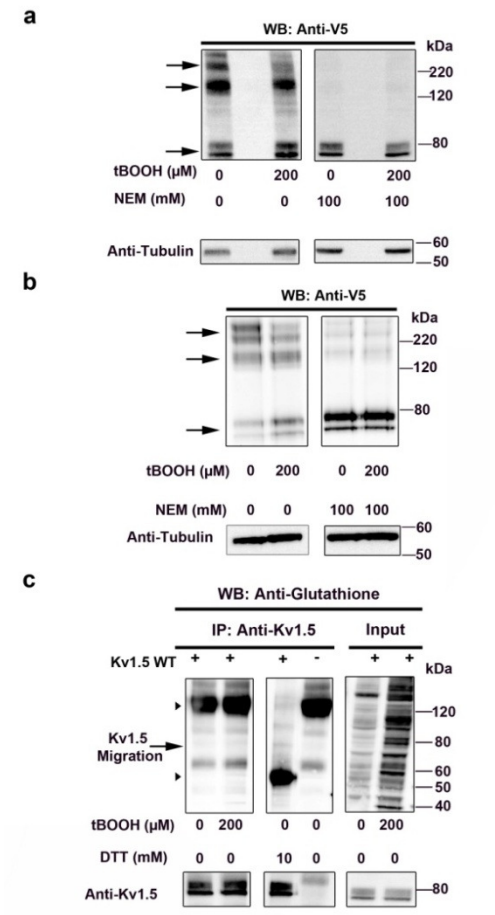

Figure 2.8: Sulfenic acid modification of Kv1.5 is not a precursor to formation of inter-molecular disulfide bonds or adducts with glutathione.
a: HL-1 cells stably expressing Kv1.5-WT were treated with *t*BOOH or vehicle for 60 min., followed by lysis in the absence (lanes 1-2) or presence (lanes 3-4) of 100 mM NEM to prevent formation of disulfide bonds during lysis. Lysates were prepared and separated *via* electrophoresis under non-reducing conditions. Arrows: Monomer and higher molecular weight oligomers.
b: LTK cells stably expressing Kv1.5-WT were treated with *t*BOOH or vehicle for 60 min., followed by lysis in the absence (lanes 1-2) or presence (lanes 3-4) of 100 mM NEM. Lysates were prepared and separated *via* electrophoresis under non-reducing conditions. Treatment with *t*BOOH does not induce formation of disulfide bonds between Kv1.5 subunits. The oxidant-mediated reduction in Kv1.5 oligomers does suggest partial hyper-oxidation of the channel, which would preclude oligomer formation. Arrows: Monomer and higher molecular weight oligomers.
c: HL-1 cells stably expressing Kv1.5-WT were treated with *t*BOOH or vehicle, or DTT (negative control) for 60 min., followed by lysis in the presence of 100 mM NEM to block thiol-disulfide exchange. Immunoprecipitated, Kv1.5 was resolved by non-reducing gel electrophoresis, and analyzed by western blot with monoclonal anti-glutathione antibody. Arrowheads: Background immunoreactivity from IP antibody.

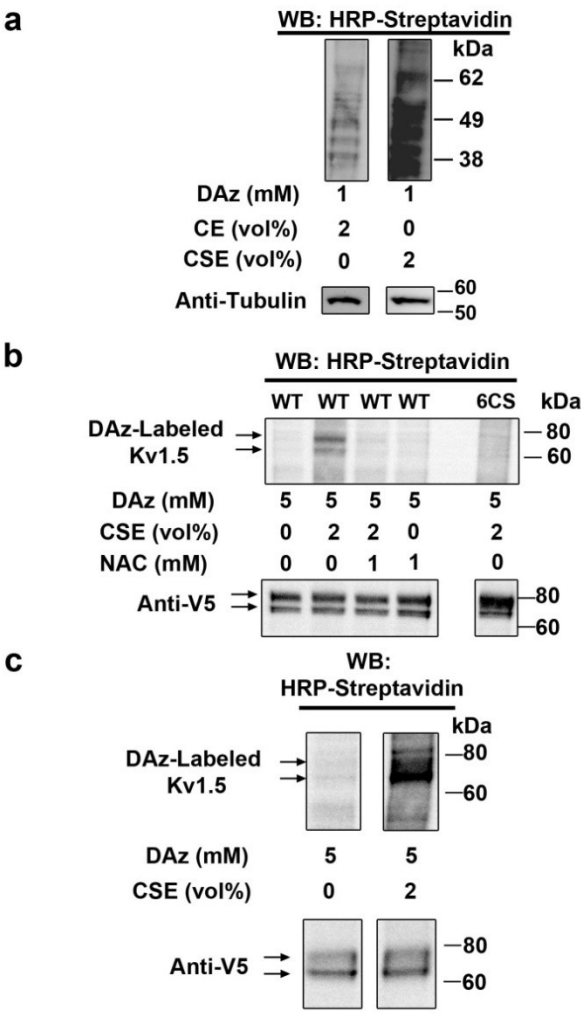

Figure 2.9: Cigarette smoke extract induces sulfenic acid modification to Kv1.5. a: LTK cells stably expressing V5-tagged Kv1.5-WT were treated with cigarette smoke extract (CSE), or unsmoked cigarette extract (CE) for 15 min and probed with DAz-1. Sulfenic acid modification was detected by HRP-streptavidin western blot.
b: LTK cells stably expressing V5-tagged WT or 6-CS Kv1.5 were treated with cigarette smoke extract or vehicle for 15 min. after a 30 min. pre-treatment with *N*-acetylcysteine (NAC) or vehicle, followed by immunoprecipitation and labeling of sulfenic acid with DAz-1. Sulfenic acid modification was detected by HRP-streptavidin western blot.
c: HL-1 cells transiently expressing V5-tagged wild-type Kv1.5 were treated with cigarette smoke extract or vehicle for 15 min., followed by immunoprecipitation and labeling of sulfenic acid with DAz-1 Sulfenic acid modification was detected by HRP-streptavidin western blot.

CHAPTER III: SULFENIC ACID MODIFICATION OF KV1.5 REGULATES CHANNEL TRAFFICKING AND EXPRESSION

Introduction

The voltage-gated potassium (Kv) channel Kv1.5, which underlies IKur, the major repolarizing current in the human atrium, is widely reported to be sensitive to changes in cellular redox state [6, 47, 50, 154]. Disease states such as atrial fibrillation and hypoxic pulmonary hypertension are strongly associated with down-regulation of Kv1.5 expression as well as with oxidative stress [6, 8]. Although both oxidative stress and altered expression of Kv1.5 have been reported to occur in disease states, a mechanistic link between these observations remains unclear. We have demonstrated that Kv1.5 undergoes modification with sulfenic acid on a single COOH-terminal cysteine (Chapter 2). Reversible formation of cysteine sulfenic acid (Cys-SOH) is generating significant excitement in the field of redox biochemistry, as it represents a critical mechanism for dynamic regulation of protein function in response to changes in cellular redox status [127]. We have shown that there is a basal level of sulfenic acid modification to Kv1.5, which increases during conditions of cellular oxidative stress (Chapter 2).

Although significant evidence indicates that oxidative stress modulates Kv channel current in the cardiovascular system, the effects of cellular redox state

on cell surface levels of Kv1.5 have not been explored. We have recently reported that Kv1.5 undergoes dynamic internalization and recycling, and that these processes may be regulated by both physiological and pharmacological stimuli [29, 85]. Acute modulation of cell surface levels of Kv1.5 is thus an important means by which cells regulate excitability in response to external stimuli. Furthermore, several reports suggest that protein endocytosis and trafficking may be redox-sensitive [166-168]. Therefore, we hypothesized that sulfenic acid modification of Kv1.5 modulates its cell surface expression and function in atrial myocytes under conditions of oxidative stress. In this work, we demonstrate that oxidative stress significantly reduced IKur current in dissociated cardiac myocytes. Using HL-1 atrial myocytes, a murine cell line that maintains a differentiated cardiac phenotype in culture [169], we show that sulfenic acid decreases Kv1.5 cell surface expression and channel current during acute oxidative stress. Importantly, we observe a similar decrease in channel surface density after treatment with cigarette smoke, an environmental pollutant that correlates strongly with atrial fibrillation [75], and induces sulfenic acid modification to Kv1.5 (Chapter 2). Furthermore, sulfenic acid modification impaired recycling of internalized Kv1.5 back to the cell surface, and promoted channel degradation after prolonged oxidative stress. We propose that sulfenic acid modification is a fate switch, leading to altered myocyte excitability in response to oxidative stress, and triggering protein degradation in the face of chronic insult.

Experimental Procedures

Materials

Mouse anti-V5 (used at 1:5000), polyclonal anti-GFP (used at 1:500), and goat-anti-mouse IgG labeled with Alexa 405, Alexa 594, or Alexa 647 were purchased from Invitrogen (Carlsbad, CA). Biotin-conjugated goat anti-rabbit secondary antibody was purchased from Jackson Immunoresearch, Inc. (West Grove, PA). Cy5-streptavidin secondary antibody was purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). Rabbit anti-alpha tubulin (used at 1:1000) was purchased from Abcam (Cambridge, MA). HRP-streptavidin conjugate (used at 1:10,000) was purchased from Pierce (Rockford, IL). Mouse monoclonal HSP70/HSC70 antibody from abcam, was kindly provided by Dr. Yoichi Osawa. Mouse monoclonal ubiquitin antibody was from Santa Cruz Biotechnology. Complete protease inhibitor cocktail tablets were obtained from Roche Applied Science (Indianapolis, IN). Protein G-agarose beads, tertiary-butyl hydroperoxide (*t*BOOH), diamide, hydrogen peroxide (H_2O_2) and dimedone were purchased from Sigma (St. Louis, MO). HL-1 cells were a generous gift from Dr. William Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA).

Transient Transfection

HL-1 cells in 35-mm culture dishes were transfected at 50-60% confluence with 0.65µg of DNA combined with 1.5µL of lipofectamine 2000 reagent (Invitrogen) in Opti-Mem (Gibco) for 3–5 h, changed to normal medium, and allowed 48h for protein expression.

Stable Cell Lines

Stable cell lines expressing human Kv1.5 [156] wild type and cysteine mutant constructs were created in LTK (mouse fibroblast) cells using the Retro-X Universal Packaging System from Clontech (Mountain View, CA), according to manufacturer's instructions. Stable cell lines expressing the following mutant constructs were generated: Kv1.5 wild type (WT), Kv1.5 6CS, in which the six NH_2-and COOH-terminal cysteines were mutated to serine, and Kv1.5 4CS, in which the COOH terminal cysteines were mutated to serine. Additionally, four Kv1.5 constructs were created beginning with Kv1.5 6CS, re-introducing individual cysteines on the COOH terminus: Kv1.5S604C, Kv1.5S586C, Kv1.5S581C and Kv1.5S564C.

DAz-1 Labeling of Sulfenic Acid, Staudinger Ligation, and Immunoprecipitation of Kv1.5

HL-1 cells were harvested in non-denaturing lysis buffer containing 150 mM NaCl, 50 mM Tris-Cl, 10 mM EDTA, 1% Triton X-100, and 5 mM DAz-1, pH 7.5 with protease inhibitors (Roche), followed by gentle rocking at 4℃ for 20 min. Lysates were then dounce homogenized, followed by centrifugation at 16,000 g for 4 min at 4℃ to remove cell debris. Supernatant was then incubated at 37℃ for 2h with gentle rocking to allow labeling of sulfenic acid modified proteins with DAz-1. Protein (250µg) was immunoprecipitated overnight at 4℃ with 1 µg of anti-V5 antibody conjugated to Protein G-agarose beads. The next day, the beads were washed twice with wash buffer containing protease inhibitors: 150 mM NaCl, 50 mM Tris-Cl, 10 mM EDTA, and .1% Triton X-100. For the ligation reaction, beads were resuspended in wash buffer with 250 µM p-biotin and

incubated 2h at 37℃ with gentle rocking, prior to electrophoresis and streptavidin-HRP Western blot.

Western Blot

LTK or HL-1 cells (mouse atrial cardiomyocytes) were harvested in denaturing lysis buffer containing 50mM tris-Cl, 10% glycerol, and 2% SDS containing complete protease inhibitors (Roche Applied Science). Membranes were isolated and separated by SDS-PAGE on a NuPAGE Novex 4–12% bis-tris gel (Invitrogen). Proteins were transferred to nitrocellulose and probed with the indicated primary antibody for 1 h at room temperature. Blots were then incubated with secondary antibodies conjugated to horseradish peroxidase (1:5000), and visualized using Western lightning enhanced chemiluminescent reagent according to the manufacturer's protocol (PerkinElmer Life Sciences). Images were captured using the EpiChemi3 darkroom (UVP, Inc., Upland, CA).

Immunocytochemistry and Confocal Imaging

For surface, internalization and recycling assays, immunocytochemistry was performed as described previously, with minor modifications.

Surface labeling of Kv1.5 in HL-1 cells: 48h post transfection, HL-1 cells transiently expressing Kv1.5-GFP were administered treatments, followed by live cell staining on ice to stop further internalization/recycling of Kv1.5. Cells were washed twice with ice-cold PBS, incubated with a polyclonal anti-GFP antibody (1:500) in 2% goat serum for 30 minutes, washed three times with PBS, incubated with goat anti-rabbit AlexaFluor 594 secondary antibody (1:500) in 2% goat serum for 30 minutes, washed three times with PBS, fixed with 4%

paraformaldehyde and mounted with ProLong Gold anti-fade reagent (Invitrogen).

Internalization of Kv1.5: HL-1 cells transiently expressing Kv1.5-GFP were live-cell stained with anti-GFP antibody (1:500) for 30 min on ice. Following this incubation, the cells were administered treatments at 37℃. Cells were then placed back on ice and any remaining surface labeled channels were saturated with goat anti-rabbit AlexaFluor 405 secondary antibody (1:200) for 30 minutes. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 2% goat serum/PBS. Internalized, labeled channel was then detected by incubating with biotin-conjugated goat anti-rabbit secondary antibody (1:500) 30 minutes, followed by incubation with Cy5-conjugated streptavidin antibody (1:500) 30 minutes.

Recycling of Kv1.5: HL-1 cells transiently expressing Kv1.5-GFP were live cell stained with an anti-GFP antibody (1:500) for 30 min on ice. Following this incubation, treatments were administered for 30 minutes at 37℃, with the remaining treatment duration performed on ice. Cells were then stained with goat anti-rabbit AlexaFluor 405 secondary antibody (1:200) for 30 min on ice to saturate remaining surface labeled Kv1.5, then returned to 37℃ for 60 minutes to allow recycling of Kv1.5. After this, recycled Kv1.5 was labeled with biotin-conjugated goat anti-rabbit secondary antibody (1:500) for 30 min on ice, followed by incubation with Cy5-conjugated streptavidin antibody (1:500) for 30 min on ice. Cells were then fixed, washed, and mounted with ProLong Gold.

Staining for colocalization of Kv1.5 with HSP70 or EEA1: For colocalization with HSP70, HL-1 cells transiently expressing Kv1.5-GFP were live-cell stained with anti-GFP antibody (1:500) for 30 min on ice. Following this incubation, the cells were administered treatments at 37℃. Cells were then placed back on ice and any remaining surface labeled channels were saturated with goat anti-rabbit AlexaFluor 405 secondary antibody (1:200) for 30 minutes. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 2% goat serum/PBS. Internalized, labeled channel was then detected by incubating with biotin-conjugated goat anti-rabbit secondary antibody (1:500) 30 minutes, followed by incubation with Cy5-conjugated streptavidin antibody (1:500) 30 min. Cells were then stained with anti-HSP70 antibody (1:500) overnight at 4℃, followed by goat anti-mouse AlexaFluor 594 secondary antibody (1:500) for 60 min at room temperature. Cells were then washed and mounted with ProLong Gold. For colocalization with EEA1, HL-1 cells transiently expressing Kv1.5-GFP were administered treatments at 37℃. After treatment, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 2% donkey serum/PBS, followed by incubation with goat anti-EEA1 antibody (1:100) in 2% donkey serum/PBS. Cells were then incubated with donkey anti-goat AlexaFluor 594 secondary antibody (1:500) for 30 min at room temperature and mounted with ProLong Gold. Staining for internalized Kv1.5 was omitted because of cross-reactivity between donkey anti-goat secondary antibody and the goat antibodies used for the internalization assay.

Imaging: Transfected cells displaying fluorescent signals were acquired on an Olympus Fluoview 500 confocal microscope using a 60X oil objective. Images were obtained by taking a series of stacks every 0.5 µm through the cell and combining the images into a composite stack. To measure oxidant-induced changes in Kv1.5 surface expression, internalization and recycling, Z-stacks were compressed, and total fluorescence was calculated for total Kv1.5 (eGFP), surface Kv1.5 (Texas Red or DAPI), and internalized/recycled Kv1.5 (Cy5) using NIH ImageJ software. Background fluorescence was determined by measuring the background signal for all channels tested. To determine specific fluorescence, the background signal was subtracted from the total fluorescent signal. For quantification, Kv1.5 fluorescent signal (surface, internalized and recycled) was normalized to total Kv1.5-GFP fluorescence in each cell.

Electrophysiology

Ionic currents were recorded at room temperature using the whole cell configuration of the patch clamp technique, as described previously, with minor modifications. HL-1 cells transiently expressing WT or 6CS Kv1.5-GFP 48h post-transfection were administered treatments at 37℃, trypsinized and allowed to settle in the recording chamber. The bath solution contained (in mmol/L): NaCl 110, KCl 4, MgCl2 1, CaCl2 1.8, HEPES 10, and glucose 10. The pH was adjusted to 7.35 with NaOH. Borosilicate glass pipettes with resistance of 2-4 Mohm were filled with a solution containing (in mmol/L): KCl 20, NaCl 8, HEPES 10, K2BAPTA 10, K2ATP 4, potassium aspartate 110, CaCl2 1, MgCl2 1. The pH was adjusted to 7.2 with KOH. Transfected cells were identified by GFP fluorescence, and a gigaohm seal was obtained by gentle suction. To measure

Kv channel activation, the membrane voltage was stepped from -80 to +60 mV in 10-mV increments. Eighty percent of series resistance was compensated. Steady state currents at the end of each depolarizing pulse were measured and normalized to cell capacitance to obtain current density. Rat cardiac myocytes were isolated sterilely [170], cultured, and used within 24 hrs. IKur currents were recorded following 10 min. treatment with M199+ medium alone or with diamide (n=4 cells per treatment). Data were analyzed using unpaired t-test , with * denoting $p<0.05$, and ** denoting $p<0.01$.

Proteasome Activity Assay

An optimized method was used for determining heart tissue chymotrypsin-like activity [171]. Reported values are without ATP and were averaged from 3 independent experiments. Values for dimedone-treated cell lysates were expressed as a percentage of values for untreated cells for each experiment.

Statistics

Statistics were performed using Prism software from Graphpad Prism Software Version 5 (San Diego, CA). All data are expressed as mean +/- SEM. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test, or unpaired, two-tailed t-test. A p value of <0.05 was considered significant.

Results

Given our discovery that Kv1.5 undergoes sulfenic acid modification, we first investigated the effect of altered cellular redox status on IKur, the repolarizing current in atrial myocytes encoded by Kv1.5, using dissociated rat cardiac myocytes. These experiments employed the oxidant diamide, which increases intracellular peroxide levels [172], induces sulfenic acid modification of Kv1.5 in our atrial myocyte cell system (Chapter 2), and inhibits other Kv currents *in vivo* [13]. Figure 3.1a shows that increased oxidative stress was accompanied by a significant decrease in IKur current. These data indicate that perturbation of cellular redox equilibrium decreases IKur current in native cardiac myocytes. Preliminary experiments demonstrated that oxidant exposure does not alter the biophysical properties of Kv1.5 in a heterologous expression system (data not shown). Therefore, we turned our attention to the effects of sulfenic acid modification on channel trafficking.

To explore the functional effects of sulfenic acid modification, we used HL-1 cells transiently expressing Kv1.5 with an extracellular GFP tag inserted between the S1 and S2 segments (Kv1.5-GFP, Figure 3.2a), and measured changes in cell surface expression of Kv1.5 using our previously published live cell staining technique [85]. Importantly, we found that Kv1.5-WT-GFP is modified with sulfenic acid to the same extent as untagged Kv1.5-WT, suggesting that sulfenic acid modification of the GFP tag itself is minimal (Figure 3.2b). A 60 minute treatment with the organic hydroperoxide, *tertiary* butyl hydroperoxide (*t*BOOH), caused a significant, time-dependent reduction in surface levels of Kv1.5 (Figure 3.2c). Furthermore, we found no difference in the

level of GFP fluorescence in oxidant- treated cells compared to vehicle (Figure 3.2d), indicating that the integrity of GFP itself was not altered by oxidation, and that oxidant exposure does not alter total Kv1.5 protein expression. As an additional measure to ensure that the reduction in surface channel was not due to changes in total Kv1.5 protein expression, we performed Western blotting experiments in HL-1 cells stably expressing Kv1.5-GFP, and found no difference in channel expression with acute *t*BOOH treatment (Figure 3.3a). This reduction in channel surface expression was also observed in cells treated with hydrogen peroxide or with diamide, indicating that the effect is not specific for a particular oxidant (Figure 3.4 a-b). Furthermore, treatment with an aqueous extract from cigarette smoke (CSE, Figure 3.5a), but not from unsmoked cigarettes (CE, Figure 3.5b), caused a significant reduction in Kv1.5 surface levels, which was abrogated by concurrent treatment with the thiol antioxidant, *N*-acetylcysteine (NAC). Collectively, these results demonstrate a general mechanism for redox modulation of channel surface expression, in which acute oxidant exposure induces sulfenic acid modification of Kv1.5 in atrial myocytes and significantly decreases the stability of the channel at the plasma membrane.

To determine a role specifically for sulfenic acid modification of Kv1.5 in modulation of channel surface expression, we used the sulfenic acid-specific alkylating agent, dimedone [138]. Our rationale for using dimedone was based on the fact that covalent modification of the sulfenic acid precludes its reduction back to the thiol form (Figure 3.6a). Using live cell staining in HL-1 cells transiently expressing Kv1.5-GFP, Figure 3.6b shows that treatment with

dimedone decreased surface Kv1.5, analogous to treatment with *t*BOOH, and that combining both agents potentiated this effect. To attribute the reduction in surface expression to modification of intracellular cysteines on the channel, we performed the same set of experiments in cells expressing a GFP-tagged Kv1.5 construct in which all six intracellular cysteines have been mutated to serine (Kv1.5-GFP-6CS). Mutation of the intracellular cysteines abolished the *t*BOOH- and dimedone-mediated reduction in surface Kv1.5 (Figure 3.6c), implicating modification of these cysteines in sulfenic acid modulation of channel surface density. Importantly, there was no significant change in total channel protein expression during this time, as indicated by total GFP fluorescence (Figure3.3c-d). As a functional correlate to our immunostaining results, we performed whole cell voltage clamping in cells expressing Kv1.5-GFP. Treatment with *t*BOOH in the presence of dimedone caused a significant reduction in Kv1.5 steady-state current density (Figure 3.6d), which paralleled the effects observed in our immunofluorescence assay. As expected, there was no significant change in Kv1.5 current density with treatment upon mutation of the six intracellular cysteines (Figure 3.6e). We have previously reported that Kv1.5 undergoes thioacylation on intracellular cysteines, and that this modification regulates expression of newly synthesized channel on the cell surface [109]. In HL-1 cells, mutation of intracellular cysteines abrogates thioacylation, leading to lower steady-state levels of channel on the cell surface (compare current density of WT and 6CS in Figures 3.6d-e). Therefore, we compared current density for wild-type channel to that of the C581S mutant using whole-cell voltage clamp. In this

mutant, cysteine 581,which undergoes sulfenic acid modification (Chapter 2) is substituted, while the sites for fatty acylation are retained. As expected, we observed no difference in steady-state current density (Figure 3.7). Therefore, our results indicate that sulfenic acid modification is a mechanism for *acute* regulation of Kv1.5 surface expression.

Given that Kv1.5 is modified with sulfenic acid on C581 (Chapter 2), we developed two additional GFP-tagged Kv1.5 constructs to determine a role specifically for C581 in sulfenic acid regulation of channel surface expression. Further surface labeling experiments revealed that mutation of C581 was sufficient to abrogate this reduction in channel surface expression (termed Kv1.5-GFP-C581S, Figure 3.8a), and re-introduction of this cysteine alone (termed Kv1.5-GFP-S581C) restored the effect (Figure 3.8b). Because the Kv1.5-S581C mutant possesses a single intracellular cysteine, this finding further implicates sulfenic acid modification of C581, rather than an intra-molecular disulfide bond or other modification, in modulation of channel surface expression. In agreement with our immunofluorescence data, mutation of C581 abolishes the redox sensitivity of channel current (Figure 3.9a-b). Thus, inducing sulfenic acid modification on C581of Kv1.5 with oxidizing agent, or trapping Kv1.5 in the sulfenic acid-modified state, causes a reduction in channel surface density and current.

Several acute mechanisms exist for reduction of Kv1.5 surface expression, including increased channel internalization, impaired recycling of internalized channel back to the cell surface, and enhanced channel degradation.

Our western blotting and immunofluorescence data showed no channel degradation with acute oxidative stress (Figure 3.3). Using our live cell, immunofluorescence-based internalization assay [85](Figure 3.10a), we first measured the effects of *t*BOOH or dimedone on channel internalization. As expected, increasing sulfenic acid with *t*BOOH, or trapping this modification with dimedone, significantly increased internalization of Kv1.5 (Figure 3.10b). Importantly, mutation of the six intracellular cysteines on Kv1.5 abolished this oxidant-induced internalization (Figure 3.10c). These effects were not due to differences in overall channel protein expression, as indicated by total GFP fluorescence (Figure 3.3e). We previously reported that a population of internalized Kv1.5 recycles back to the cell surface [85]. Therefore, we determined whether sulfenic acid modification of Kv1.5 interferes with this dynamic recycling process. Using our immunofluorescence-based recycling assay [85] (Figure 3.11a), we treated cells with dimedone, followed by a 60-minute period to allow internalized channel to recycle back to the cell surface. Notably, we observed that trapping sulfenic acid modification significantly reduced recycling of internalized Kv1.5 back to the cell surface (Figure 3.11b). This effect was not due to changes in total channel protein expression, as total GFP levels for the treatment groups were not significantly different (Figure 3.3f). Consistent with our previous report [85], intracellularly-retained channel colocalized with EEA1, a marker for early endosomes (Figure 3.11c). This interaction was more pronounced after a 60 min treatment with *t*BOOH, an observation that likely reflects the larger pool of internalized channel. Therefore,

under acute oxidative stress, sulfenic acid modification triggers internalization of Kv1.5, and significantly reduces channel recycling back to the cell surface.

Given these findings, we sought to determine the intracellular fate of internalized channel. After performing co-immunostaining experiments with a number of intracellular markers, we uncovered a previously unreported, oxidative stress-induced colocalization of Kv1.5 with the molecular chaperone, HSP70 (Figure 3.12). HSP70, which is highly inducible under conditions of cellular stress, plays a pivotal role in stabilizing proteins, including ion channels, and in targeting misfolded proteins for ubiquitination and proteasomal degradation [173]. Consistent with a role for this protein in cell stress, HSP70 acquires a punctate pattern after *t*BOOH treatment and colocalizes with a substantial population of internalized Kv1.5 (Figure 3.12).

The observed colocalization of Kv1.5 with HSP70, combined with data suggesting that over-oxidation of proteins may target them for degradation [148, 174, 175], led us to hypothesize that prolonged oxidative stress diverts the channel to a degradation pathway. To investigate this possibility, we treated HL-1 cells stably expressing WT Kv1.5 to a 15 hour treatment with *t*BOOH, alone or in the presence of the proteasomal inhibitor, MG132. To prevent oxidant-induced changes in Kv1.5 transcription [87], we performed all treatments in the presence of the protein synthesis inhibitor, cycloheximide. Prolonged treatment with *t*BOOH caused degradation of Kv1.5 in as little as 15 hours, which was rescued by inhibition of the proteasome (Figure 3.13a), but was insensitive to the lysosomal inhibitor, leupeptin (Figure 3.13b). Importantly, oxidant-induced

channel degradation was not observed in the Kv1.5-6CS mutant protein (Figure 3.13c), implicating oxidation of intracellular cysteines on the channel in the degradation. Since sulfenic acid can oxidize further to sulfinic (Cys-SO_2H) acid [68], we hypothesized that irreversible hyperoxidation of Kv1.5 might promote degradation. To test this possibility, we used dimedone to trap the sulfenic acid modification on Kv1.5, thereby preventing further oxidation. Figure 3.13d shows that dimedone attenuates Kv1.5 degradation under oxidative stress conditions, as predicted. Although dimedone blocked channel degradation, it was necessary to rule out direct effects of dimedone and oxidant treatments on the activity of the proteasome. To this end, we first treated HL-1 cells with dimedone and *t*BOOH, alone and in the presence of the proteasomal inhibitor MG132, followed by western blotting with an anti-ubiquitin antibody. In contrast with the positive control samples treated with MG132, treatment of cells with *t*BOOH or dimedone caused no accumulation of ubiquitinated proteins in the cells (Figure 3.14a). To directly assess the effects of dimedone on proteasomal activity, we measured the chymotrypsin-like activity of the proteasome using a -procedure [171] after 15 hour treatments with dimedone. Importantly, dimedone treatment alone did not affect activity of the proteasome (Figure 3.14b). Collectively, these results indicate that oxidant and dimedone treatments do not alter proteasomal activity. These results further support our hypothesis that hyper-oxidation of Kv1.5 may enhance channel degradation by diverting Kv1.5 from a recycling pathway to the proteosome (Figure 3.15).

Discussion

Numerous reports suggest that Kv1.5, which comprises the major repolarizing current in the atrium of the human heart, is sensitive to cellular redox state. Furthermore, disease states associated with oxidative stress, such as atrial fibrillation and chronic pulmonary hypertension, are characterized by down regulation of channel protein expression, with no change in channel transcription [6, 8]. In Chapter II, we demonstrated that Kv1.5 is modified in a redox-sensitive fashion with sulfenic acid on C581, a novel, redox-sensitive modification to an ion channel. In this work, we explored the functional effects of this modification. We hypothesized that sulfenic acid modulates channel trafficking and expression under conditions of oxidative stress.

Our finding that diamide treatment significantly reduces IKur currents in native cells is agreement with others. In ventricular myocytes, diamide treatment significantly reduces both the transient outward (Ito) and the slowly inactivating steady-state (IKss) Kv channel currents, effects which were mediated by alteration of the thioredoxin and glutaredoxin systems [13]. Similar effects on Ito were observed after pharmacological inhibition of thioredoxin, glutathione reductase, or glutathione synthesis *in vivo* [13]. Our results demonstrate a previously unreported functional consequence of altered redox status on IKur. These findings collectively suggest that redox regulation of Kv channels may be a general mechanism for modulating channel function in the heart.

Although currents encoded by Kv1.5 are reported to be redox-sensitive, the molecular mechanism for this redox sensitivity is poorly understood. Initial electrophysiological experiments using hydrogen peroxide treatments in cells

transiently expressing Kv1.5 showed no change in the biophysical properties of the channel (data not shown). Therefore, we turned our attention to exploring the effects of oxidative stress on channel trafficking. The results of our immunofluorescence-based assay for detection of surface Kv1.5 indicate that sulfenic acid modification of the channel reduces its levels on the cell surface. A significant reduction in surface expression occurs after accumulation of sulfenic acid via two distinct means: induction of oxidative stress with *t*BOOH, or trapping of the modification with dimedone, effects which are more pronounced when both agents are combined (Figure 3.6b). Mutation of the six intracellular cysteines on the channel abolished the reduction in surface expression (Figure 3.6c). Interestingly, there was a significant increase in surface Kv1.5 6CS with *t*BOOH or dimedone treatment, an effect that may be due to sulfenic acid modification of other cellular pathways which regulate Kv1.5 surface expression. Kv1.5 associates with multiple scaffolding proteins *in vivo*, which may also be redox-sensitive [156, 176, 177]. We observe similar increases in sulfenic acid modification (Chapter II) and decreased Kv1.5 surface expression (Figure 3.4) with multiple oxidants, demonstrating that the effects are not specific to one particular oxidant and likely represent a general cellular mechanism for redox-regulation of channel expression and trafficking. Although our *in vitro* system may not fully simulate the conditions in an intact organism, the use of hydrogen peroxide [150, 178] *t*BOOH [179] and diamide [172] are well-accepted methods for simulating oxidative stress. Our whole cell voltage clamping results provide confirmation of our immunofluorescence data via a second, parallel method.

Importantly, we localized these functional effects using both approaches to a single COOH-terminal cysteine (Figures 3.8 and 3.9). Furthermore, we demonstrated that an aqueous extract derived from cigarette smoke, a well-established source of oxidative stress [162], induces a similar reduction in Kv1.5 surface expression. This finding is significant; cigarette smoking is strongly linked to atrial fibrillation [75] as well as to reduced expression of Kv1.5 [154], although mechanistic data elucidating these associations are lacking.

In accordance with our surface labeling experiments, we find a similar increase in channel internalization after treatment with *t*BOOH or dimedone. Although both agents combined result in a more pronounced reduction in channel surface levels, we did not observe the same corresponding effect on internalization (Figure 3.10b). This is likely due to a technical limitation of the internalization assay, which requires labeling of surface channel with anti-GFP antibody *prior* to treatment. *t*BOOH treatment, but not treatment with dimedone, appears to interfere with association of the anti-GFP antibody to the channel. This finding was confirmed in control experiments (data not shown), and literature evidence indicates that factors such as pH and redox environment can affect the function and stability of antibodies [180]. Nevertheless, this implies that the observed levels of internalization achieved with *t*BOOH, alone or in the presence of dimedone, are actually an *underestimation* of the true effect. The finding that dimedone treatment alone causes internalization suggests that there is a basal tone of sulfenic acid-modified channel in cardiac myocytes. This is supported by data outlined in Chapter II, showing a low level of sulfenic acid-

modified channel in cells that have not been challenged with oxidant. Accordingly, the increase in internalization is accompanied by impairment in channel recycling (Figure 3.11b), indicating that sulfenic acid modification diverts channel away from the normal recycling pathway. The effects of *t*BOOH on channel recycling could not be assessed because of similar technical limitations to the recycling assay, which would have resulted in *overestimation* of the true effect. To our knowledge, this is the first report demonstrating that cellular redox state, via a redox-sensitive posttranslational modification, can acutely regulate cell surface levels of Kv1.5. These results are in agreement with other studies showing that oxidative stress can interfere with trafficking of other membrane proteins, such as the transferrin receptor [168], or alter the molecular machinery involved in this trafficking process [166]. The molecular means by which sulfenic acid modification of Kv1.5 induces internalization requires further exploration. Kv1.5 sub-cellular localization is modulated by N- and COOH-terminal PDZ domains, interaction with caveolin proteins, and fatty acylation [109, 176, 177, 181]. Sulfenic acid modification may disrupt association with scaffolding proteins at the membrane, or it may induce a conformational change in the channel that promotes the channel to associate with the internalization machinery. As the molecular mechanisms governing Kv1.5 endocytosis are elucidated, this may lead to further insight on how sulfenic acid triggers internalization.

The use of dimedone as a tool to assess a role for sulfenic acid in cellular processes is not without precedent [144, 182]. Our results clearly demonstrate that trapping sulfenic acid modification with dimedone promotes channel

internalization, an effect which is lost upon mutation of intracellular cysteines on the channel. However, given that dimedone forms a covalent adduct with sulfenic acid, we cannot rule out that this newly formed adduct possesses chemical properties which are distinct from sulfenic acid. Sulfenic acid modifications are regulated by multiple cellular reducing systems, including the thioredoxin and glutaredoxin systems [127]. Given the significant redundancy in these systems, and lack of knowledge regarding the enzymes involved in catalyzing the sulfenic acid modifications, a definitive determination of cause and effect is beyond the current state-of-the art in the field. However, we provide very strong evidence of a causative role for sulfenic acid in regulation of Kv1.5, with the following lines of evidence:

1. We show that acute oxidative stress induces formation of sulfenic acid, but does not stimulate disulfide formation or glutathionylation (Chapter II). In addition, the primary site of this modification has been localized to C581 (Chapter II).
2. We demonstrate that dimedone treatment decreases surface channel expression (analogous to treatment with *t*BOOH). Importantly, mutation of C581 is sufficient to abrogate this reduction in channel surface expression and re-introduction of this cysteine alone restored the effect (Figure 3.8). Collectively these data provide a direct link between sulfenic acid modification of C581 and channel surface expression.

In Figure 3.12, we demonstrate that Kv1.5 colocalizes with HSP70 under conditions of oxidative stress. Several lines of evidence indicate that HSP70

recognizes nascent hydrophobic clefts in proteins that have begun to unfold under oxidative stress [173]. HSP70 plays an important role in targeting these misfolded proteins for ubiquitination and proteasomal degradation [173]. These findings, combined with our observation that sulfenic acid impairs channel trafficking, led us to hypothesize that sulfenic acid modification may initiate a cascade of events culminating in channel degradation. Indeed, our data showing that sustained oxidative stress promotes degradation are consistent with the well-established role for HSP70 in protein degradation. In contrast with our findings, work from others suggests that HSP70 stabilizes Kv1.5 expression at the cell membrane [183]. However, unlike our studies, these experiments were conducted in non-cardiac cell lines overexpressing HSP70, without exploring the effects of oxidative stress. We maintain that our results in the HL-1 cell line more accurately reflect the role of oxidative stress and HSP70 in Kv1.5 degradation in the cardiovascular system. Further experiments are necessary to determine the nature of the interaction between Kv1.5 and HSP70 under oxidative stress. HSP70 interacts with specific ubiquitin E3 ligases, including ChIP and parkin, promoting specificity in ubiquitination of substrate proteins [173, 184]. Thus, these E3 ligases may be a logical starting point for experiments exploring the pathway leading to ubiquitination of Kv1.5. Additionally, while HSP70 targets proteins for degradation, HSP90 has a protective role [173]. As both chaperones are involved in triage of damaged proteins, identifying a role for HSP90 in protecting Kv1.5 from degradation under oxidative stress would provide additional evidence in support of our model.

The finding that Kv1.5 undergoes degradation which is sensitive to MG132 suggests that it may be targeted to a proteasomal pathway. This result is consistent with previous work published in our laboratory [29, 109]. This finding is of interest, given that it is traditionally accepted that membrane proteins which enter the endocytic pathway undergo degradation by the lysosome [185]. However, proteasomal degradation of membrane proteins is not without precedent, as several other transmembrane ion channels are degraded by the ubiquitin-proteasome system, including KCNQ1, CFTR, and the inositol 1,4,5-trisphosphate receptor [184, 186, 187]. Recent evidence suggests that mechanisms exist for transport of membrane proteins to the cytosol, where they are accessible to the proteasome, including retrotranslocation [188]. Alternatively, MG132 may inhibit protein degradation through another mechanism, such as depletion of the free cellular pool of ubiquitin. Thus, further experiments are necessary to definitively determine the cellular pathway by which Kv1.5 undergoes degradation. Notably, our data show that concurrent treatment with dimedone, which blocks progression of sulfenic acid to further oxidation states, significantly blocks Kv1.5 degradation. These results are consistent with our hypothesis that irreversible hyper-oxidation of sulfenic acid leads to protein degradation. There are several possible explanations for why dimedone did not fully block channel degradation. Although cells were pre-treated for 30 minutes prior to *t*BOOH treatment, it is possible that there was an insufficient amount of intracellular dimedone present to fully alkylate all sulfenic acid-modified channel. Concentrations exceeding those used in our experiments

were cytotoxic. It is also plausible that the kinetics of channel hyperoxidation *in vivo* exceed those for binding of dimedone to sulfenic acid. Varying experimental conditions may yield more complete alkylation of sulfenic acid-modified channel. Additionally, it is important to note that dimedone blocks progression of sulfenic acid to other stably oxidized states, such as mixed disulfide bonds and adducts with glutathione. We demonstrated that sulfenic acid modified Kv1.5 does not form inter-molecular disulfide bonds or adducts with glutathione under acute oxidative stress (Chapter II), but whether this occurs after sustained oxidative stress requires further experimentation. Currently, experiments are underway to identify formation of sulfinic or sulfonic acid modification to Kv1.5 under prolonged oxidative stress, and determine the role for cysteine hyper-oxidation in channel degradation. To do this, we have recently developed an antibody which recognizes the COOH-terminal, sulfinylated/sulfonylated (SO_2H/SO_3H) C581 form of Kv1.5. Additionally, our previous work demonstrated that thioacylation prevents nascent Kv1.5 from being diverted to a degradation pathway [109]. Therefore, it would be interesting to determine whether sustained oxidative stress inhibits channel thioacylation, and ascertain a role for this inhibition in the observed channel degradation. Importantly, our results clearly demonstrate that treatment with *t*BOOH or dimedone alone does it inhibit proteasomal activity, ruling out direct, non-specific effects on the proteasome.

As further evidence for sulfenic acid regulation of Kv1.5 in diseases and environmental exposures, we demonstrate that cigarette smoke extract in a dose sufficient to induce sulfenic acid modification to Kv1.5 (Chapter II) reduces

channel expression on the cell surface, which is blocked by concurrent treatment with the antioxidant, *N*-acetylcysteine. Importantly, cigarette smoke exposure is strongly correlated with fibrillation of the atria, where Kv1.5 controls cardiac repolarization [75]. These data provide the first molecular link between environmental exposure to cigarette smoke, oxidative stress and altered channel expression/function, which may lead pathophysiological states such as atrial fibrillation.

In summary, we have shown that oxidative stress can lead directly to internalization and protein degradation of Kv1.5, and that sulfenic acid modification of COOH-terminal C581 alone is sufficient to trigger these events (Figure 3.15). Redox modulation of Kv1.5 current is associated with numerous pathophysiological states that involve loss of channel protein [6, 8, 50]. We show, for the first time, that oxidative stress acutely regulates Kv1.5 surface expression. To our knowledge, this is the first report of sulfenic acid modification to an ion channel, which provides a molecular explanation for the observed oxidative stress-mediated decreases in channel current. While the roles of ROS and modulation of Kv1.5 in the pathogenesis of disease continue to be intensely studied, it is clear that other factors peripheral to the channel itself, including expression of modulatory Kv beta subunits, cannot fully account for channel redox sensitivity [50]. Indeed, our results indicate that oxidative modification of the Kv1.5 channel itself has a profound effect on stability and functional density. By extension, sulfenic acid modification may also regulate surface levels of other

ion channels, thereby linking oxidative stress to altered cellular excitability and disease.

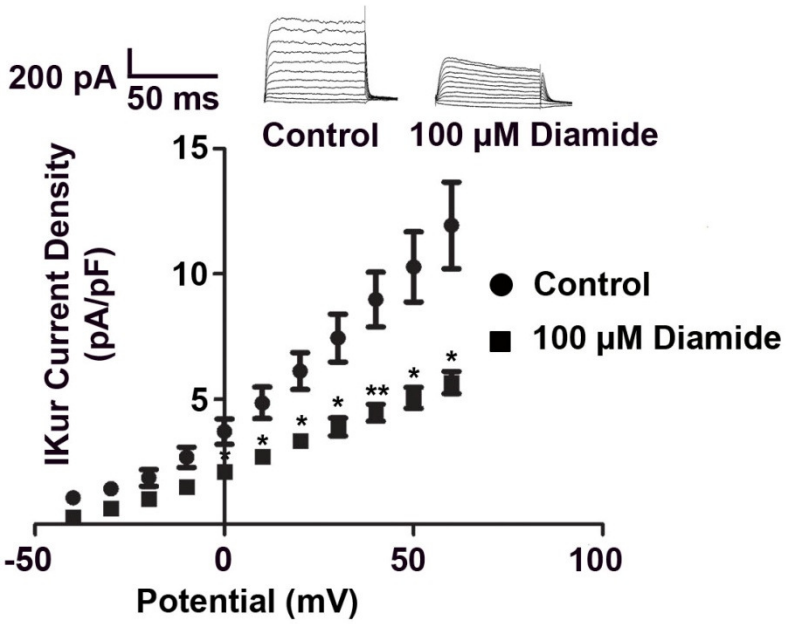

Figure 3.1: Diamide significantly reduces IKur currents in native cardiac myocytes.
Rat cardiac myocytes were isolated sterilely, cultured, and used within 24 hrs. IKur currents were recorded following 10 min. treatment with M199+ medium alone or with diamide (n=4 cells per treatment). Data were analyzed using unpaired t-test * denotes $p<0.05$, ** denotes $p<0.01$.

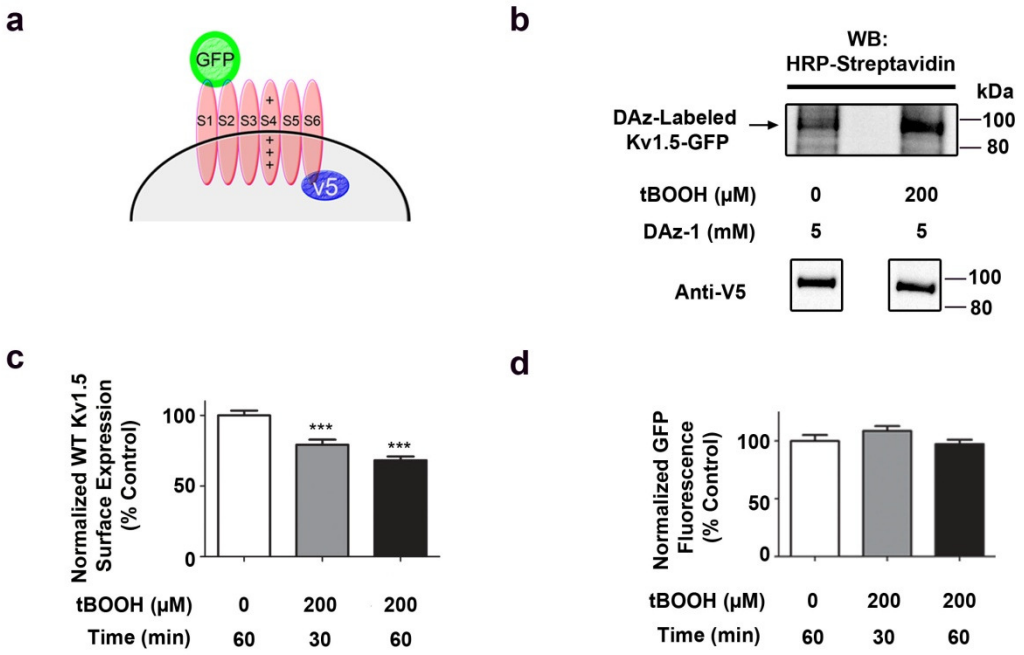

Figure 3.2: Oxidant treatment causes a significant, time-dependent reduction in surface levels of Kv1.5 in HL-1 atrial myocytes.
a: Cartoon illustrating the Kv1.5-GFP construct.
b: HL-1 cells stably expressing wild type Kv1.5-GFP were treated with *t*BOOH or vehicle 60 min., followed by immunoprecipitation and labeling with DAz-1 and HRP-streptavidin western blot.
c: Cells expressing Kv1.5-GFP were treated with *t*BOOH or vehicle for 30 or 60 min. Surface channel (red) was quantified using NIH ImageJ software, normalized to total GFP (*i.e.* total Kv1.5) fluorescence, and analyzed via one-way ANOVA. *** indicates $p<0.0001$ relative to vehicle.
d: Levels of total GFP fluorescence (total Kv1.5 expression) were quantified and analyzed as in (c.) Levels are not significantly different, ($p>0.05$).

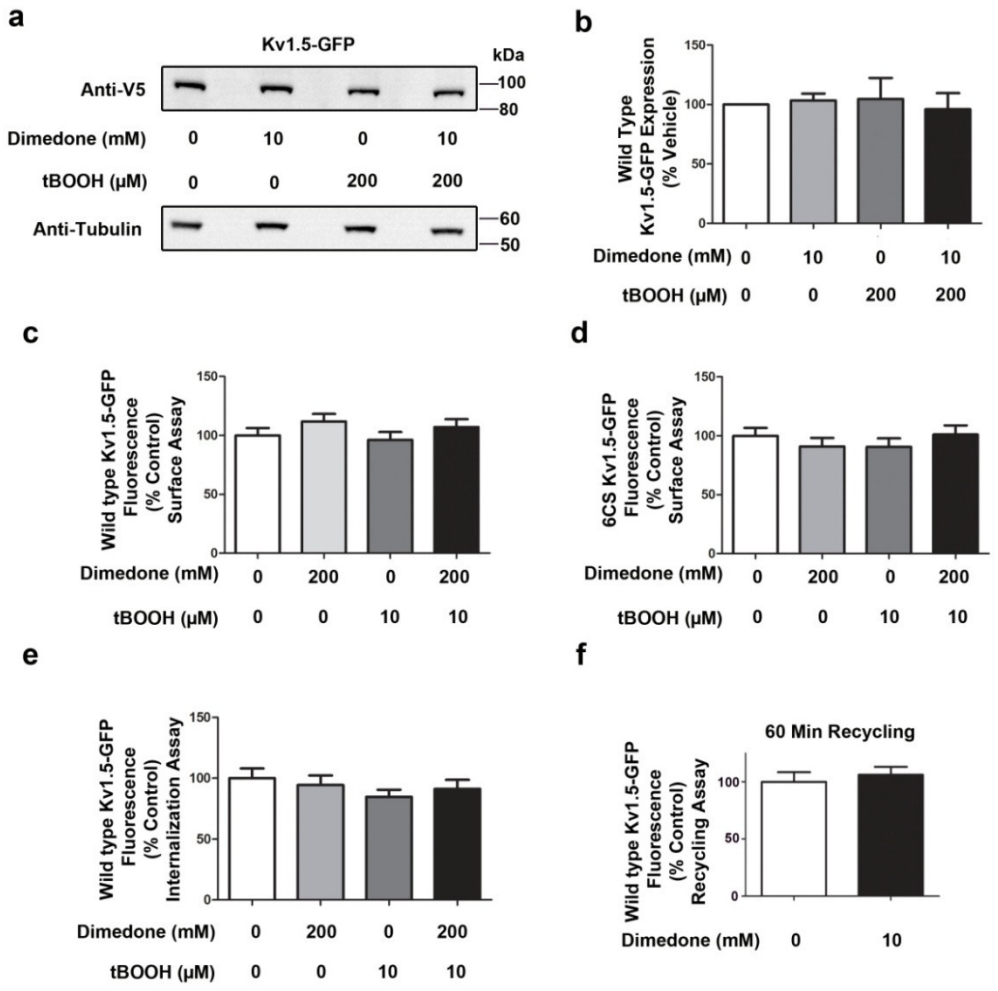

Figure 3.3: Acute exposure to oxidative stress does not significantly affect Kv1.5 protein expression.
a: Representative western blot image of HL-1 cells stably expressing V5-tagged Kv1.5-pHluorin (a pH sensitive variant of GFP). Cells were treated with vehicle or *t*BOOH for 60 min. An additional sample received dimedone alone for 90 min. A final sample was pre-treated with dimedone for 30 min., followed by 60 min. *t*BOOH treatment in the continued presence of dimedone.
b, Data from three separate experiments in (a) were quantified via densitometry. V5 band (Kv1.5-pHluorin) area density was normalized to alpha tubulin, converted to ratios (relative to vehicle) and analyzed via 1-way ANOVA, followed by Tukey's post-hoc test. $p>0.05$, non-significant.
c-f GFP fluorescence, used as an internal control for Kv1.5-GFP expression, was quantified using NIH ImageJ software. Results were analyzed via Kruskal-Wallis test, followed by Dunn's post test (c-e), or unpaired t-test (f). Although Kv1.5-GFP-WT and Kv1.5-GFP-6CS differ from each other in their steady-state expression levels, total protein expression is not significantly different across the treatment groups in surface, internalization or recycling assays ($p>0.05$).

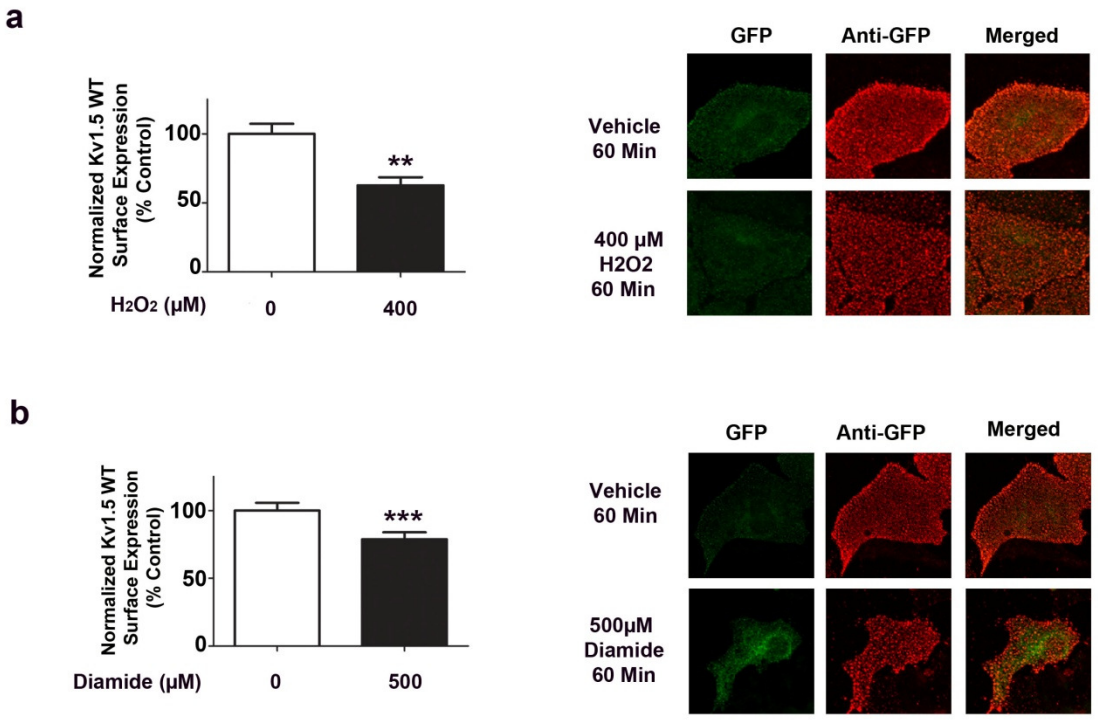

Figure 3.4: The reduction in Kv1.5 surface expression in HL-1 atrial myocytes is not oxidant-specific.
a-b, HL-1 cells transiently expressing Kv1.5-GFP were treated with H_2O_2 or vehicle for 60 min. (a) or diamide or vehicle for 30 min. (b), followed by labeling of surface Kv1.5 (red). Surface channel was quantified using NIH ImageJ software, normalized to total GFP (i.e. total Kv1.5) fluorescence, and analyzed via unpaired t-test. ** indicates $p<.01$, *** indicates $p<.001$ relative to vehicle. GFP levels are not significantly different ($p>0.05$), data not shown.

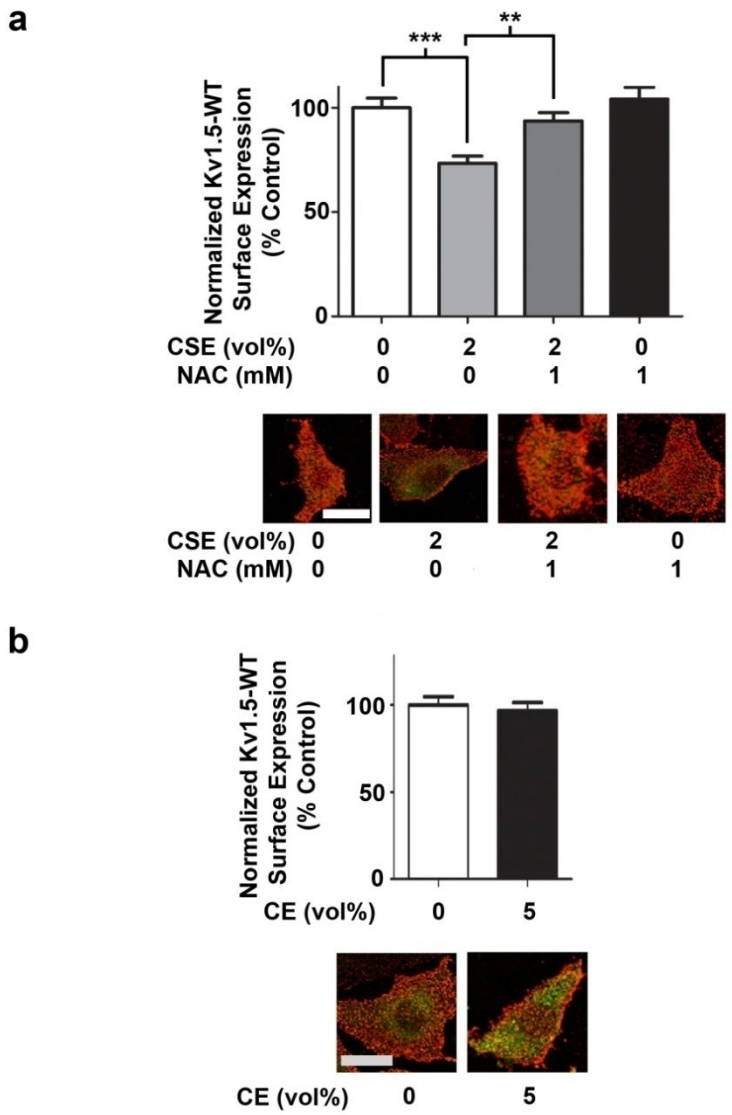

Figure 3.5: Cigarette smoke extract significantly reduces surface levels of Kv1.5. a: HL-1 cells transiently expressing Kv1.5-GFP were treated with vehicle or CSE for 15 min. Cells were then stained live to label surface populations of Kv1.5 (red). Surface channel was quantified using NIH ImageJ software, normalized to total GFP (i.e. total Kv1.5) fluorescence, and analyzed via Kruskal-Wallis test, followed by Dunn's post test, n=100+ cells per condition, three experiments. ***indicates $p<.0001$, **indicates $p<.001$. Scale bar: 20 microns.
b: HL-1 cells transiently expressing Kv1.5-GFP were treated with vehicle or CE for 30 min. Cells were then stained live to label surface populations of Kv1.5 (red). Surface channel was quantified using NIH ImageJ software, normalized to total GFP (i.e. total Kv1.5) fluorescence, and analyzed via Kruskal-Wallis test, followed by Dunn's post test, n=100+ cells per condition, three experiments, $p>0.05$. Scale bar: 20 microns.

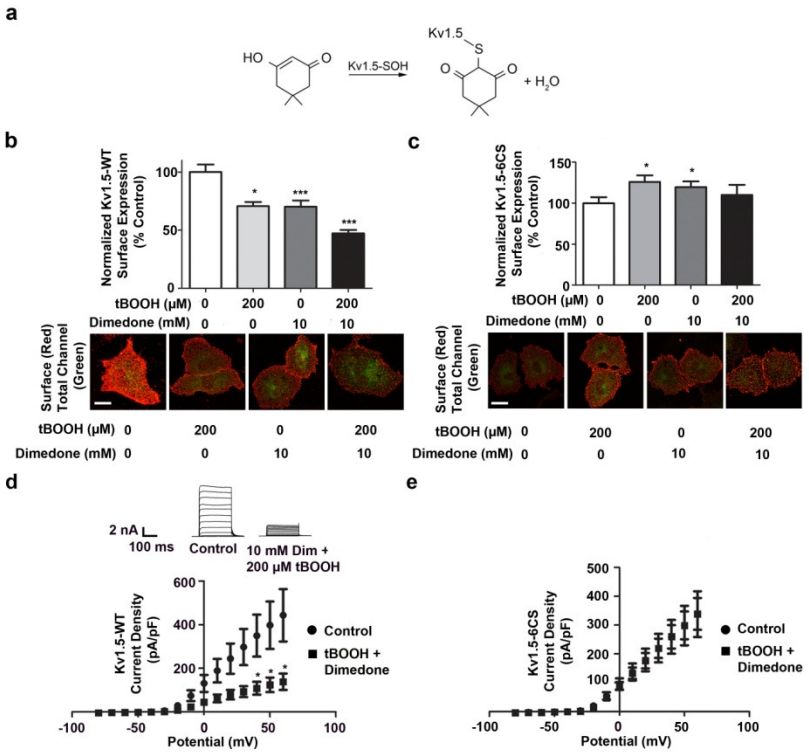

Figure 3.6: Sulfenic acid modification to intracellular cysteines on Kv1.5 reduces channel expression on the cell surface.
a: Reaction of dimedone with sulfenic acid-modified Kv1.5, not drawn to scale.
b: HL-1 cells transiently expressing Kv1.5-GFP were treated with vehicle or *t*BOOH for 60 min. or dimedone for 90 min. An additional sample was pre-treated with dimedone for 30 min., followed by 60 min. *t*BOOH treatment in the continued presence of dimedone. Cells were then stained live to label surface populations of Kv1.5 (red, collapsed Z-stacks) Surface channel was quantified using NIH ImageJ software, normalized to total GFP (green, i.e. total Kv1.5) fluorescence, and analyzed via Kruskal-Wallis test, followed by Dunn's post test, n=100+ cells per condition, three experiments. ***indicates p<.0001, *indicates p<.05, relative to vehicle. Scale bar: 30 microns.
c: Experiment outlined in (b) was performed in HL-1 cells transiently expressing Kv1.5-6CS *indicates p<.05 relative to control. Scale bar: 30 microns.
d: Results of whole cell voltage clamping experiments in HL-1 cells transiently transfected with Kv1.5-GFP-WT and pretreated 30 min. with dimedone, followed by 60 min. *t*BOOH in the presence of dimedone (n=7), or vehicle (n=11). Cells were administered 10 mV step changes in voltage from a holding potential of -80 mV. Steady state potassium current was measured at the end of each test pulse and normalized to cell capacitance. Inset: Sample outward Kv channel current traces for vehicle and dimedone/*t*BOOH treated cells. Results were analyzed via unpaired t-test. *indicates p<.05 relative to control.
e: Experiment outlined in (d) was performed in HL-1 cells transiently expressing Kv1.5-6CS (n=8 control, n=7 for dimedone + *t*BOOH).

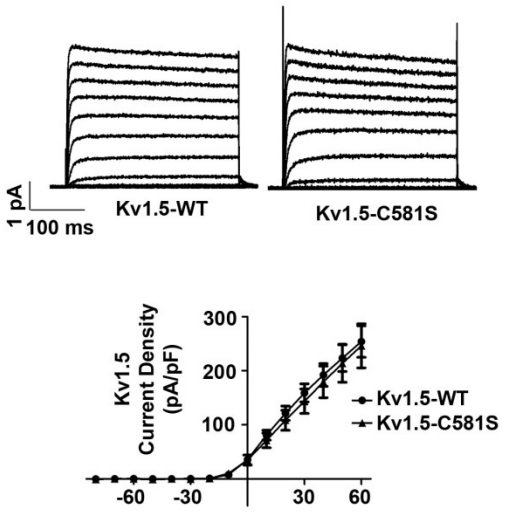

Figure 3.7: Steady-state current density of Kv1.5 C581S is equivalent to WT channel.
Comparison of steady-state current density in HL-1 cells transiently transfected with Kv1.5-GFP-WT or Kv1.5-GFP-C581S. Cells were administered 10 mV step changes in voltage from a holding potential of -80 mV, as shown in protocol. Steady state potassium current was measured at the end of each test pulse and normalized to cell capacitance. Top: Sample outward Kv channel current traces for WT and C581S. Bottom: Current density, n=5 cells each. Results were analyzed via unpaired t-test, $p>0.05$. Kv1.5-C581S exhibits steady-state current levels that are indistinguishable from Kv1.5-WT.

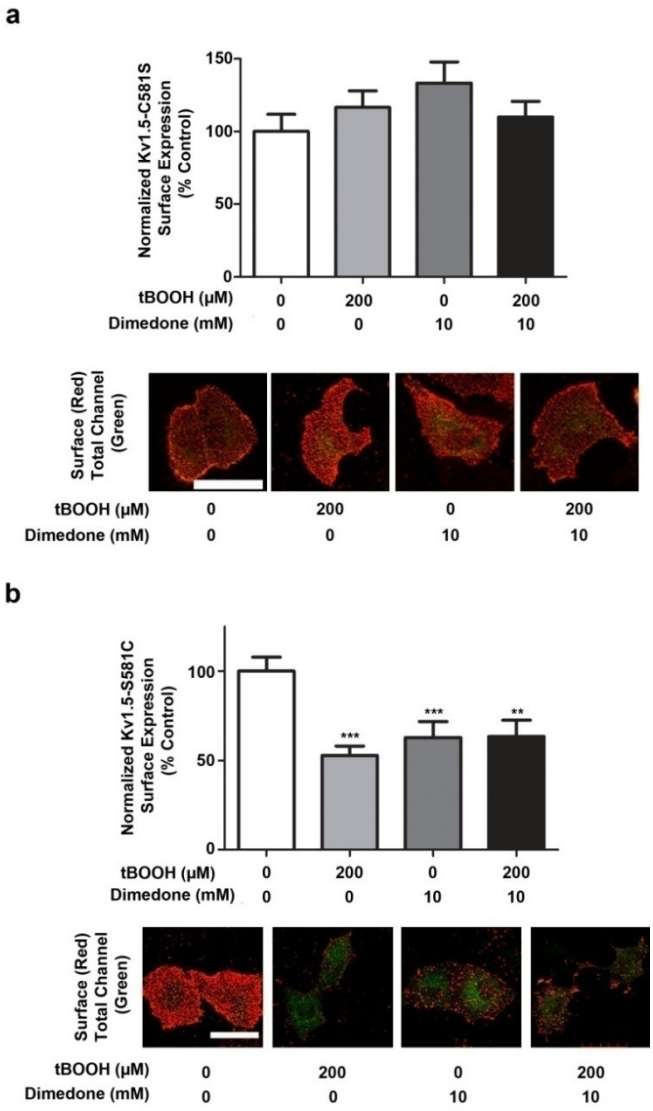

Figure 3.8: Sulfenic acid modification of C581 is necessary and sufficient to cause a reduction in Kv1.5 surface expression.
a: HL-1 cells transiently expressing Kv1.5-C581S-GFP were treated with vehicle or *t*BOOH for 60 min. or dimedone for 90 min. An additional sample was pre-treated with dimedone for 30 min., followed by 60 min. *t*BOOH treatment in the continued presence of dimedone. Cells were then stained live to label surface populations of Kv1.5 (red, collapsed Z-stacks). Surface channel was quantified as outlined in Figure 2 and analyzed via Kruskal-Wallis test, followed by Dunn's post test, n=60+ cells per condition, three experiments. $p>0.05$ relative to vehicle, not significant. Scale bar: 30 microns.
b: HL-1 cells transiently expressing Kv1.5-S581C-GFP were treated and surface Kv1.5 was quantified as outlined in a. Data were analyzed as in (a),n=60+ cells per condition, three experiments. ***indicates $p<.0001$, **indicates $p<.001$, relative to vehicle. Scale bar: 30 microns.

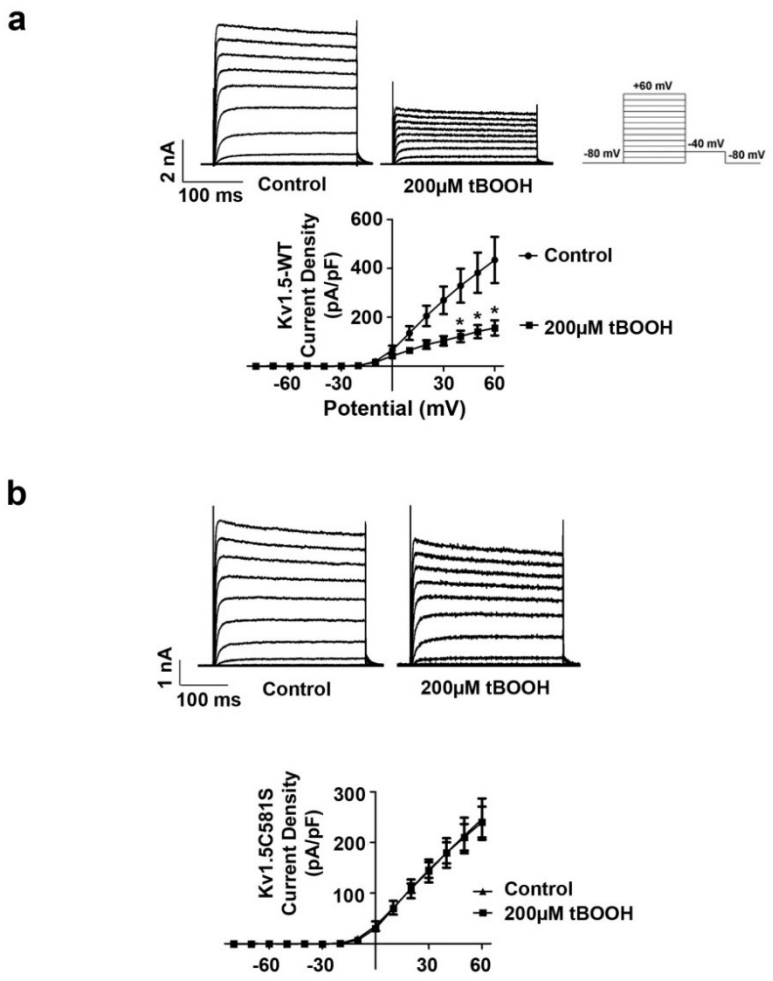

Figure 3.9: Mutation of C581 abolishes the redox sensitivity of Kv1.5 current.
a: Results of whole cell voltage clamping experiments in HL-1 cells transiently transfected with Kv1.5-GFP-WT and treated with vehicle (n=6) or 200μM *t*BOOH (n=4) for 60 min. Cells were administered 10 mV step changes in voltage from a holding potential of -80 mV, as shown in protocol. Steady state potassium current was measured at the end of each test pulse and normalized to cell capacitance. Top: Sample outward Kv channel current traces for vehicle and *t*BOOH treated cells. Bottom: Current density. Results were analyzed via unpaired t-test. *indicates $p<.05$ relative to control.
b: Results of whole cell voltage clamping experiments in HL-1 cells transiently transfected with Kv1.5-GFP-C581S and treated with vehicle (n=5) or 200μM *t*BOOH (n=4) for 60 min. Cells were administered 10 mV step changes in voltage from a holding potential of -80 mV, as shown in protocol. Steady state potassium current was measured at the end of each test pulse and normalized to cell capacitance. Top: Sample outward Kv channel current traces for vehicle and *t*BOOH treated cells. Bottom: Current density. Results were analyzed via unpaired t-test, $p>0.05$.

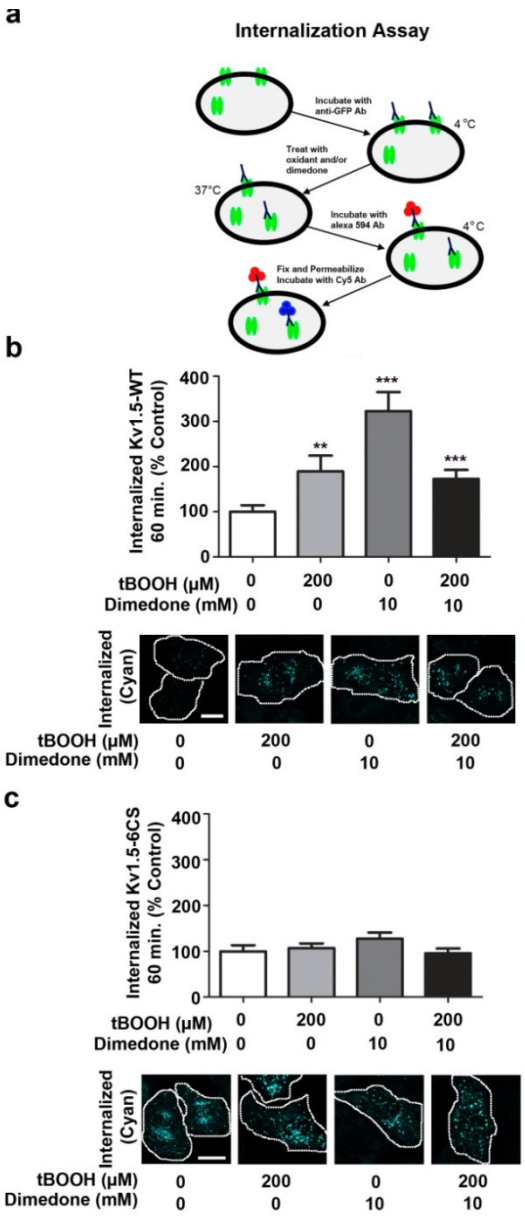

Figure 3.10: Sulfenic acid modification of intracellular cysteines on Kv1.5 significantly increases channel internalization.
a: Diagram of live-cell internalization assay in HL-1 atrial myocytes.
b: HL-1 cells expressing Kv1.5-GFP were treated as in Fig. 2c. Internalized channel (cyan) was visualized using confocal microscopy, and was quantified using NIH ImageJ software, normalized to total GFP (i.e. total Kv1.5) fluorescence, and analyzed via Kruskal-Wallis test, followed by Dunn's post test, n= 85+ cells per condition, three experiments. ** indicates p<.001, *** indicates p<.0001, relative to vehicle. Scale bar: 30 microns.
c: Experiment outlined in (b) was repeated in cells expressing Kv1.5-6CS-GFP, n= at least 62 cells per condition. * indicates p>0.05 relative to vehicle.

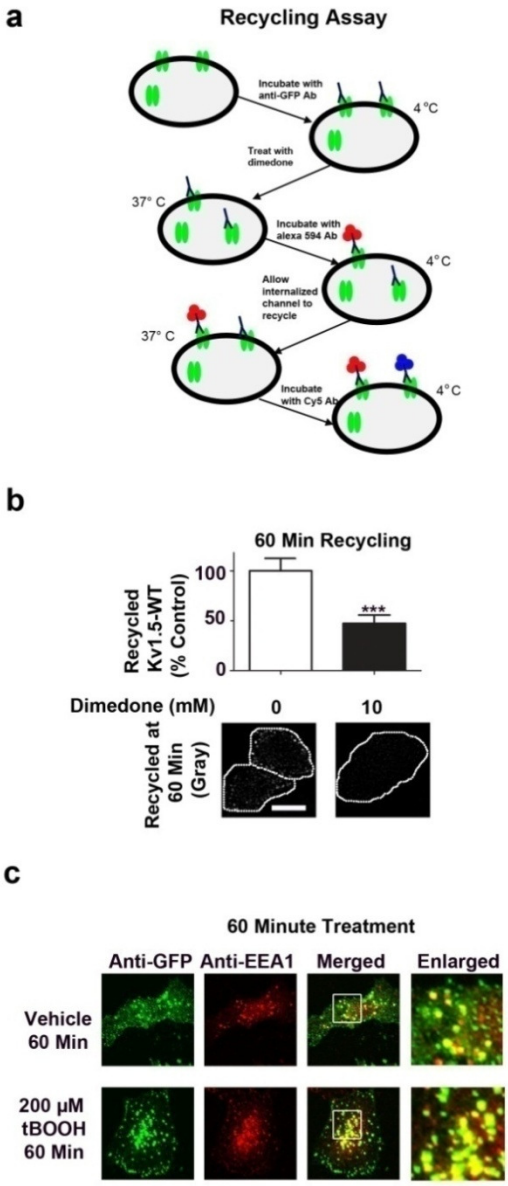

Figure 3.11: Sulfenic acid modification blocks Kv1.5 recycling after internalization to EEA1-positive compartments.
a: Diagram of live-cell recycling assay in HL-1 atrial myocytes.
b: In HL-1 cells expressing Kv1.5-GFP, recycled channel (gray) at 60 min. was measured after vehicle or dimedone treatment for 90 min. Data from 3 experiments were quantified using NIH ImageJ software and analyzed via unpaired t- test with Welch's correction, n= at least 77 cells per condition. *** indicates $p<.001$ relative to vehicle.
c: Internalized Kv1.5-GFP was detected as outlined in Figure 3.10, followed by labeling with anti-EEA1 overnight at 4℃. Internalized Kv1.5 (green) and EEA1 (red) were visualized using confocal microscopy. Yellow puncta indicate colocalization.

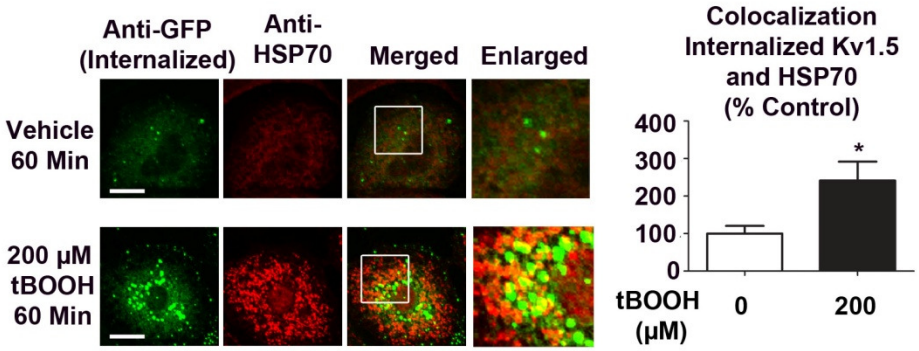

Figure 3.12: Internalized Kv1.5 colocalizes with HSP70 under acute oxidative stress.
Left: Internalized Kv1.5-GFP was detected as in Figure 3.10, followed by monoclonal anti-HSP70 (Stressgen) overnight at 4℃. Internalized Kv1.5 (here, colored green) and HSP70 (red) were visualized using confocal microscopy.
Right: Quantification of colocalization between HSP70 and Kv1.5 (yellow puncta), n=15 cells per condition * designates $p<.05$.

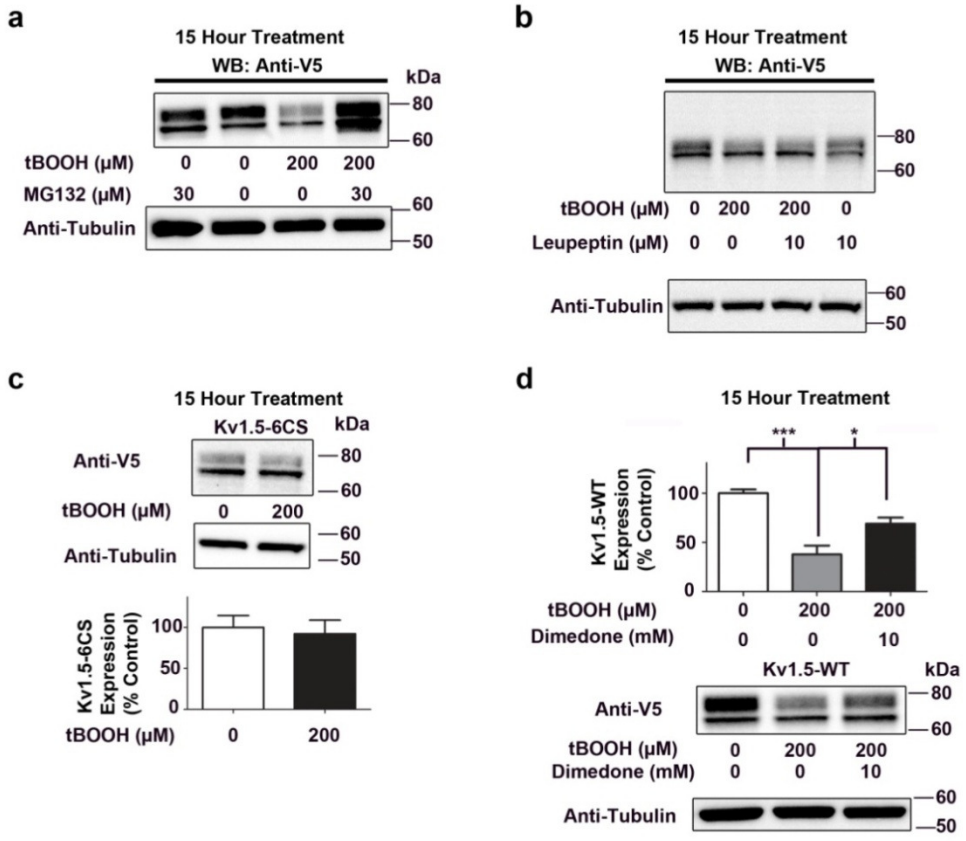

Figure 3.13: Sulfenic acid modification of Kv1.5 diverts the channel to a degradation pathway.
a: Top: HL-1 cells stably expressing Kv1.5-WT were treated with vehicle or *t*BOOH, alone or concurrently with the proteasome inhibitor, MG132 in the presence of 5 μM cycloheximide to block protein synthesis. After 15 hrs, cell lysates were generated and analyzed by western blotting with anti-V5 antibody.
b: HL-1 cells expressing Kv1.5-WT were treated with *t*BOOH, alone or in the presence of the lysosomal inhibitor, leupeptin. After 15 hrs, cell lysates were generated and analyzed by western blotting with anti-V5 antibody. The blot was stripped and re-probed with alpha-tubulin antibody as a loading control. Kv1.5 degradation under conditions of oxidative stress is insensitive to lysosomal inhibition.
c: HL-1 cells transiently expressing Kv1.5-6CS were administered the same treatments as WT. After 15 hrs, cell lysates were generated and analyzed by western blotting with anti-V5 antibody. Kv1.5-6CS expression (anti-V5 signal) was normalized to tubulin expression. Results were analyzed via 1-way ANOVA, $p>0.05$
d: HL-1 cells stably expressing Kv1.5-WT were administered *t*BOOH +/- dimedone treatments in the presence of 5 μM cycloheximide to block protein synthesis. After 15 hrs, cell lysates were generated and analyzed by western blotting with anti-V5 antibody. Results were analyzed via 1-way ANOVA. *designates $p< .05$, ***designates $p<.0001$.

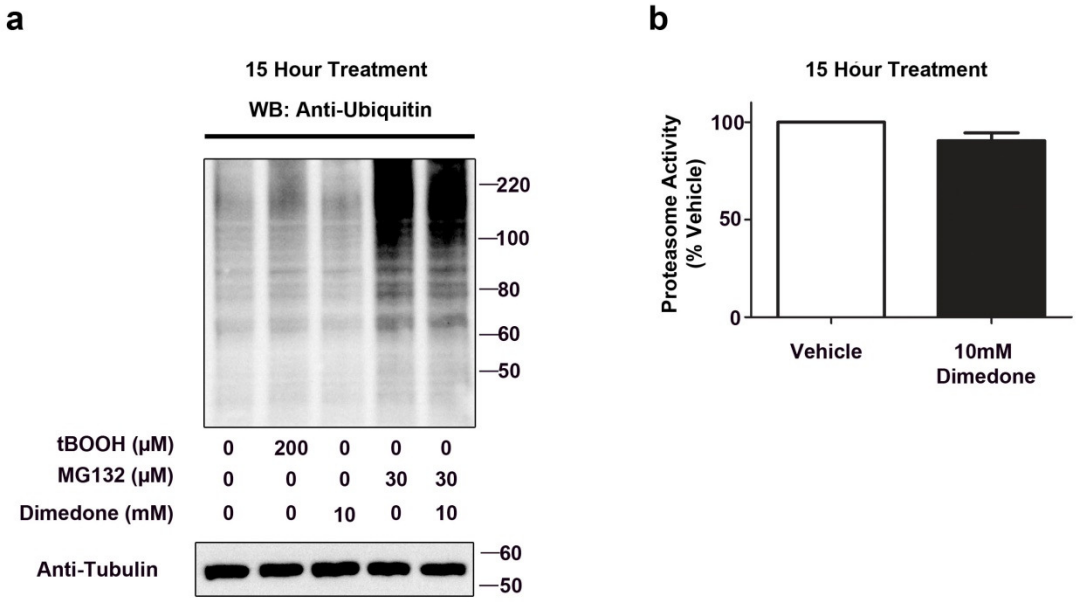

Figure 3.14 Oxidant and dimedone treatments do not interfere with function of the proteasome.
a: HL-1 cells were treated with vehicle, *t*BOOH, dimedone, MG132, or MG132 + dimedone. After 15 hrs, cell lysates were generated and analyzed *via* western blotting with monoclonal anti-ubiquitin antibody (Santa Cruz 8017). Blot was stripped and re-probed with alpha-tubulin antibody as a loading control. *t*BOOH and dimedone treatment do not inhibit the proteasomal degradation of ubiquitinated proteins.
b: HL-1 cells were treated with dimedone or vehicle. After 15 hrs, cell lysates were generated and an optimized proteasomal activity method was used for determining heart tissue chymotrypsin-like activity [171]. Reported values are without ATP and were averaged from three independent experiments. Values for dimedone-treated cell lysates were expressed as a percentage of values for untreated cells for each experiment and analyzed via unpaired t-test, $p>0.05$

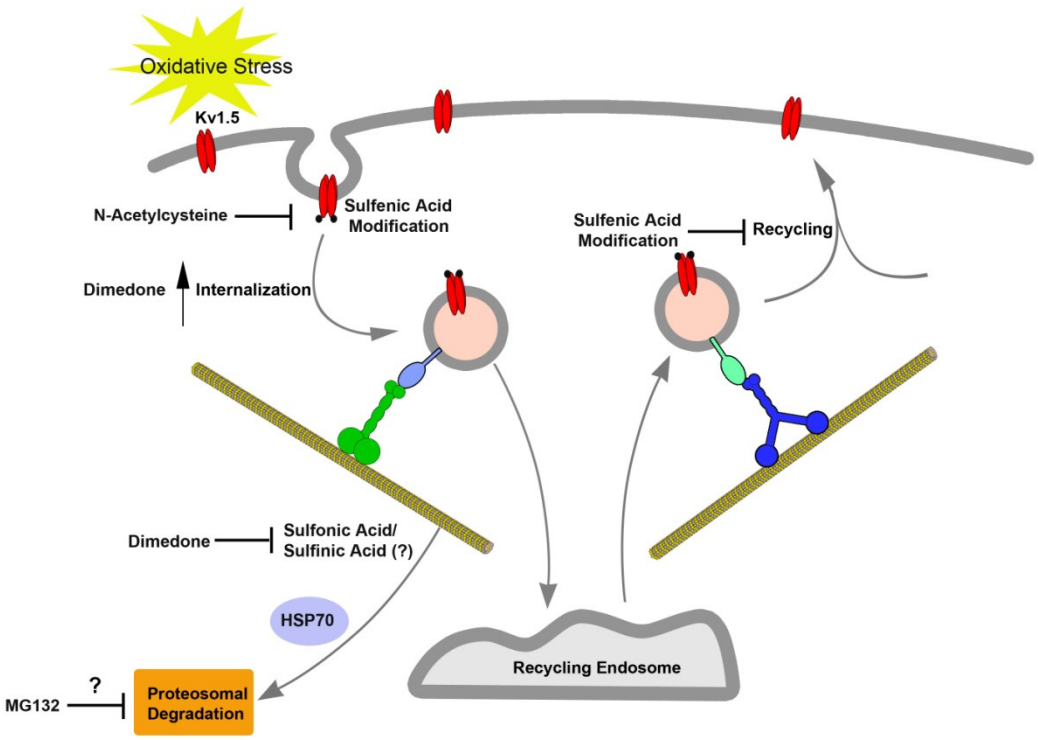

Figure 3.15: Model
Oxidative stress induces formation of sulfenic acid on COOH-terminal cysteine residue 581 of Kv1.5, which may be reduced back to the thiol form by antioxidant enzymes. Sulfenic acid modification triggers Kv1.5 internalization, and inhibits its recycling. Internalized Kv1.5 colocalizes with HSP70 under acute oxidative stress. Sulfenic acid ultimately results in channel degradation, which may occur via a proteasomal pathway. Trapping sulfenic acid with dimedone and preventing its progression to other cysteine modifications blocks this degradation.

CHAPTER IV: CONCLUSION

Introduction

Redox modulation of Kv1.5 current is associated with numerous disease states. In spite of this, the putative molecular mechanism(s) linking dysregulation of Kv1.5, oxidative stress, and disease remain unclear. Through the work of this thesis, we have demonstrated that Kv1.5 undergoes modification with sulfenic acid, a novel, redox-sensitive, modification that has not been previously reported on an ion channel or any other membrane protein. Furthermore, we demonstrate for the first time that oxidative stress acutely regulates Kv1.5 surface expression, an effect which is mediated by COOH-terminal sulfenic acid modification. Under conditions of acute oxidative stress, sulfenic acid is not a precursor to formation of a disulfide bond or adduct with glutathione, and it does not interfere with channel thioacylation. Importantly, sulfenic acid modification is sufficient to induce channel internalization and block channel recycling. Under acute oxidative stress, Kv1.5 colocalizes with HSP70, and ultimately undergoes degradation via a pathway which is sensitive to proteasomal, but not lysosomal inhibitors. To our knowledge, this report is first to provide a mechanistic link between oxidation of Kv1.5 and its degradation.

This work is significant because Kv1.5 is known to be the putative oxygen sensor in the pulmonary vasculature [6], and Kv channels regulate coronary

vessel tone in response to hydrogen peroxide or hyperglycemia-induced oxidative stress [42, 59]. Furthermore, Kv1.5 plays an important role in the pathogenesis of diseases such as atrial fibrillation and pulmonary arterial hypertension [6, 8, 50, 77]. By demonstrating that Kv1.5 undergoes redox-sensitive regulation in atrial myocytes, our work suggests that Kv1.5 also functions in oxygen sensing in the heart.

Sulfenic Acid Modification of Kv1.5 *In Vivo*

The extent to which sulfenic acid modification occurs on native channels *in vivo*, and the stoichiometry of this modification, are currently unknown. Modeling studies of cardiac action potentials suggest that very small changes in the biophysical properties or expression levels of ion channels can have substantial effects on the duration of the action potential, rhythmicity, and tissue refractoriness [3]. Thus, modification of a relatively small population of native channels may have significant effects on the function of the heart *in vivo*. Several factors make the study of native Kv channels *in vivo* challenging. Studies of atrial fibrillation in rodent models are difficult due to the small amount of tissue, and the electrophysiological differences between rodents and humans make extrapolation of results difficult [189]. Furthermore, although antibodies to endogenous channel are available [155, 190], they are not always effective in all models. Recent attempts to develop a transgenic mouse expressing Kv1.5 with an extracellular GFP tag would have allowed us the ability to study native channel posttranslational modifications and trafficking in intact animals; however, these efforts thus far have been unsuccessful. In spite of these difficulties, several additional experiments could be conducted to further expand our

understanding of the role for sulfenic acid in modulation of Kv1.5 expression. Recent mass spectrometry approaches have been developed, which may enable detection and quantification of posttranslational modifications to Kv channels in cells [191]. Stable isotope labeling with amino acids in cell culture (SILAC), which has been used successfully for quantifying phosphorylation of Kv2.1 [191], could be employed for quantification of the proportion of sulfenic or sulfinic/sulfonic acid-modified Kv1.5. Additionally, we have developed a Kv1.5 construct which contains a COOH-terminal TEV cleavage site and 6X-His tag, enabling selective enrichment of the COOH-terminal fragment of channel with the redox-sensitive C581 prior to mass spectrometry. Although our initial attempts to identify sulfenic acid modification of Kv1.5 via mass spectrometry were unsuccessful, this method would likely yield useful results with refinements to the purification procedure. Immunoisolation of Kv1.5 from native cells, followed by mass spectrometry analysis, would enable detection of cysteine oxidation on Kv1.5 in intact animals [191]. Recent evidence suggests that rapid pacing of atrial myocytes induces oxidative stress [38, 72]. Thus, it would be interesting to determine whether rapid pacing of myocytes also induces sulfenic acid modification and degradation of Kv1.5. These experiments may yield insight into the timing of channel degradation after the onset of disease. Sulfenic acid modification is regulated by multiple, redundant enzyme systems [45]. Additional mechanistic experiments could be conducted, using pharmacologic [15] or siRNA knockdown [192] of endogenous antioxidant systems to demonstrate the predominant system regulating sulfenic acid modification of Kv1.5. These

experiments would also provide an alternative approach to ectopic addition of oxidizing agents, which is well-accepted, yet not without controversy [193]. Additional experiments could be conducted to identify sulfenic acid modification of Kv1.5 in tissues after exposure of intact animals to oxidants such as cigarette smoke, or in tissue from human atrial fibrillation patients. This technique could be done using DAz labeling, streptavidin-biotin immunoprecipitation of sulfenic acid-modified protein, followed by immunodetection of sulfenic acid-modified Kv1.5. In summary, work in this thesis demonstrates a novel new mechanism for redox regulation of Kv1.5, and presents the opportunity for new experiments designed to further investigate the numerous unanswered questions that remain.

Therapeutic Implications for Atrial Fibrillation

Atrial fibrillation is an enigmatic condition, with numerous factors influencing its occurrence. Initiation of the arrhythmia can occur as a result of fibrosis and structural abnormalities due to pre-existing heart disease [189]. Alternatively, it is currently unclear, but plausible, that it could be triggered by acute alterations in ion channel function, brought on by inflammatory disease states or environmental exposures [7, 40, 75]. Significant evidence suggests that atrial fibrillation is strongly associated with oxidative stress [7, 40], although the molecular mechanism(s) linking oxidative stress and disease development are unknown. Atrial fibrillation is self-perpetuating; once the arrhythmia is initiated, rapid pacing of atrial myocytes increases oxidative stress, thus promoting further alterations in ion channel function and expression, and perpetuating additional rhythm disturbance [72]. Recent evidence suggests that oxidative stress is involved in the early electrophysiological remodeling that occurs upon rapid atrial

pacing [38]. Several clinical studies have demonstrated beneficial effects of administering antioxidant agents, including *N*-acetylcysteine [194] and ascorbic acid [38, 195] in the prevention of post-operative atrial fibrillation. However, interpretation of clinical trial data is complicated by the fact that patients undergoing open heart surgery are often taking other medications, such as ACE inhibitors [196]. Independently of their antioxidant effects, antioxidants may synergize with ACE inhibitors in protecting against atrial fibrillation [196].

Although antioxidant therapies hold promise in the treatment of atrial fibrillation, additional interventions are needed to fully address this complex, multifactorial condition. Work in this thesis, which demonstrates a functional effect for sulfenic acid modification of Kv1.5, provides a basis for development of more targeted pharmacological therapies. We have furthermore identified a specific region of this channel as a molecular target for oxidative stress. This finding presents the opportunity for developing peptide-based pharmacological therapies, which could be administered at the onset of disease, to attenuate or prevent channel oxidation and degradation. This, in turn, would be expected to alleviate the pathological remodeling that permanently alters Kv1.5 expression in the disease state. Although peptide-based therapies are limited by several factors, including their bio-availability and efficacy, recent successes with therapies for heart failure and cardiomyopathy encourage optimism [197, 198]. Other pathways peripheral to Kv1.5, which promote increased oxidative stress, may also be important targets for development of novel therapies for atrial fibrillation. Substantial evidence suggests that activation of angiotensin-II is a

significant source of oxidative stress, and is an important contributor to atrial fibrillation [199]. Accordingly, polymorphisms in the renin-angiotensin system were discovered to be associated with non-familial, structural atrial fibrillation [200]. Given that the condition is multi-factorial, a combination of therapies will likely provide optimal benefit.

Another interesting area for development of future therapies involves identifying drugs which can target Kv1.5 trafficking. Recent work in our laboratory demonstrates that conventional antiarrhythmic drugs can alter channel trafficking, in addition to their traditional pore blocking effects [29]. This is a significant finding, because one disadvantage to traditional antiarrhythmic drugs is that they have potentially lethal pro-arrhythmic side effects [29]. Our work in Chapter 3 demonstrates that sulfenic acid modification of Kv1.5, which occurs under oxidative stress, is the trigger for channel internalization, and ultimately leads to channel degradation. Therefore, to develop a therapeutic strategy it is necessary to intervene in this process during oxidative stress. It is imperative that pharmacological therapies be tailored to the specific pathophysiological state, without interfering with normal physiological function. It would thus be interesting to develop future experiments to study the differential effects of antiarrhythmic drugs on channel trafficking under oxidative stress, hypoxia, and in normal cellular conditions. Such experiments could pave the way for development of therapies which target Kv1.5 trafficking specifically under oxidative stress.

Public Health Implications

It has been several decades since the Framingham study and others like it identified important risk factors for cardiovascular disease [201]. In spite of this, cardiovascular events continue to be the leading cause of morbidity and mortality in the US [202]. Exposure to cigarette smoke and other air pollutants is a leading cause of cardiovascular disease, including cardiac arrhythmias [203, 204]. The pathogenesis of cigarette smoking-induced cardiovascular disease is complex, and includes impairment in autonomic regulation of heart rate variability and heart rate, as well as increased platelet aggregation and endothelial dysfunction [204]. On a molecular level, significant evidence indicates that inhalation of air pollutants, including cigarette smoke, induces pulmonary as well as systemic oxidative stress [203]. Cigarette smoke contains over 4,000 components, myriad of which induce oxidative stress, including soluble transition metals, polycyclic aromatic hydrocarbons, and nicotine [205, 206]. In Chapter II of this thesis, we demonstrate that exposure to an aqueous extract of cigarette smoke induces a global increase in sulfenic acid-modified proteins in cardiac myocytes. Specifically, we find a robust increase in sulfenic acid modification to Kv1.5. In Chapter III, we further demonstrate that exposure to cigarette smoke extract, in a dose sufficient to induce sulfenic acid, causes a reduction in Kv1.5 surface expression. This effect is blocked by concurrent treatment with the thiol antioxidant, *N*-acetylcysteine, indicating that the reduction in channel surface expression is due to oxidative stress. Our findings are consistent with those of Hong et al., who found decreased Kv1.5 mRNA and protein synthesis in bronchial smooth muscle cells of rats exposed to cigarette smoke [154]. To our

knowledge, however, our results provide the first report that cigarette smoke exposure can alter channel expression posttranslationally, via a mechanism involving oxidative stress. These findings are significant, because smoking has been linked to both atrial fibrillation [75] and pulmonary arterial hypertension [207], diseases characterized by significant down-regulation of Kv1.5 [6, 8]. It is difficult to extrapolate these findings to achieve an estimation of the relative risk of cardiovascular events in the general population. However, these results warrant further studies to investigate the role for toxicant exposures, particularly indoor and outdoor air pollution, in the alteration of ion channel expression and cardiovascular disease. Given the prevalence of cigarette smoke exposure and cardiovascular disease, basic scientific, clinical, and epidemiological studies should be used to promote legislative action, such as laws designed to reduce exposure of the general population, especially children, to secondhand smoke. Clearly, with regard to the pathogenesis of toxicant-induced cardiovascular disease, numerous scientific questions remain unanswered. Furthermore, current efforts to improve public health in this area remain insufficient: 15 states in the US currently enforce only meager restrictions on tobacco smoke exposure [208], and government restrictions on air quality are often poorly enforced [203]. With this information in mind, it is clear that significant work on the part of scientists and regulatory authorities remains to be done.

Summary

In summary, through work in this thesis, we have demonstrated a novel mechanism by which sulfenic acid modification of Kv1.5 regulates channel trafficking and expression in atrial myocytes. We demonstrate that the channel is

modified with sulfenic acid on a single, COOH-terminal cysteine, C581, under oxidative stress. Furthermore, sulfenic acid modification of C581 is sufficient to cause a significant reduction in channel surface expression, which occurs through an increase in internalization and a decrease in recycling of channel back to the cell surface. Under prolonged oxidative stress, sulfenic acid is a trigger for channel degradation, which may be mediated by HSP70 and the ubiquitin-proteasomal pathway. Thus, we have demonstrated a molecular mechanism linking oxidative stress, cysteine oxidation on the channel itself, and channel degradation. Given the association between oxidative stress and Kv channel dysregulation in disease, this finding may have important therapeutic and public health implications.

Additional experiments are necessary to further elucidate the mechanisms by which oxidative stress, through cysteine oxidation, regulates Kv1.5 expression in the cardiovascular system. Hopefully the work of this thesis will provide a starting point for further studies, and help influence understanding of the mechanism of Kv1.5 oxygen sensitivity and its implications in disease. I am thrilled that I was able to contribute to the rapidly growing and exciting fields of cellular and redox biology.

APPENDIX A: SUMMARY OF MAMMALIAN KV CHANNELS

Information provided by [1].

Channel	Gene	Family	Current Phenotype	Predominant Tissue Expression
Kv1.1	KCNA1	Shaker	Delayed Rectifier	Brain, Retina
Kv1.2	KCNA2	Shaker	Delayed Rectifier	Brain, Spine, Retina
Kv1.3	KCNA3	Shaker	Delayed Rectifier	T- and B- Lymphocytes, Microglia, Kidney
Kv1.4	KCNA4	Shaker	A-type, Fast Inactivating	Heart, Brain, Pancreatic Islet Cells
Kv1.5	KCNA5	Shaker	Delayed Rectifier	Heart, Arterial Smooth Muscle, Microglia
Kv1.6	KCNA6	Shaker	Delayed Rectifier	Brain, Spinal Cord
Kv1.7	KCNA7	Shaker	Delayed Rectifier	Heart, Skeletal Muscle, Lung
Kv1.8	KCNA10	Shaker	Delayed Rectifier	Kidney
Kv2.1	KCNB1	Shab	Delayed Rectifier	Brain, Pulmonary Arterial Smooth Muscle, Pancreatic Islet Cells
Kv2.2	KCNB2	Shab	Slowly Activating, Non-Inactivating	Brain, Pancreatic Islet Cells
Kv3.1	KCNC1	Shaw	Delayed Rectifier	Brain, Skeletal Muscle
Kv3.2	KCNC2	Shaw	Delayed Rectifier	Brain
Kv3.3	KCNC3	Shaw	A-type, Fast Inactivating	Brain, Corneal Epithelium
Kv3.4	KCNC4	Shaw	A-type, Fast Inactivating	Brain, Skeletal Muscle
Kv4.1	KCND1	Shal	A-type, Fast Inactivating	Brain, Heart
Kv4.2	KCND2	Shal	A-type, Fast Inactivating	Brain, Heart
Kv4.3	KCND3	Shal	A-type, Fast Inactivating	Heart, Brain
Kv5-6	KCNF KCNG	eag	Modifiers of Kv2.1 Channels	Varied
Kv7.1	KCNQ1	KvLQT	Delayed Rectifier	Heart, pancreas, kidney, lung
Kv7.2	KCNQ2	KVLQT	Neuronal M Current	Brain
Kv7.3	KCNQ3	KVLQT	Neuronal M Current	Brain
Kv7.4	KCNQ4	KVLQT	Neuronal M Current	Brain, Hair cells of the cochlea
Kv7.5	KCNQ5	KVLQT	Neuronal M Current	Brain, Skeletal Muscle
Kv8-9	KCNV KCNS	N/A	Modifiers of Kv2 Channels	Varied
Kv10.1	KCNH1	eag	Delayed Rectifier	Brain, Stem Cells

Channel	Gene	Family	Current Phenotype	Predominant Tissue Expression
Kv10.2	KCNH5	eag	Outwardly Rectifying, Non-Inactivating	Brain, Skeletal Muscle
Kv11.1	KCNH2	eag	Delayed Rectifier	Central Nervous System, Heart
Kv11.2	KCNH6	eag	Delayed Rectifier	Nervous System
Kv11.3	KCNH7	eag	Delayed Rectifier	Nervous System
Kv12.1	KCNH8	eag	Delayed Rectifier	Nervous System
Kv12.2	KCNH3	eag	Delayed Rectifier	Brain
Kv12.3	KCNH4	eag	Delayed Rectifier	Lung, Brain

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