Redox-Sensitive Sulfenic Acid Modification Regulates Surface Expression of the Cardiovascular Voltage-Gated Potassium Channel Kv1.5

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Rationale: Kv1.5 (KCNA5) is expressed in the heart, where it underlies the IKur current that controls atrial repolarization, and in the pulmonary vasculature, where it regulates vessel contractility in response to changes in oxygen tension. Atrial fibrillation and hypoxic pulmonary hypertension are characterized by downregulation of Kv1.5 protein expression, as well as with oxidative stress. Formation of sulfenic acid on cysteine residues of proteins is an important, dynamic mechanism for protein regulation under oxidative stress. Kv1.5 is widely reported to be redox-sensitive, and the channel possesses 6 potentially redox-sensitive intracellular cysteines. We therefore hypothesized that sulfenic acid modification of the channel itself may regulate Kv1.5 in response to oxidative stress.

Objective: To investigate how oxidative stress, via redox-sensitive modification of the channel with sulfenic acid, regulates trafficking and expression of Kv1.5.

Methods and Results: Labeling studies with the sulfenic acid–specific probe DAz and horseradish peroxidase–streptavidin Western blotting demonstrated a global increase in sulfenic acid–modified proteins in human patients with atrial fibrillation, as well as sulfenic acid modification to Kv1.5 in the heart. Further studies showed that Kv1.5 is modified with sulfenic acid on a single COOH-terminal cysteine (C581), and the level of sulfenic acid increases in response to oxidant exposure. Using live-cell immunofluorescence and whole-cell voltage-clamping, we found that modification of this cysteine is necessary and sufficient to reduce channel surface expression, promote its internalization, and block channel recycling back to the cell surface. Moreover, Western blotting demonstrated that sulfenic acid modification is a trigger for channel degradation under prolonged oxidative stress.

Conclusions: Sulfenic acid modification to proteins, which is elevated in diseased human heart, regulates Kv1.5 channel surface expression and stability under oxidative stress and diverts channel from a recycling pathway to degradation. This provides a molecular mechanism linking oxidative stress and downregulation of channel expression observed in cardiovascular diseases. (Circ Res. 2012;111:842-853.)

Key Words: oxidative stress □ Kv1.5 □ sulfenic acid □ atrial fibrillation □ trafficking □ voltage-gated potassium channels □ posttranslational modification

Atrial fibrillation is the most common cardiac arrhythmia and is predicted to rise dramatically over the next several years. Treatment includes the use of antiarrhythmic drugs and/or electric cardioversion. These treatments, although initially successful in managing atrial fibrillation, often become ineffective over time. One likely contributing factor to this resistance is the electric and structural remodeling that occurs in the heart with the persistence of atrial fibrillation. Chronic atrial fibrillation–induced electrophysiological remodeling of the atria includes changes to action potential duration and refractory period that result from alterations in the function and expression of ion channels in cardiac myocytes.

In the atria, the balance of inward Ca2+ and outward K+ currents determines action potential duration and refractoriness. Potassium channel–blocking drugs have been used as antiarrhythmics to treat patients for many years; however, these agents...
are most effective in treating recent-onset atrial fibrillation and are less effective in patients with chronic atrial fibrillation. One of the predominant repolarizing $K^+$ currents in the atria is the ultrarrapid delayed-rectifier current $I_{Kur}$. This current is encoded by the $Kv1.5$ channel subunit, which in the human heart is selectively expressed in the atria and is considered an important therapeutic target for treatment of atrial fibrillation.\(^1,2\) Paradoxically, $I_{Kur}$ and $Kv1.5$ protein are reduced in chronic human atrial fibrillation, which may limit its potential therapeutic efficacy in patients with persistent or permanent arrhythmias.\(^3\) This pathology-specific decrease in $Kv1.5$ protein level is also observed in the pulmonary vasculature during chronic hypoxic pulmonary hypertension (HPH).\(^4\) Although it is an important therapeutic consideration, the precise mechanisms underlying this ion channel remodeling and decrease in channel protein remain unknown.

One element common to both HPH and chronic atrial fibrillation is a metabolic imbalance leading to a pro-oxidant shift in cellular redox state, which causes cellular oxidative stress. Oxidative stress, defined as excessive production of reactive oxygen species (ROS), and/or diminished antioxidant capacity, is a newly recognized hallmark of cardiovascular disease. Rapid activation of the atrium, which occurs during atrial fibrillation, leads to increased ROS and a decrease in tissue levels of antioxidants.\(^5,6\) Multiple $K^+$ channels and currents, including the $Kv1.5$-encoded $I_{Kur}$, are regulated by oxidizing agents.\(^4,7–9\) Therefore, it is likely that the altered oxidative state of myocytes contributes to electric remodeling. However, despite significant evidence linking conditions of oxidative stress to reduced $Kv1.5$ expression, the molecular link(s) between these observations is unclear.

One potential direct mechanism for $Kv1.5$ regulation is via redox-sensitive posttranslational modifications to the channel. 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.\(^10\) 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 (oxoforms) have been identified in vivo that play a crucial role in protein stability and function.\(^11\) 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.\(^10,11\) 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 a more stable disulfide or sulfenic acid form.\(^11\) $Kv1.5$ has 6 intracellular cysteines divided among the NH$_2$ and COOH termini. This, combined with the widely reported redox sensitivity of the channel, led to the hypothesis that sulfenic acid modification to the channel may regulate its function and expression. In the current study, we show that there is a global increase in the level of sulfenic acid–modified proteins in the atria of human patients with chronic atrial fibrillation, compared with healthy control subjects. We further demonstrate that $Kv1.5$ is a substrate for sulfenic acid modification that, under conditions of oxidative stress, functions as a fate switch to divert the channel from a recycling to a degradation pathway in myocytes.

**Methods**

**Human Heart Tissue Procurement**

Atrial myocardial tissue from patients with chronic atrial fibrillation was collected at the time of transplantation at the University of Michigan or the University of California, San Francisco. Atrial myocardial tissue from nonfailing hearts was collected from unmatched...
donors from the University of Michigan under approval from the Gift of Life: Michigan Organ and Tissue Donation Program. Before tissue retrieval, all hearts were perfused with ice-cold cardioplegia. Samples from each heart were snap-frozen in liquid N2 in the operating room and stored at -80°C. Tissue collection from human hearts used in this study has the approval of the University of Michigan Institutional Review Board, and subjects gave informed consent.

Stable Cell Lines

Stable cell lines expressing Kv1.5 wild-type (WT) 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 the manufacturer’s instructions. HL-1 cells were a gift from William Claycomb.

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

Rodent or human tissue samples, LTK or HL-1 cells, were harvested in non-denaturing lysis buffer containing D Az, followed by Staudinger ligation and horseradish peroxidase (HRP)-streptavidin Western blotting (Online Data Supplement). Blots were stripped and reprobed with anti-V5 or anti-Kv1.5 antibody to assess immunoprecipitation efficiency.

Perfusion of Isolated Rat Heart

Rat hearts were excised and Langendorff-perfused with Krebs-Henseleit buffer (KHB) with or without 200 μmol/L diamide for 55 minutes, followed by isolation of membranes and labeling with D Az (Online Data Supplement).

Immunocytochemistry and Confocal Imaging

For surface, internalization, and recycling assays, immunocytochemistry was performed as described previously, with minor modifications (Online Data Supplement).

Electrophysiology

Ionic currents were recorded at room temperature in HL-1 cells transiently expressing Kv1.5 using the whole-cell configuration of

Figure 2. Sulfenic acid modification of Kv1.5. A, Topology of a single α-subunit of WT human Kv1.5, showing all 10 cysteine residues. Aligned vertebrate Kv1.5 sequences are centered on human C-terminal cysteines. Conserved cysteines are highlighted, with CS81 in dark gray. B, LTK cells stably expressing V5-tagged WT were labeled with D Az-1 and conjugated to p-biotin (lane 3). Sulfenic acid modification was detected by HRP-streptavidin Western blot (WB). C, Top, LTK cells stably expressing V5-tagged WT Kv1.5, Kv1.5 to 4CS, or Kv1.5 to 6CS were labeled with D Az-1. Bottom, Averaged quantified densitometry data from 3 experiments normalized to WT Kv1.5 and analyzed using 1-way ANOVA, followed by Tukey post hoc comparison. ***P<0.0001 relative to WT. D, Top, COOH-terminal cysteines were individually reintroduced into the null background of Kv1.5 6CS. Sulfenic acid modifications were detected by labeling with D Az. Bottom, Summary of 3 experiments quantified via densitometry, normalized to WT Kv1.5, and analyzed using 1-way ANOVA, followed by Tukey post hoc comparison. ***P<0.0001 relative to WT.
the patch-clamp technique, as described previously, with minor modifications10 (Online Data Supplement). For measurement of \( I_{\text{Kur}} \), rat cardiac myocytes were isolated steriley, and currents were recorded as outlined in the Online Data Supplement.17

**Proteasome Activity Assay**

An optimized method was used for determining heart tissue chymotrypsin-like activity.18 Reported values are without ATP and were averaged from 3 independent experiments. Values for dimedone-treated cell lysates are expressed as a percentage of values for untreated cells for each experiment.

**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 1-way ANOVA followed by Tukey post hoc test or unpaired, 2-tailed \( t \) test. A probability value of \(<0.05\) was considered significant.

**Results**

**Human Chronic Atrial Fibrillation Is Accompanied by a Global Increase in Sulfenic Acid–Modified Proteins**

Significant evidence indicates that atrial fibrillation is associated with oxidative stress5,6,19 and that this may play an important role in the pathologic remodeling that occurs in chronic disease; however, a role for sulfenic acid modification in this process has not been investigated. We therefore hypothesized that atrial fibribilation in human atria has been accompanied by an increase in sulfenic acid–modified proteins. To test this hypothesis, we used the novel chemical probe DAz. When coupled to a biotinylated secondary agent, DAz enables detection of sulfenic acid–modified protein in cells and tissue12 (Online Figure 1A). We first used this technique in oxidized mouse heart and detected a robust increase in sulfenic acid, confirming that DAz labels sulfenic acid–modified proteins in tissue (Online Figure 1B). Consistent with our hypothesis, the level of sulfenic acid modification in the human atria from patients with chronic atrial fibrillation was substantially higher than that of healthy control patients (Figure 1A and 1B and Online Figure II). This finding, combined with the evidence that Kv1.5 current is redox sensitive, led us to explore a mechanistic role for sulfenic acid regulation of Kv1.5 in atrial fibrillation.

**Kv1.5 Is Modified by Sulfenic Acid on a Single COOH-Terminal Cysteine**

Kv1.5 has 6 intracellular cysteines: 2 on the NH2 terminus and 4 on the COOH terminus, which may be candidates for oxidation to sulfenic acid (Figure 2A). Sequence comparison revealed that all 4 COOH-terminal sites are conserved in mammalian sequences (Figure 2A). Therefore, we hypothesized that Kv1.5 may be a substrate for sulfenic acid modification and that this may account for its reported redox sensitivity. Kv1.5 appears as a doublet comprised as a nascent, nonglycosylated form and a mature, glycosylated form.16 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 (Figure 2B, lane 3). Omitting DAz or p-biotin resulted in few nonspecific bands, indicating that DAz is specific for sulfenic acid–modified proteins and that p-biotin is specific for proteins labeled with DAz (Figure 2B, lanes 1–2). To confirm that the observed bands were indeed Kv1.5, we performed DAz labeling and immunoprecipitation, followed by deglycosylation of the channel with PNGase. Consistent with literature indicating that Kv1.5 undergoes glycosylation,16 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 (Online Figure IIIA). To test the specificity of this labeling, we used the parent compound and competitive inhibitor of DAz, 5,5-dimethyl-1,3-cyclohexanedione (dimedone).12 Including dimedone with DAz in the lysis buffer blocked labeling of sulfenic acid–modified Kv1.5 (Online Figure IIIB), demonstrating the specificity of DAz for sulfenic acid–modified Kv1.5.

To determine the specific sulfenic acid modification profile of Kv1.5, we first used LTK cells stably expressing Kv1.5-WT, as well as 2 additional cysteine mutant proteins: (1) Kv1.5 to 4CS, in which all 4 COOH-terminal cysteines were mutated to serine, and (2) Kv1.5 to 6CS, in which all 6 intracellular 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.16 As expected, mutating all 6 intracellular cysteines substantially reduced sulfenic acid modification of Kv1.5. However, sulfenic acid modification of Kv1.5 to 4CS was reduced to the same extent (Figure 2C), indicating that sulfenic acid modification occurs primarily on the COOH-terminus of the channel. To resolve the specific locus of this modification, we developed 4 additional stable cell lines, each expressing Kv1.5 with a different COOH-terminal cysteine reintroduced individually into the null background of the 6-cysteine mutant (Kv1.5-S564C, S581C, S586C, and S604C). As shown in Figure 2D, reintroducing CS81 restored sulfenic acid modification of Kv1.5 to a level that was similar to WT. Taken together, these studies show that CS81, located on the cytoplasmic portion of Kv1.5 near the COOH-terminus, is the principal site for oxidation to sulfenic acid.

**Oxidative Stress Induces Formation of Sulfenic Acid on Kv1.5**

To explore the redox sensitivity of sulfenic acid modification, we induced oxidative stress in cells stably expressing Kv1.5-WT, using the organic hydroperoxide tertiary butyl hydroperoxide (tBOOH). Using DAz labeling, immunoprecipitation of Kv1.5 and HRP-streptavidin Western blotting, we found that 30-minute treatment with 200 \( \mu \)mol/L tBOOH caused a significant increase in the fraction of sulfenic acid–modified-Kv1.5 (\( P<0.01 \), Figure 3A). Importantly, concurrent treatment with the thiol-specific antioxidant N-acetylcysteine (NAC) abolished the tBOOH-induced increase in sulfenic acid, while having no significant effect alone. We also observed an increase in sulfenic acid modification to Kv1.5 after treatment with the more physiologically relevant oxidizing agent, hydrogen peroxide, or by depletion of reduced intracellular glutathione with diamide20 (Figure 3B and 3C), indicating that the modification is not specific to a particular oxidant. We also examined the oxidant-induced modification of Kv1.5 in HL-1 atrial myocytes, which maintain a differentiated, contractile phenotype in vitro.21 Importantly, we observed a similar increase in sulfenic acid modification to Kv1.5 in myocytes after treatment with either...
Figure 3. Redox sensitivity of Kv1.5 sulfenic acid modification. A, Top. HRP-streptavidin Western blot (WB) depicting LTK cells stably expressing Kv1.5-WT, treated for 30 minutes with H2O2 or vehicle, and analyzed using 1-way ANOVA, followed by Tukey post hoc comparison. **P<0.01, *P<0.05. **B and C, Top. LTK cells stably expressing Kv1.5-WT were treated for 30 minutes with H2O2 (B), diamide (C), or vehicle, followed by labeling with DAz and HRP-streptavidin Western blot. Bottom. Data from 3 separate experiments were quantified via densitometry and analyzed via unpaired t test with Welch correction. **P<0.05.

β-BOOH or diamide (Online Figure IVA and B), indicating that this modification was not cell type–specific. Together, these data show that sulfenic acid modification of Kv1.5 reflects changes in cellular redox state induced by multiple oxidants in varying cell types, including HL-1 cardiac myocytes.

Endogenous Kv1.5 Is Modified With Sulfenic Acid, Which Regulates Channel Current and Surface Levels in Cardiac Myocytes

To further explore a role for sulfenic acid modification in regulating Kv1.5 in vivo, we perfused freshly isolated, intact rat hearts with normal KHB solution, or with KHB solution containing 200 μmol/L diamide, which increases intracellular peroxide levels,22 induces sustained arrhythmia,13 inhibits IKur current, indicating that endogenous Kv1.5 undergoes sulfenic acid modification in modulation of IKur currents, we used the sulfenic acid–specific alkylating agent dimedone.24 Our rationale for using dimedone was based on the fact that this covalent modification of sulfenic acid precludes its reduction back to the thiol form and prevents further oxidation to sulfonic or sulfenic acid (Figure 4B, top). As shown in Figure 4B, treatment with dimedone significantly reduced IKur currents in dissociated cardiac myocytes. These results collectively show that Kv1.5 is a substrate for sulfenic acid in the heart, which regulates endogenous Kv1.5 mediated IKur currents.

Our previous work demonstrated that changes in current density can result from acute regulation of channel trafficking, leading to altered Kv1.5 levels on the cell surface.15,25 This evidence, combined with the findings shown in Online Figure VI and Figure 4A and 4B, led us to explore the effects of sulfenic acid modification on channel trafficking. To this end, we used HL-1 cells transiently expressing Kv1.5 with an extracellular green fluorescent protein (GFP) tag inserted between the S1 and S2 segments (Kv1.5-GFP, Online Figure VIIA) and measured changes in cell surface expression of Kv1.5 using live-cell immunofluorescent labeling.15 This technique utilizes an antibody against the external GFP tag in live, nonpermeabilized cells, therefore allowing the discrimination between surface and total cellular populations of channel (total GFP florescence). Our previous work demonstrated that the GFP tag has no effect on the electrophysiological properties or glycosylation of the channel.15 In control experiments, we found that Kv1.5-WT-GFP was modified with sulfenic acid to the same extent as untagged Kv1.5-WT, suggesting that potential sulfenic acid modification of the GFP tag itself does not contribute significantly to the signal (Online Figure VIIIB). In live-cell surface labeling experiments, we found that oxidant treatment resulted in a significant decrease in the surface levels of Kv1.5 in HL-1 myocytes (Figure 4C). This reduction was time-dependent (Online Figure VIIIC), with no difference in the
level of GFP fluorescence in oxidant-treated cells compared with vehicle (Online Figure VIII).

To determine a specific role for sulfenic acid modification of Kv1.5 in modulation of channel surface expression, we again used dimedone. Treatment with dimedone decreased surface Kv1.5 (Figure 4C), analogous to treatment with tBOOH, and combining both agents potentiated this effect. This is consistent with our biochemical and in vivo data (Figure 2B and 2D and Figure 4A and 4B) showing that there is a basal level of sulfenic acid modification in nonoxidant treated cells and suggests that hyperoxidation of cysteine to sulfinic or sulfonic acid does not trigger the decrease in channel surface levels. This
reduction in channel surface expression was also observed in cells treated with either hydrogen peroxide or diamide, indicating that the effect is not oxidant-specific (Online Figure VIIIA and B). As a functional correlate to our immunostaining results, we performed whole-cell voltage-clamp measurements in HL-1 myocytes expressing Kv1.5-GFP. Treatment with tBOOH caused a significant reduction in Kv1.5 steady-state current density (Figure 4D), which paralleled the effects observed in our immunofluorescence assay. Importantly, we found that oxidant exposure does not alter the biophysical properties of Kv1.5 (Online Table I), suggesting that a reduction in channel surface expression underlies the reduction in current. This is the first report to show that oxidative stress regulates the surface density of ion channel proteins, providing insight into the molecular mechanisms underlying the effects of ROS on channel current.

Oxidation of Kv1.5 C581 Is Necessary and Sufficient for Reduction in Channel Current and Surface Density

Our data show that C581 is necessary for sulfenic acid modification of Kv1.5 (Figure 2D). Therefore, we tested the requirement of C581 for oxidant-induced changes in channel function. Mutation of C581 on Kv1.5-GFP (Kv1.5-GFP-C581S) was sufficient to abrogate the reduction in channel surface expression and prevent the decrease in Kv1.5 channel current induced by tBOOH or dimedone in HL-1 atrial myocytes (Figure 5A and 5B). Significantly, reintroduction of this cysteine alone to the Kv1.5 6-cysteine mutant (Kv1.5-GFP-S581C) restored the effect on surface levels and channel current (Figure 5C and 5D). Importantly, there was no significant change in total channel protein expression in these experiments, as indicated by Western blotting for WT Kv1.5-GFP, as well as total GFP fluorescence in all experiments (Online Figure IXA through E). Because the Kv1.5-S581C mutant possesses a single intracellular cysteine, this finding further implicates sulfenic acid modification of C581, rather than an intramolecular disulfide bond or other modification, in modulation of channel surface expression. Thus, inducing sulfenic acid modification on C581 of Kv1.5 with oxidizing agents, or trapping Kv1.5 in the sulfenic acid–modified state, causes a reduction in channel surface density and current. 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 levels of the channel at the plasma membrane.

Oxidation Triggers Internalization of Kv1.5 and Diverts Channel Protein From a Recycling Pathway

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 short-term oxidative stress (Online Figure IXA and B). Therefore, using a live-cell immunofluorescence-based internalization assay (Online Figure XA), we first measured the effects of tBOOH or dimedone on channel internalization. To directly measure Kv1.5 internalization, surface channels were initially labeled at low temperature...
with a primary antibody directed against the extracellular GFP epitope tag, followed by treatment at 37°C with rBOOH, dimedone, or vehicle. Cells were then removed from the treatment, and channels remaining at the plasma membrane were labeled at 4°C with a saturated concentration of AlexaFluor 405–conjugated secondary antibody. Cells were subsequently fixed, permeabilized, and incubated with a second biotinylated secondary antibody, which was detected with streptavidin Cy5. Using this strategy, only those channels originally present at the cell surface can be detected. Of these, only the fraction that remained at the plasma membrane was labeled with the first AlexaFluor secondary antibody, whereas the fraction that was internalized was detected with the second Cy5-labeled secondary antibody. At 37°C, we observed no internalized channel (gray) was visualized using confocal microscopy, quantified using NIH ImageJ software, normalized to GFP fluorescence (ie, total Kv1.5), and analyzed via Kruskal-Wallis test, followed by Dunn posttest (n=85± cells per condition, 3 experiments). **P<0.001, ***P<0.0001 relative to vehicle. Scale bar: 30 μm. B, In HL-1 cells, recycled channel (gray) at 60 minutes was measured after vehicle or dimedone treatment for 90 minutes. Data were quantified using NIH ImageJ software and analyzed via 1-way ANOVA, followed by Tukey post hoc comparison (n=at least 70 cells per condition, 3 experiments). **P<0.0001 relative to vehicle control.

**Figure 6. Effect of sulfenic acid on Kv1.5 internalization and recycling.** A. HL-1 cells transiently expressing Kv1.5-WT-GFP were treated with vehicle, rBOOH, or dimedone for 60 minutes. Internalized channel (gray) was visualized using confocal microscopy, quantified using NIH ImageJ software, normalized to GFP fluorescence (ie, total Kv1.5), and analyzed via Kruskal-Wallis test, followed by Dunn posttest (n=85± cells per condition, 3 experiments). **P<0.001, ***P<0.0001 relative to vehicle. Scale bar: 30 μm. B, In HL-1 cells, recycled channel (gray) at 60 minutes was measured after vehicle or dimedone treatment for 90 minutes. Data were quantified using NIH ImageJ software and analyzed via 1-way ANOVA, followed by Tukey post hoc comparison (n=at least 70 cells per condition, 3 experiments). **P<0.0001 relative to vehicle control.**

**Sulfenic Acid Modification of Kv1.5 in Response to Oxidative Stress Results in Channel Degradation**

On the basis of the preceding results, we sought to determine the intracellular fate of internalized channel after exposure to oxidant. Consistent with our previous report, intracellularly retained channel colocalized with early endosomal antigen 1 (EEA1), a marker for early endosomes (Figure 7A). This interaction was more pronounced after a 60-minute treatment with rBOOH, an observation that probably reflects the larger pool of internalized channel (Figure 7A). After performing coimmunostaining experiments with a number of intracellular markers, we uncovered a previously unreported, oxidative stress–induced colocalization of Kv1.5 with the molecular chaperone heat shock protein (HSP)70 (Figure 7B). 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 ubiquitina-
oxidation of proteins may target them for degradation, 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 with tBOOH, alone or in the presence of the N-(benzyloxycarbonyl) leucinylleucinylleucinal protease inhibitor MG132. To prevent oxidant-induced changes in Kv1.5 transcription, we performed all treatments in the presence of the protein synthesis inhibitor cycloheximide. Treatment with tBOOH caused degradation of Kv1.5 in as little as 15 hours, which was rescued by inhibition of the proteasome (Figure 8A). Importantly, oxidant-induced channel degradation was not observed in the Kv1.5-C581S mutant protein (Figure 8B), implicating oxidation of this cysteine on the channel in the degradation. Because sulfenic acid can oxidize further to sulfonic (Cys-SO$_3$H) or sulfenic (Cys-SO$_2$H) acid, 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 8C shows that dimedone attenuates Kv1.5 degradation under oxidative stress conditions, as predicted. Although dimedone blocked channel degradation, it remained possible that these effects were mediated by depletion of the intracellular pool of ubiquitin, or direct inhibition of the proteasome. To rule out direct effects of dimedone and oxidant treatments on the activity of the proteasome, we first treated HL-1 cells with dimedone and tBOOH, alone and in the presence of the proteasomal inhibitor MG132, followed by Western blotting with an antibasquin antibody. In contrast with the positive control samples treated with MG132, treatment of cells with tBOOH or dimedone alone caused no accumulation of ubiquitinated proteins in the cells (Online Figure XIA). To directly assess the effects of dimedone on proteasomal activity, we measured the chymotrypsin-like activity of the proteasome using a previously published procedure after 15-hour treatments with dimedone. Dimedone treatment alone did not affect activity of the proteasome (Online Figure XIB). These results indicate that oxidant and dimedone treatments do not alter proteasomal activity. Taken together, these findings support the hypothesis that sulfenic acid diverts Kv1.5 from a recycling to a degradation pathway (Figure 8D).

Discussion

Numerous reports suggest that Kv1.5, which comprises an important 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 downregulation of channel protein expression, with no change in channel transcription. Despite these observations, the molecular mechanism(s) underlying this pathological remodeling have remained elusive. In the present study, we show that Kv1.5 is modified with sulfenic acid in the heart and that this increases under oxidative stress. We further show that this modification occurs on a single, COOH-terminal cysteine and that it regulates $I_{Kur}$ currents in native myocytes. To our knowledge, this is the first report demonstrating sulfenic acid modification to an ion channel. We demonstrate that peroxide-induced stress leads to increased internalization and impaired recycling of the channel in cardiac myocytes and that oxidation of COOH-terminal C581 is sufficient to mediate these effects. Importantly, sulfenic acid modification ultimately leads to channel degradation. These results therefore provide a molecular mechanism by which oxidative stress can lead to Kv1.5 degradation in cardiac myocytes via oxidation of the channel itself.

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 nonenzymatic mechanism, it is not a random occurrence. Recent functional site profiling has revealed that sulfenic acid–modified proteins are flanked by polar amino acid residues capable of forming hydrogen bonds. It is interesting that the modified cysteine C581 (Figure 2A, dark gray) 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. The conservation of this cysteine may provide insight into the unique role for Kv1.5 in oxygen sensing in the heart and probably the pulmonary vasculature. Indeed, our results show that oxidation of this cysteine to sulfenic acid enables cells to translate acute changes in redox state into altered cellular excitability.

A link between oxidative stress and downregulation of Kv channels is currently the subject of intense research.\(^4,9,32\) 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\(^33\) and pharmacological agents.\(^25\) Importantly, our work provides the first evidence that oxidative stress, via sulfenic acid modification to the channel itself, can acutely regulate the level of channel at the cell surface.

In accordance with our surface labeling experiments, we find a similar increase in channel internalization after treatment with tBOOH or dimedone. The finding that dimedone treatment alone causes internalization suggests that there is a basal level of sulfenic acid–modified channel in cardiac myocytes. This is supported by data in Figures 2 to 4 and Online Figure IV, showing a basal level of sulfenic acid–modified channel in cells and myocytes that have not been challenged with oxidant. Accordingly, the increase in internalization is accompanied by impairment in channel recyling (Figure 6B), indicating that sulfenic acid modification diverts channel away from the normal recycling pathway. These results are in agreement with other studies showing (Figure 5). We observe similar increases in sulfenic acid modification (Figure 3) and decreased Kv1.5 surface expression (Online Figure VIII) with multiple oxidants, demonstrating that the effects are not specific to one particular oxidant and probably represent a general cellular mechanism for redox regulation of channel expression and trafficking. Our whole-cell voltage-clamping results provide confirmation of our immunofluorescence data via a second, parallel method. Importantly, we localized these functional effects by using both approaches to a single COOH-terminal cysteine (Figure 5).
that oxidative stress can interfere with trafficking of other membrane proteins, such as the transferrin receptor, or alter the molecular machinery involved in this trafficking process. As the molecular mechanisms governing Kv1.5 endocytosis are elucidated, this may lead to further insight into how sulfenic acid triggers internalization.

Our results show that human atrial fibrillation is accompanied by a global increase in sulfenic acid–modified protein in the atrium and that endogenous Kv1.5 is modified with sulfenic acid. The findings that Kv1.5 is a substrate for sulfenic acid modification and that chronic oxidative stress results in channel degradation suggest that this is at least 1 mechanism linking ion channel remodeling to persistent atrial fibrillation. The loss of channel protein associated with atrial fibrillation precludes the direct measurement of sulfenic acid–modified channel in the pathophysiological state. However, our results are consistent with reports showing that chronic heart failure is a substrate for atrial fibrillation, resulting in the oxidative stress–induced loss of Kv1.5 channel protein. Importantly, it was shown that this ion channel remodeling was sensitive to the antioxidant NAC, indicating that redox-sensitive remodeling in the atria may be prevented or reversed. Targeting sulfenic acid modification to ion channel proteins may provide a novel therapeutic approach to treat persistent atrial fibrillation.

In summary, we demonstrate that human chronic atrial fibrillation is associated with a global increase in sulfenic acid. We further show 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. Redox modulation of Kv1.5 current is associated with numerous pathophysiological states that involve loss of channel protein, including atrial fibrillation and pulmonary hypertension. We show, for the first time, that oxidative stress acutely regulates Kv1.5 surface expression. This is the first report of sulfenic acid modification to an ion channel, which provides a potential molecular explanation for the pathological remodeling that occurs in cardiovascular diseases.

Although 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 β-subunits, cannot fully account for channel redox sensitivity. 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.

Acknowledgments

We would like to thank Dr William Pratt for his advice and critical review of the manuscript. We thank Dr Yoichi Osawa for providing the anti-HSP70 antibody and Dr Stephen Ragsdale for providing diamide.

Sources of Funding

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Disclosures

None.

References


Novelty and Significance

**What Is Known?**

- Multiple potassium channels, which play an essential role in maintaining normal cardiac rhythm and blood vessel contractility, are regulated by oxidative stress.
- Modification of cysteine residues of proteins to sulfenic acids alters the expression and function of many proteins under oxidative stress.
- Diseases such as chronic atrial fibrillation and hypoxic pulmonary hypertension (HPH) are characterized by both oxidative stress and reduced expression of the oxygen-sensitive, voltage-gated potassium (Kv) channel Kv1.5.

**What New Information Does This Article Contribute?**

- Chronic atrial fibrillation in human patients is accompanied by a global increase in sulfenic acid–modified proteins in the heart.
- Sulfenic acid containing Kv1.5 protein is present in normal heart, and the level of this modification increases under oxidative stress.
- Sulfenic acid formation in Kv1.5 reduces the level of the channel protein on the cell surface, disrupts its normal trafficking, and promotes its degradation in cardiac myocytes.

Oxidative stress, characterized by excessive formation of reactive oxygen species or insufficient antioxidant capacity, is a common denominator in a vast array of cardiovascular diseases. Another common hallmark of cardiovascular disease is a pathological change in the expression level of ion channel proteins, leading to impaired vessel contractility and cardiac rhythm. Chronic atrial fibrillation and HPH are characterized by both oxidative stress and a reduction in the level of Kv1.5 channel protein, although the molecular mechanism(s) linking these observations is unclear. In the present study, we demonstrate a global increase in the level of sulfenic acid on proteins in the atria of human patients with chronic atrial fibrillation and that oxidative stress results in formation of sulfenic acid in Kv1.5 in the rat heart. This modification interrupts the normal, dynamic trafficking of the channel and promotes its degradation. This is the first report demonstrating sulfenic acid modification of an ion channel, and it provides a molecular mechanism linking oxidative stress to reduced channel expression levels observed in atrial fibrillation and HPH. Targeting sulfenic acid modification of ion channel proteins may provide a novel therapeutic approach for treating cardiovascular diseases.
Redox-Sensitive Sulfenic Acid Modification Regulates Surface Expression of the Cardiovascular Voltage-Gated Potassium Channel Kv1.5

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SUPPLEMENTAL MATERIAL

DETAILED METHODS:

Materials
Anti-Kv1.5 antibody (1st extracellular loop, #APC-150) was purchased from Alomone Labs (Jerusalem, Israel). Mouse anti-V5 antibody (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 (tBOOH), diamide, hydrogen peroxide (H2O2) 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 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 NH2-and COOH-terminal cysteines were mutated to serine, and Kv1.5-4CS, in which the COOH terminal cysteines were mutated to serine. A COOH-terminal point mutant construct was mutating cysteine 581 to serine: Kv1.5S581S. 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 Labeling of Sulfenic Acid, Staudinger Ligation, and Immunoprecipitation of Kv1.5

For labeling of human and rodent tissue homogenates with DAz, tissue was homogenized in non-denaturing lysis buffer, pH 7.5, containing 150 mM NaCl, 50 mM Tris-Cl, 5 mM EDTA, 1% Triton X-100, 1 mM DAz, and protease inhibitors (Roche). The tissue was first cut into small pieces with a razor blade, followed by mechanical homogenization and passage through a 16-gauge needle on ice. Homogenates then underwent shaking at 265 RPM at 37°C for 2 h, followed by a 10 minute spin at 10,000 g and 4°C. 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 2 mg/mL) was incubated in 100µM p-biotin and 5 mM DTT for 2 hours, nutating at 37°C. Samples were then resuspended in SDS sample buffer and frozen at -20°C prior to streptavidin western blotting. For labeling sulfenic acid-modified Kv1.5 in cell lysates, 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, pH 7.5 with protease inhibitors (Roche), followed by gentle rocking at 4°C for 20 min. Lysates were then dounce homogenized, followed by centrifugation at 16,000 g for 4 min at 4°C to remove cell debris. Supernatant was then incubated at 37°C for 2-2.5h with gentle rocking to allow labeling of sulfenic acid modified proteins with DAz². Protein (250 µg) was immunoprecipitated overnight at 4°C 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°C with gentle rocking, prior to electrophoresis and streptavidin-HRP Western blotting.

Perfusion of Isolated Rat Heart and Immunoprecipitation of Kv1.5

Rat hearts were excised and Langendorff perfused with Krebs-Henseleit buffer (KHB) with or without 200µM diamide for 55 min., followed by isolation of membranes as previously described³. Briefly, 200-300 mg tissue was minced with a razor blade and homogenized in buffer containing 50 mM tris, 10 mM EGTA, 5 mM DAz, and protease inhibitors (pH 7.4). Homogenate was then centrifuged at 4°C for 10 min. at 2000 RPM. Supernatant was collected and membranes were isolated via ultracentrifugation at 45000g for 90 min. at 4°C. The supernatant was then discarded and the pellet resuspended in homogenization buffer (see above) + 1% NP-40, followed by incubation at 37°C for 2 hours to allow DAz to bind sulfenic acid-modified proteins. Kv1.5 immunoprecipitation was then performed overnight using 1.5 µL anti-Kv1.5 antibody (Alomone Labs APC-150), 75 µL protein A-sepharose beads, and 1 mg total protein. Staudinger ligation and electrophoresis were performed the next day as outlined above.

N-Glycosidase Treatment of Kv1.5

Vehicle-treated cells were lysed in the presence of DAz, 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.

Immunocytochemistry and Confocal Imaging

For surface, internalization and recycling assays, immunocytochemistry was performed as described previously⁴, 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 minutes on ice. Following this incubation, the cells were administered treatments at 37°C. 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°C, 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°C 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°C. 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°C, 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°C. After treatment, cells were fixed with 4% paraformaldehyde, permeabllized 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\(^5\), with minor modifications. HL-1 cells transiently expressing WT or cysteine mutant Kv1.5-GFP 48h post-transfection were administered treatments at 37°C, trypsinized and allowed to settle in the recording chamber. The bath solution contained (in mmol/L): NaCl 110, KCl 4, MgCl\(_2\) 1.8, CaCl\(_2\) 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, CaCl\(_2\) 1, MgCl\(_2\) 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. For IKur experiments, rat cardiac myocytes were isolated and recordings were conducted following treatment with dimedone, diamide or vehicle. Ca\(^{2+}\) current was inhibited by the addition of 200uM CdCl\(_2\) to our bath solution mM (110 NaCl, 4 KCl, 1 MgCl\(_2\)•H\(_2\)O, 1.8 CaCl\(_2\), 10 HEPES, 1.8 glucose, pH 7.35). Pipette solution consisted of mM (20 KCl, 8 NaCl\(_2\), 1 MgCl\(_2\)•H\(_2\)O, 1 CaCl\(_2\), 110 L-Aspartic acid potassium salt, 10 HEPES, 10 K\(_2\)BAPTA, 4 K\(_2\)ATP, pH to 7.2). \(I_{K_{slow}}\) \((I_{Kur} + I_{ss})\) was obtained using a 100ms pre-pulse to -40mV from a holding potential of -80mV, to inhibit Na\(^{+}\) current and current mediated by Kv4 channels \((I_{to})\), followed by a 500ms pulse to +30mV. \(I_{ss}\), current mediated by Kv2.1 and hERG, was obtained using the same protocol as above with the addition of 50uM 4-Aminopyridine (4-AP), to inhibit \(I_{Kur}\). Current specific to Kv1.5 \((I_{Kur})\) was then obtained by determining the difference between \(I_{K_{slow}}\) and \(I_{ss}\).

Proteasome Activity Assay
An optimized method was used for determining heart tissue chymotrypsin-like activity\(^6\). 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 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.
Online Figure I: Labeling sulfenic acid-modified proteins with DAz. A, Strategy for detecting sulfenic acid-modified Kv1.5 using the novel chemical probe, DAz. Cells are lysed in the presence of DAz, which covalently binds to sulfenic acid-modified proteins. DAz labeled proteins are then conjugated to a secondary, biotinylated agent, enabling detection of the sulfenic acid modification via streptavidin western blot. B, HRP-streptavidin western blot depicting DAz-labeled sulfenic acid in whole tissue lysate from mouse heart treated with tBOOH or vehicle.
Online Figure II: Human atrial fibrillation is accompanied by a global increase in sulfenic acid-modified proteins. Representative HRP-streptavidin western blot depicting global sulfenic acid-modified proteins in a human patient with chronic atrial fibrillation, compared to an unmatched, healthy donor.
Online Figure III: DAz recognizes sulfenic acid-modified Kv1.5. A, LTK cells stably expressing Kv1.5-WT were labeled with DAz. Samples were then treated overnight at 37°C with reaction buffer (control, lane 1) or PNGase (lane 2) to deglycosylate Kv1.5, which abolished the doublet, leaving a single, non-glycosylated band. The blot was then stripped and re-probed with anti-V5 antibody to verify efficient immunoprecipitation. B, LTK cells stably expressing Kv1.5-WT were labeled with DAz, alone or concurrently with dimedone, the parent compound of DAz. Dimedone blocks DAz labeling of sulfenic acid-modified Kv1.5.
Online Figure IV: Sulfenic acid modification of Kv1.5 is redox-sensitive in cardiac myocytes. A, HL-1 cells stably expressing Kv1.5-WT were treated for 60 min. with tBOOH, followed by labeling with DAz. Results from three separate experiments were quantified via densitometry analyzed via unpaired t-test. *indicates p<.05 relative to control. B, HL-1 cells stably expressing Kv1.5-WT were treated for 60 min. with diamide, followed by labeling with DAz. Results were quantified via densitometry and analyzed via unpaired t-test. *indicates p<.05 relative to control.
Online Figure V: Diamide treatment produces a global increase in sulfenic acid-modified proteins in freshly isolated rat heart.  

A, Western blot depicting lysates from HEK cells transfected with Kv1.5-WT or Kv1.5-GFP and probed for Kv1.5 using a commercially available antibody (Alomone Labs).  

B, Representative HRP-streptavidin western blot depicting sulfenic acid-modified proteins in isolated rat hearts perfused with normal KHB solution, or KHB solution containing diamide.
Online Figure VI: IKur, the Kv1.5-encoded current, is redox-sensitive. Rat cardiac myocytes were isolated steriley, 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 $p<0.01$. 
Online Figure VII: 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 tBOOH or vehicle 60 min., followed by immunoprecipitation and labeling with DAz and HRP-streptavidin western blot. C, Cells expressing Kv1.5-GFP were treated with tBOOH 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. n=60+ cells per condition, three experiments. *** indicates p<.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>.05).
Online Figure VIII: 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 H2O2 or vehicle (A) for 60 min. or diamide or vehicle (B) for 30 min., 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. n=60+ cells per condition, three experiments. ** indicates p<.01, *** indicates p<.001 relative to vehicle. GFP levels are not significantly different (p>.05) data not shown. Scale bars: 30 microns.
Online Figure IX: Short-term 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-GFP. Cells were treated with vehicle or tBOOH 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. tBOOH treatment in the continued presence of dimedone.  

B, Data from three separate experiments in (A) were quantified via densitometry. V5 band (Kv1.5-GFP) 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>.05, non-significant.  

C-G, 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, or unpaired t-test (G), p>.05 for all samples.
Online Figure X: Internalization and recycling assays. Diagram of live-cell internalization (A) or recycling (B) assays in HL-1 atrial myocytes. C, HL-1 cells transiently expressing Kv1.5-GFP were live cell stained and treated with dimedone or vehicle as outlined in Detailed Methods. Zero minutes recycling is the time point after dimedone or vehicle treatment, but prior to returning cells to 37°C to allow recycling of internalized Kv1.5. At this point, as expected, there is no recycled Kv1.5 (middle panel).
Online Figure XI: Oxidant and dimedone treatments do not interfere with function of the proteasome.  

A, HL-1 cells were treated with vehicle, tBOOH, dimedone, MG132, or MG132 + dimedone. After 15 hrs, cell lysates were generated and analyzed via western blotting with monoclonal anti-ubiquitin antibody. Blot was stripped and re-probed with alpha-tubulin antibody as a loading control. tBOOH 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. 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>.05
SUPPLEMENTAL TABLE

Online Table I: Oxidant treatment does not significantly alter the biophysical properties of Kv1.5. Currents were recorded from HL-1 atrial myocytes following vehicle or tBOOH treatment, using the patch clamp technique as described in the detailed methods.

<table>
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<th>$V_{50}$ Activation (+60mV)</th>
<th>$V_{50}$ Inactivation (+60mV)</th>
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<tr>
<td>Kv1.5-GFP</td>
<td>428.52 +/- 187.5</td>
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