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 (*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[1] 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. A COOH-terminal point mutant construct was mutating cysteine 581 to serine: Kv1.5C581S. 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 DAz[2]. 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 described[3]. 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 previously[4], 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°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, 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[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, 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. For IKur experiments, rat cardiac myocytes were isolated and recordings were conducted following treatment with dimedone, diamide or vehicle. Ca^+ current was inhibited by the addition of 200uM CdCl to our bath solution mM (110 NaCl, 4 KCl, 1 $MgCl_2.H_2O$, 1.8 $CaCl_2$, 10 HEPES, 1.8 glucose, pH 7.35). Pipette solution consisted of mM (20 KCl, 8 $NaCl_2$, 1 $MgCl_2.H_2O$, 1 $CaCl_2$, 110 L-Aspartic acid potassium salt, 10 HEPES, 10 K_4BAPTA, 4 K_2ATP, pH to 7.2). I_{Kslow} (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. Iss, 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_{Kslow} and I_{ss}.

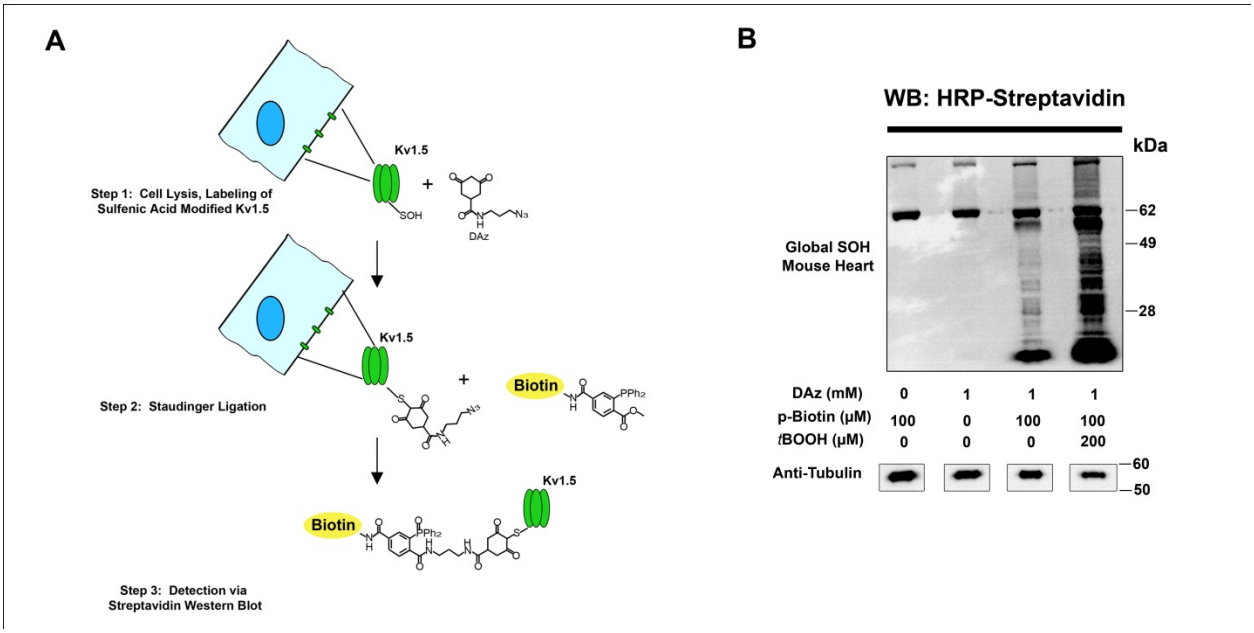
Proteasome Activity Assay

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.

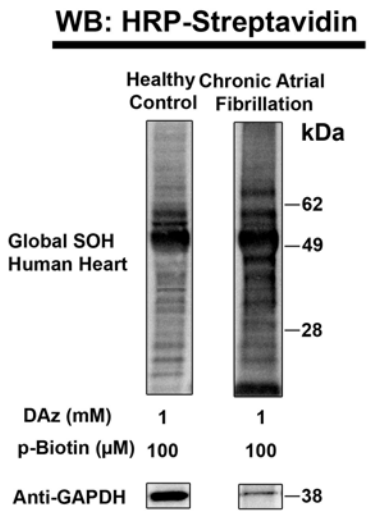
Statistics

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.

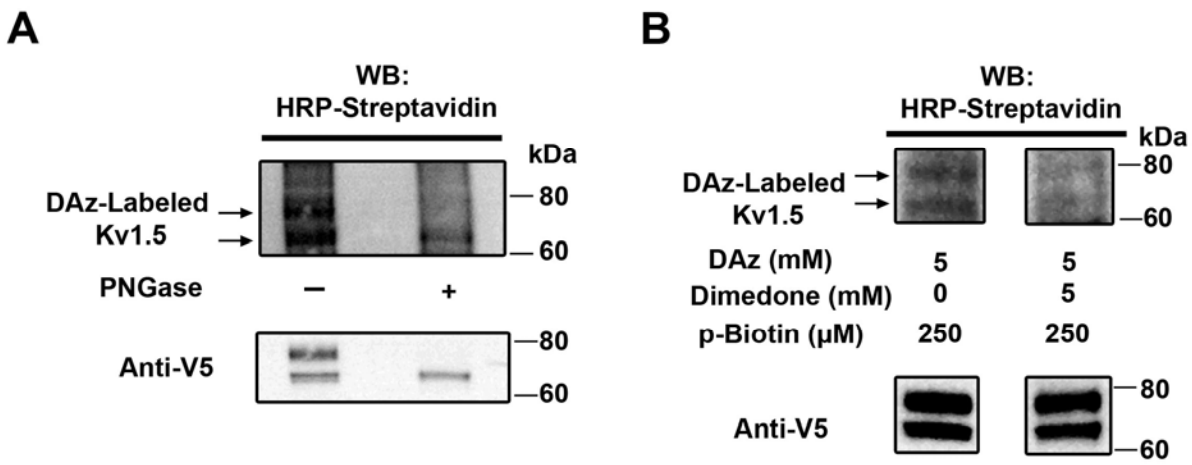
SUPPLEMENTAL FIGURES



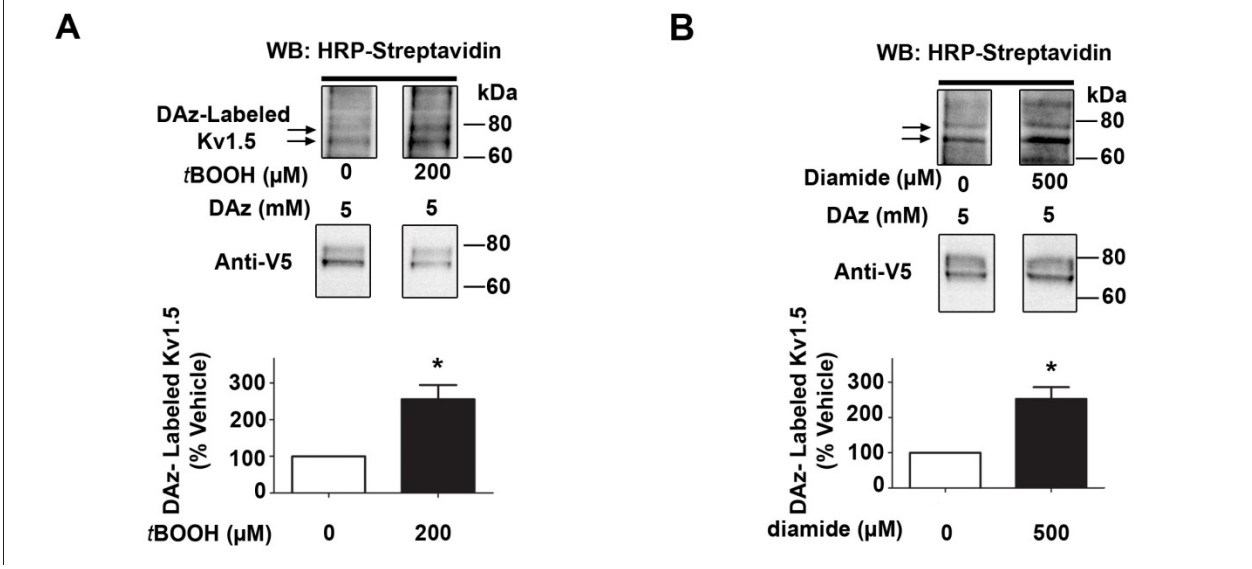
Online Figure I: Labeling sulfenic acid-modified proteins with DAz. A, Strategy for detecting sulfenic acid-modified Kv1.5 using the novel chemical probe, DAz. Cells are lysed in the presence of DAz, which covalently binds to sulfenic acid-modified proteins. DAz labeled proteins are then conjugated to 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 *t*BOOH or vehicle.



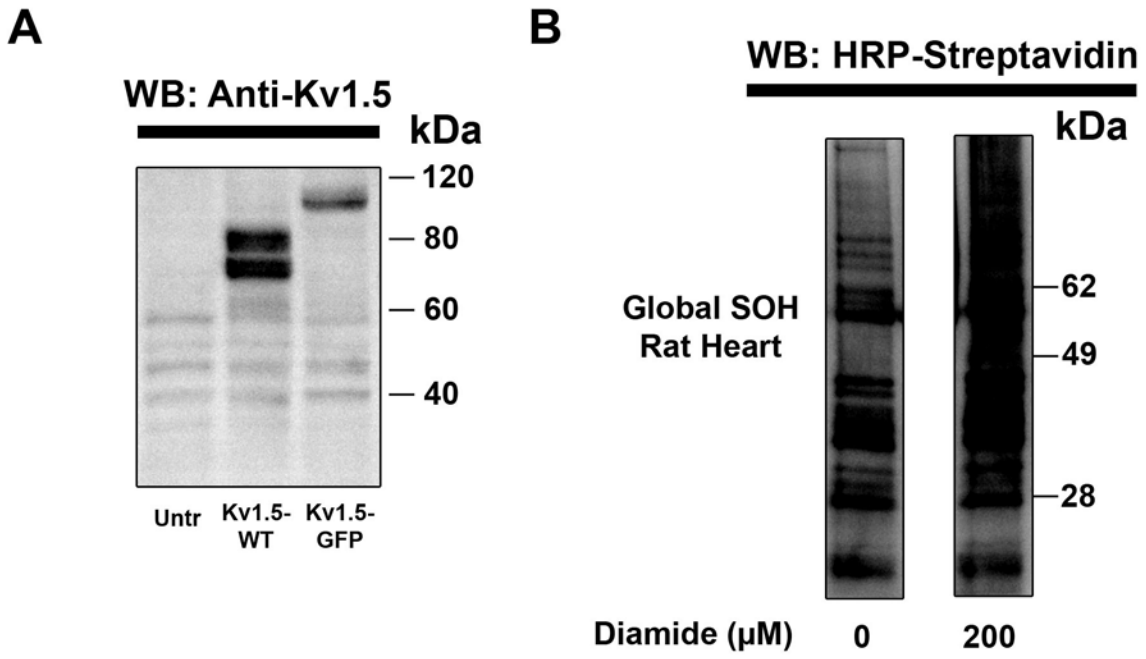
Online Figure II: Human atrial fibrillation is accompanied by a global increase in sulfenic acid-modified proteins. Representative HRP-streptavidin western blot depicting global sulfenic acid-modified proteins in a human patient with chronic atrial fibrillation, compared to an unmatched, healthy donor.



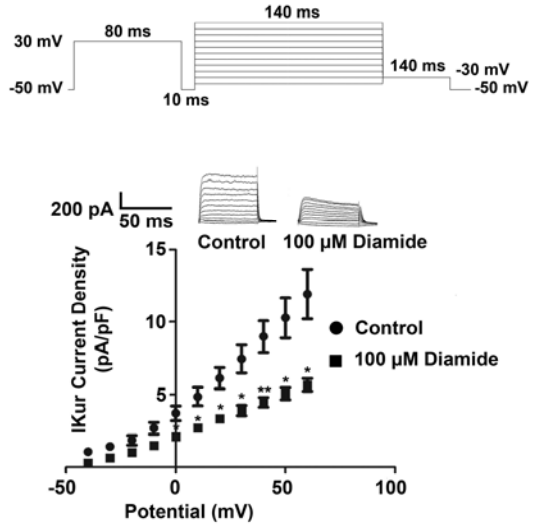
Online Figure III: DAz recognizes sulfenic acid-modified Kv1.5. **A**, LTK cells stably expressing Kv1.5-WT were labeled with DAz. Samples were then treated overnight at 37°C with reaction buffer (control, lane 1) or PNGase (lane 2) to deglycosylate Kv1.5, which abolished the doublet, leaving a single, 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.



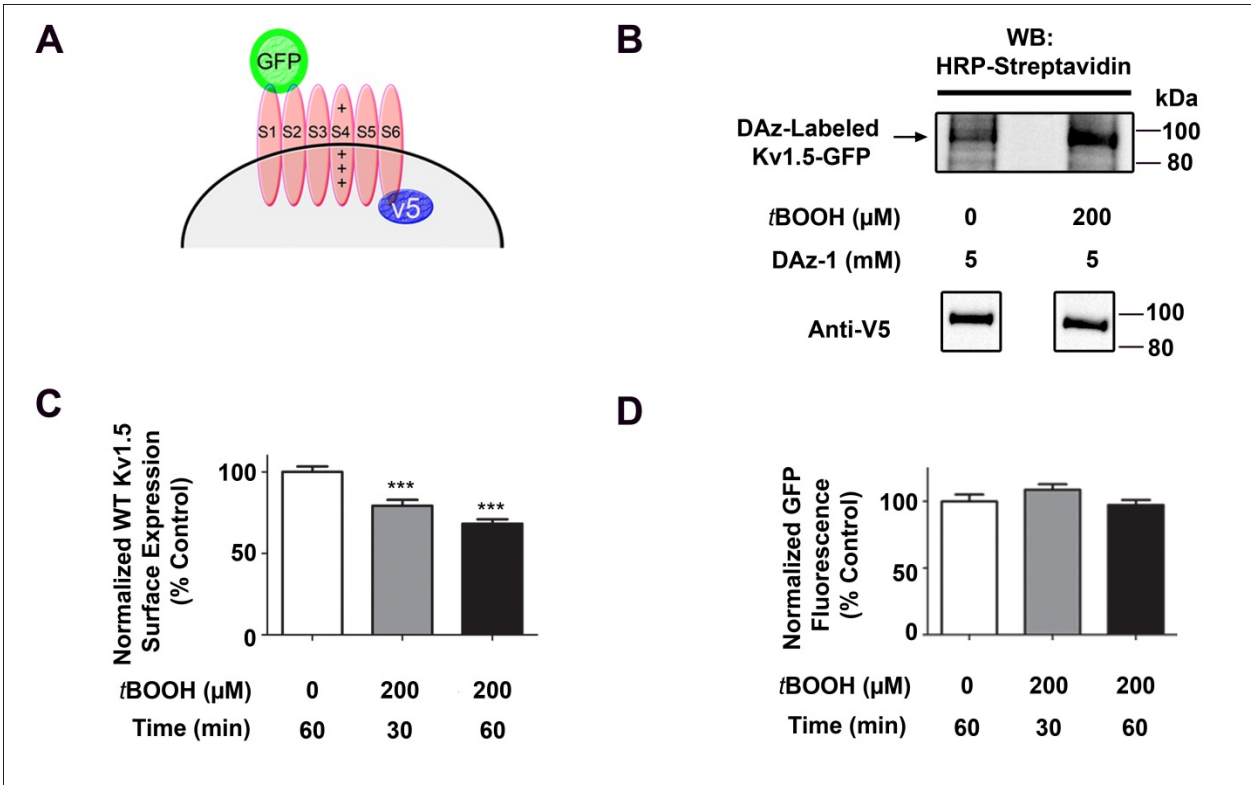
Online Figure IV: Sulfenic acid modification of Kv1.5 is redox-sensitive in cardiac myocytes. A, HL-1 cells stably expressing Kv1.5-WT were treated for 60 min. with *t*BOOH, followed by labeling with DAz. Results from three separate experiments were quantified via densitometry analyzed via unpaired t-test. *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.



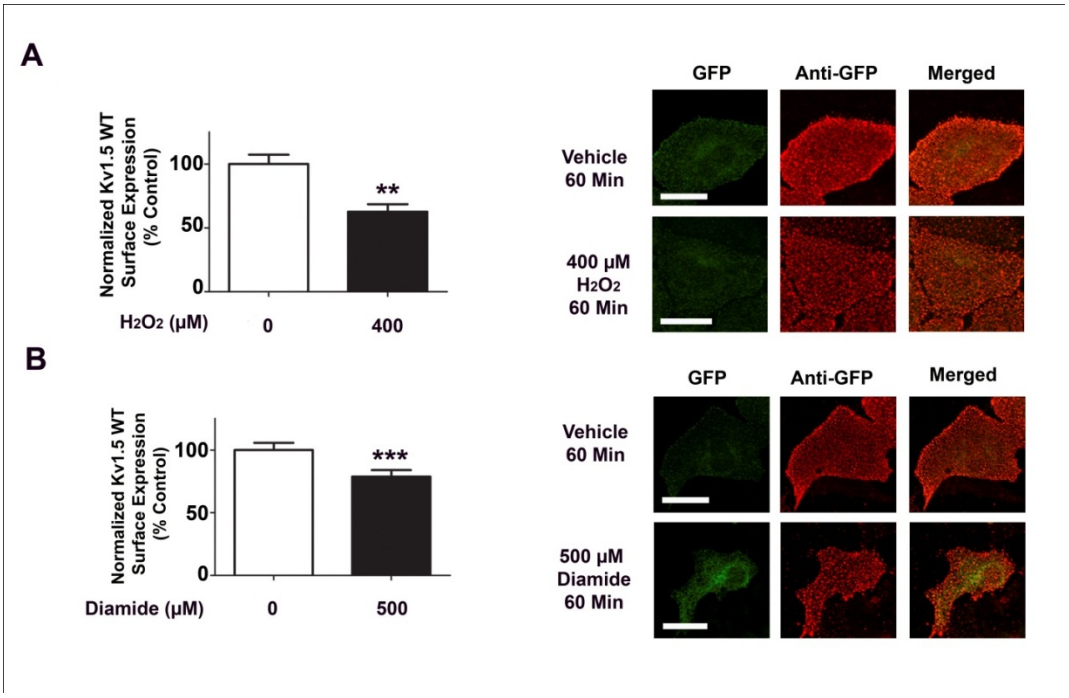
Online Figure V: Diamide treatment produces a global increase in sulfenic acid-modified proteins in freshly isolated rat heart. **A**, Western blot depicting lysates from HEK cells transfected with Kv1.5-WT or Kv1.5-GFP and probed for Kv1.5 using a commercially available antibody (Alomone Labs). **B**, Representative HRP-streptavidin western blot depicting sulfenic acid-modified proteins in isolated rat hearts perfused with normal KHB solution, or KHB solution containing diamide.



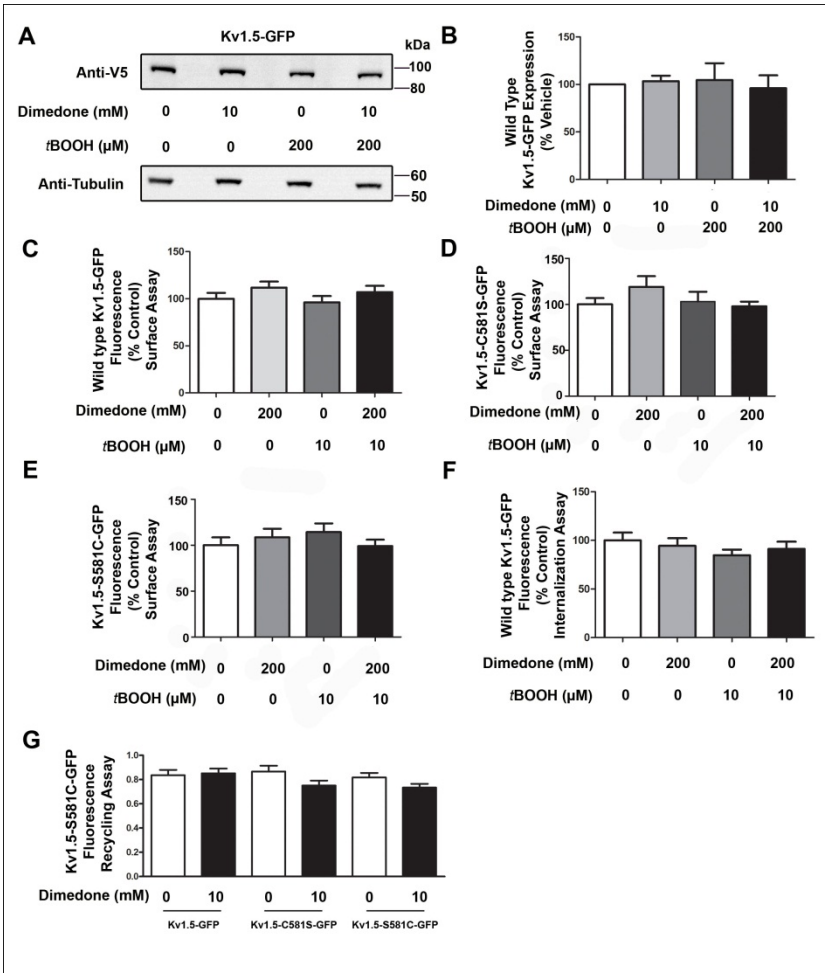
Online Figure VI: IKur, the Kv1.5-encoded current, is redox-sensitive. Rat cardiac myocytes were isolated sterilely, cultured, and used within 24 hrs. IKur currents were recorded following 10 min. treatment with M199+ medium alone or with diamide (n=4 cells per treatment). Data were analyzed using unpaired t-test * denotes $p<0.05$, ** denotes $p<0.01$.



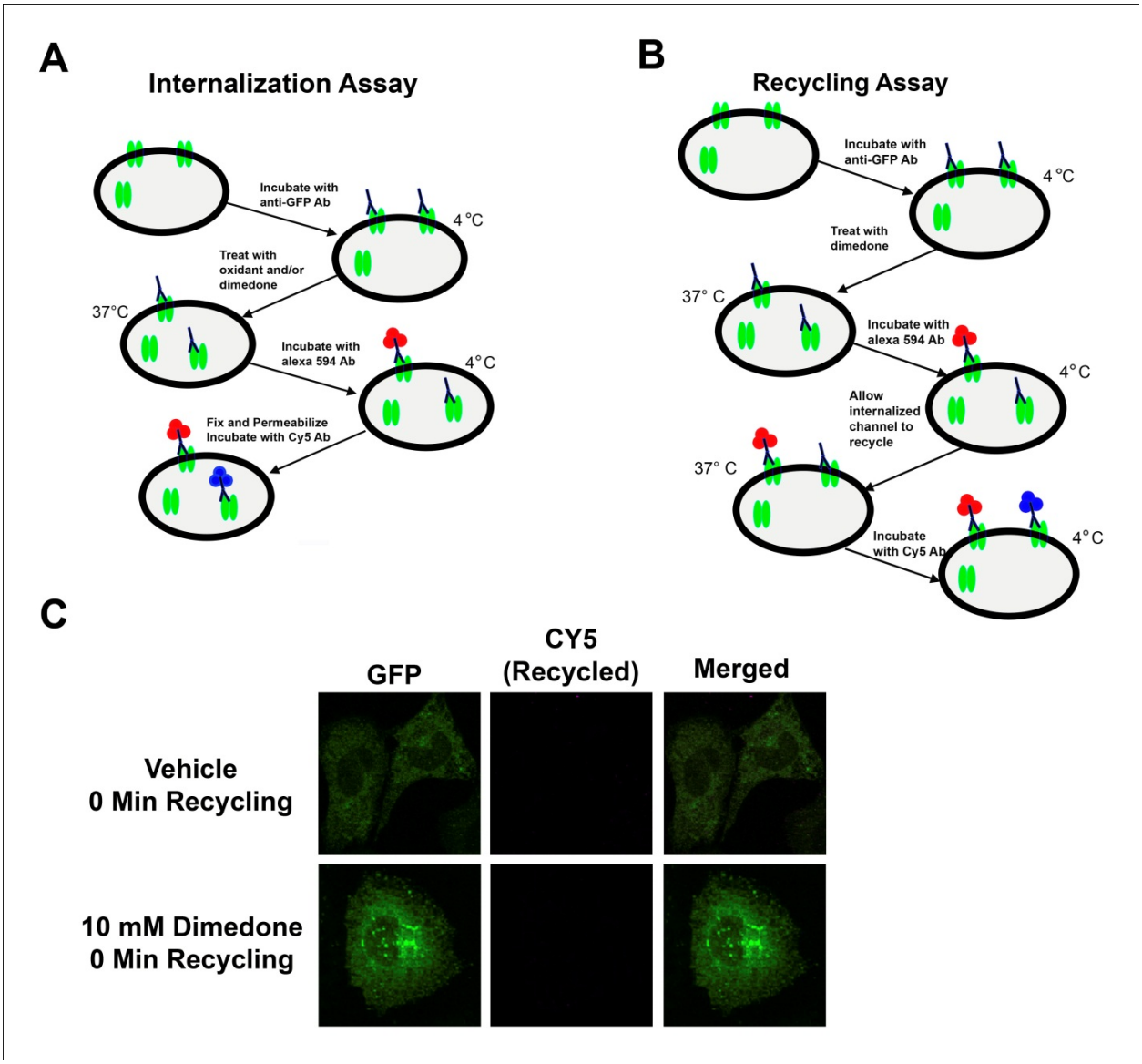
Online Figure VII: Oxidant treatment causes a significant, time-dependent reduction in surface levels of Kv1.5 in HL-1 atrial myocytes.
A, Cartoon illustrating the Kv1.5-GFP construct. **B**, HL-1 cells stably expressing wild type Kv1.5-GFP were treated with *t*BOOH or vehicle 60 min., followed by immunoprecipitation and labeling with DAz and HRP-streptavidin western blot. **C**, Cells expressing Kv1.5-GFP were treated with *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. 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).



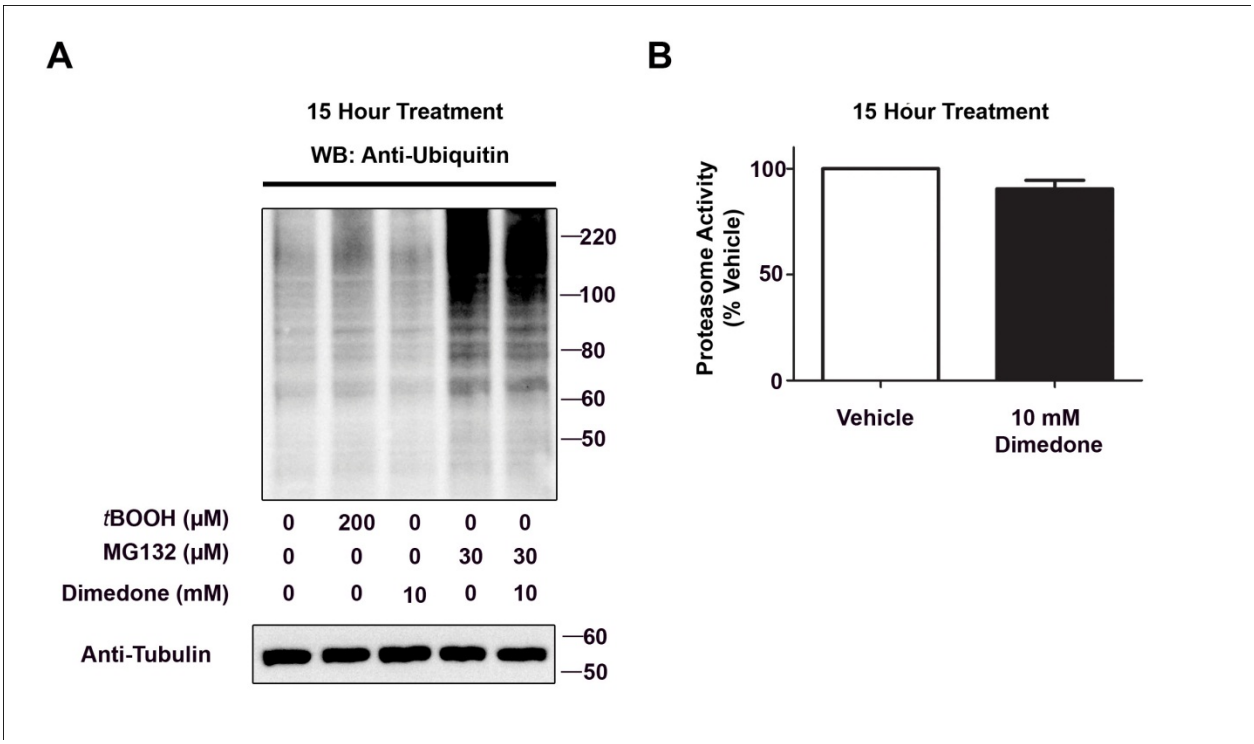
Online Figure VIII: The reduction in Kv1.5 surface expression in HL-1 atrial myocytes is not oxidant-specific. A-B, HL-1 cells transiently expressing Kv1.5-GFP were treated with H2O2 or vehicle (A) for 60 min. or diamide or vehicle (B) for 30 min., followed by labeling of surface Kv1.5 (red). Surface 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 tovehicle. 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 *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-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, *t*BOOH, 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. *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. 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-1atrial myocytes following vehicle or *t*BOOH treatment, using the patch clamp technique as described in the detailed methods.

	Current Density (+60mV)	**V_{50} Activation (+60mV)**	**V_{50} Inactivation (+60mV)**
Kv1.5-GFP	428.52 +/- 187.5	- 9.49 +/ - 1.2	-11.71 +/ - 1.6
Kv1.5-GFP + 200uM tBOOH	171.87 +/- 47.1	-10.23 +/ - 0.8	-13.38 +/ - 2.3

1. Martens, J R, Sakamoto, N, Sullivan, S A, Grobaski, T D, and Tamkun, M M. Isoform-specific localization of voltage-gated K+ channels to distinct lipid raft populations. Targeting of Kv1.5 to caveolae*. J Biol Chem.* 2001;276:8409-14.
2. Reddie, K G, Seo, Y H, Muse Iii, W B, Leonard, S E, and Carroll, K S. A chemical approach for detecting sulfenic acid-modified proteins in living cells*. Mol Biosyst.* 2008;4:521-31.
3. Brackenbury, W J, Davis, T H, Chen, C, Slat, E A, Detrow, M J, Dickendesher, T L, Ranscht, B, and Isom, L L. Voltage-gated Na+ channel beta1 subunit-mediated neurite outgrowth requires Fyn kinase and contributes to postnatal CNS development in vivo*. J Neurosci.* 2008;28:3246-56.
4. McEwen, D P, Schumacher, S M, Li, Q, Benson, M D, Iniguez-Lluhi, J A, Van Genderen, K M, and Martens, J R. Rab-GTPase-dependent endocytic recycling of Kv1.5 in atrial myocytes*. J Biol Chem.* 2007;282:29612-20.
5. Zhang, L, Foster, K, Li, Q, and Martens, J R. S-acylation regulates Kv1.5 channel surface expression*. Am J Physiol Cell Physiol.* 2007;293:C152-61.
6. Predmore, J M, Wang, P, Davis, F, Bartolone, S, Westfall, M V, Dyke, D B, Pagani, F, Powell, S R, and Day, S M. Ubiquitin proteasome dysfunction in human hypertrophic and dilated cardiomyopathies*. Circulation.* 121:997-1004.