Published in final edited form as:

J Mol Cell Cardiol. 2010 February ; 48(2): 352. doi:10.1016/j.yjmcc.2009.11.016.

Selective γ -ketoaldehyde scavengers protect Na_v1.5 from oxidant-induced inactivation

T Nakajima¹, SS Davies^{2,3}, E Matafonova^{2,3}, F Potet¹, V Amarnath⁴, KA Tallman⁵, RA Serwa⁵, NA Porter⁵, JR Balser^{1,2,3}, S Kupersmidt^{1,2}, and LJ Roberts III^{2,3}

¹Department of Anesthesiology, Vanderbilt University School of Medicine, Nashville, TN 37232

²Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232

³Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232

⁴Department of Pathology, Vanderbilt University School of Medicine, Nashville, TN 37232

⁵Department of Chemistry, Vanderbilt University School of Medicine, Nashville, TN 37232

Abstract

The cardiac sodium channel (SCN5A, Na_v1.5) is a key determinant of electrical impulse conduction in cardiac tissue. Acute myocardial infarction leads to diminished sodium channel availability, both because of decreased channel expression and because of greater inactivation of channels already present. Myocardial infarction leads to significant increases in reactive oxygen species and their downstream effectors including lipoxidation products. The effects of reactive oxygen species on Na_v1.5 function in whole hearts can be modeled in cultured myocytes, where oxidants shift the availability curve of I_{Na} to hyperpolarized potentials, decreasing cardiac sodium current at the normal activation threshold. We recently examined potential mediators of the oxidant-induced inactivation, and found that one specific lipoxidation product, the isoketals, recapitulated the effects of oxidant on sodium currents. Isoketals are highly reactive γ -ketoaldehydes formed by the peroxidation of arachidonic acid that covalently modify the lysine residues of proteins. We now confirm that exposure to oxidants induces lipoxidative modification of Na_v1.5 and that the selective isoketal scavengers block voltage dependent changes in sodium current by the oxidant tert-butylhydroperoxide, both in cells heterologously expressing Na_v1.5 and in a mouse cardiac myocyte cell line (HL-1). Thus, inhibition of this lipoxidative modification pathway is sufficient to protect the sodium channel from oxidant induced inactivation and suggests the potential use of isoketal scavengers as novel therapeutics to prevent arrhythmogenesis during myocardial infarction.

Keywords

ion channels; oxidative stress; reactive carbonyls; arrhythmia

© 2009 Elsevier Ltd. All rights reserved.

Corresponding author: Sabina Kupersmidt, Ph.D, Anesthesiology, Research Division, Vanderbilt University School of Medicine, P-445 MRB IV, 2213 Garland Ave., Nashville, TN 37232-0413, ph.: (615) 936-2586, fax: (615) 936-0456, sabina.kupersmidt@vanderbilt.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

The cardiac sodium (Na^+) current (I_{Na}), contributes to the initiation and morphology of cardiac action potentials as well as to the propagation of electrical impulses, and is thus a key factor for maintaining normal cardiac rhythm. Indeed, dysfunction of Na^+ channels in various pathophysiological conditions leads to multiple life-threatening arrhythmias. For example, genetic changes in *SCN5A*, which encodes the pore-forming subunit of the cardiac voltage-gated Na^+ channel ($\text{Na}_v1.5$), cause at least four distinct types of inherited arrhythmia disorders, including the long QT syndrome (LQTS), Brugada syndrome, atrioventricular conduction block, and familial sick sinus syndrome [1–4]. In ischemic myocardium, the normal function of I_{Na} is compromised. I_{Na} in surviving myocytes from the epicardial border zone of canine hearts at 5-days post infarct is reduced and considered to be a contributing factor to reentrant ventricular tachyarrhythmias [5,6]. Moreover, following an ischemic event, the modification of I_{Na} function combined with pharmacological blockade of I_{Na} enhances the likelihood of life-threatening arrhythmias. As documented in the CAST Study, class Ic anti-arrhythmic drugs (Na^+ channel blockers) increased the mortality of patients with recent myocardial infarction [7].

Myocardial infarction, with its concomitant ischemia and pressure overload, results in increased production of reactive oxygen species (ROS) [8,9]. In turn, ROS modify cellular lipids, proteins and nucleic acids. Ischemia/reperfusion induces ventricular tachyarrhythmias in isolated perfused hearts that can be blocked by cocktails of antioxidant enzymes and small molecular weight antioxidants [10,11]. In isolated myocytes, the oxidant tert-butylhydroperoxide (*t*-BHP) or photoinduced oxidizers dramatically reduce I_{Na} [12–14]. Oxidants induce a wide range of modifications to cellular lipids, nucleic acids, and protein, so that a large number of potential mechanisms could mediate the impact of ROS upon I_{Na} in ischemic hearts. One potential mediator of these effects are reactive lipoxidation products that modify the sodium channel, as addition of reactive lipoxidation products induce changes in I_{Na} [14,15]. However, whether reactive lipoxidation products alters I_{Na} directly via adduction to $\text{Na}_v1.5$ or indirectly by adduction to other targets is unknown; to date, there has been no direct evidence that $\text{Na}_v1.5$ is modified by lipoxidation products under oxidative conditions or that specifically blocking lipoxidative modification prevents the changes in I_{Na} .

One recent advance that facilitates the identification of lipid modified proteins in cultured cells has been the use of “click chemistry” to form a stable triazole linkage between a terminal alkyne on the lipid of interest and a biotin-linked azide that is added during the final stages of the experiments [16–22]. Modification of fatty acids with a terminal alkyne (alkynyl-fatty acids) does not significantly alter the physical properties of the fatty acid, so that alkynyl fatty acids are readily incorporated into membrane phospholipids and serve as substrate for enzymes in a manner virtually identical to unmodified fatty acids [23]. Therefore, peroxidation of alkynyl polyunsaturated fatty acids in cellular membranes likely forms reactive lipid aldehydes that retain the terminal alkyne (Fig. 1A, line 3). Proteins that are adducted by these lipid aldehydes can then be captured by ex vivo reaction of this alkyne with biotin-azide followed by streptavidin-based affinity purification [16] (Fig. 1A, last line).

Isoketals (IsoK) appear to be the most reactive lipoxidation products generated during myocardial infarction [14], making them the most likely candidates to mediate the effects of oxidants on I_{Na} . IsoKs are a series of γ -ketoaldehyde regioisomers generated by the peroxidation of arachidonic acid, an abundant polyunsaturated fatty acid found esterified in membrane phospholipids [24,25]. IsoKs form in situ on phospholipids, where they can then react with the lysine residues of proteins to form stable adducts and to modify the function of membrane proteins [26]. The rate of adduction to protein by IsoKs greatly exceeds that of other well-studied reactive lipoxidation including 4-hydroxynonenal [24]. Treatment of HEK293

cells heterologously expressing Na_v1.5 (HEK- Na_v1.5) with *t*-BHP not only induces voltage-dependent decreases in I_{Na}, but also significantly increases the levels of IsoK protein adducts [14]. Importantly, the levels of IsoK protein adducts measured under these conditions were similar to levels of IsoK protein adducts found in the epicardial border zone in experimentally induced myocardial infarction. Exogenous addition of physiologically relevant IsoK concentrations to both HEK- Na_v1.5 and HL-1 myocytes altered I_{Na} in a manner that recapitulated the effect of *t*-BHP. Interestingly, none of the other well-established lipoxidation products that we tested, including 4-hydroxynonenal, induced these same effects [14]. These findings strongly suggest that alterations in I_{Na} induced by oxidative stress in ischemic myocardium may be predominantly mediated by IsoKs.

To determine the specific contribution of IsoKs to oxidant-induced damage, we recently described the development of selective γ -ketoaldehyde scavengers. IsoKs react with pyridoxamine and lipophilic analogs of pyridoxamine including salicylamine (SA) and pentyl pyridoxamine (PPM) approximately 2000-fold faster than with lysine [27,28]. Therefore, in the presence of these scavengers, IsoK fails to adduct to protein or to inactivate enzymatic activity [28]. Although pyridoxamine can also react with α -ketoaldehydes generated by lipoxidation such as methylglyoxal [29], this reaction is much slower [27], so that the principal product scavenged during peroxidation of arachidonic acid is IsoK [28]. Two lipophilic pyridoxamine analogs, SA and PPM, provide better protection against oxidant-induced cytotoxicity than pyridoxamine itself, with SA being the most cytoprotective. The extent of cytoprotection correlates well with lipophilicity, consistent with the notion that the scavenger acts most effectively in the phospholipid bilayer where IsoK are formed.

In this study, we explored the role of IsoKs in the inactivation of I_{Na} in conditions simulating oxidative injury. We find that exposure of HEK- Na_v1.5 cells to *t*-BHP leads to lipoxidative modification of Na_v1.5. We also demonstrated that pretreatment with IsoK scavenger prevents the changes in sodium channel availability induced by *t*-BHP, both in Na_v1.5 transfected cells and in an immortalized adult mouse atrial myocytes (HL-1 cells) [30]. Thus, our current findings indicate that Na_v1.5 is directly modified by IsoK adducts in oxidative conditions, leading to its voltage-dependent inactivation, and further suggest that the potential of IsoK scavengers as a novel therapeutic method to prevent arrhythmogenesis during ischemic events.

2. Materials and Method

2.1 Reagents

Alkynyl-arachidonic acid (al-AA) and the biotin azide were synthesized as described in [23] and [16], respectively. The TBTA ligand was synthesized as described in [31]. TCEP hydrochloride salt was purchased commercially (Pierce).

15-E₂-isoketal (IsoK) was prepared from dimethoxyacetal precursor in methylene chloride as previously described [32] and a stock of 10 mM IsoK prepared in DMSO by evaporation of methylene chloride under nitrogen. Salicylamine acetate salt was prepared as previously described [28].

2.2 Detection of lipoxidative modification of Na_v1.5

Characterization of lipid modification of Na_v1.5 was performed as outlined in Fig. 1A. HEK293 cells stably expressing Na_v1.5-GFP fusion protein (HEK- Na_v1.5-GFP) [33] were a kind gift from Dr. Katherine T. Murray (Vanderbilt University School of Medicine). HEK- Na_v1.5-GFP were seeded in 10 cm plates at 2.0×10^6 cells/plate in DMEM supplemented with 10 % fetal bovine serum, 1 % antibiotic/antimycotic mixture, and 500 μ g/ml G418 in a 5 % CO₂ incubator at 37°C overnight. Cells were washed twice with 5 mls Dubecco Phosphate

Buffered Saline (DPBS) and incubated with 2 μ l vehicle (DMSO) or 100 mM alk-AA in 10 mls DMEM (without supplements) overnight. The cells were then scraped, washed twice with DPBS and resuspended in DPBS. Cells preincubated with al-AA or with vehicle were then treated with vehicle, iron reagent (20 μ M FeCl₃, 20 μ M adenosine diphosphate, 10 μ M ascorbate), 10 mM *t*-BHP, or 50 mM *t*-BHP for one hour.

2.3 Click chemistry to biotin label lipoxidatively modified proteins

Lysates were prepared by adding 0.2 volumes of 7 $\times$ lysis buffer (100 mM HEPES, pH 7.5; 150 mM NaCl; 0.1 M EDTA; 1% Igepal (NP-40)) and 0.2 volumes of 7 $\times$ commercial protease inhibitor cocktail (Roche), then incubating on ice for 20 minutes (mins). After sonication, cellular debris was removed by centrifugation at 14,000 $\times$ g at 4°C. Samples were then reduced with sodium borohydride (50 mM final concentration) on ice for 1 hour (hr). Click chemistry was performed on 5.6 mg of protein lysate by adding 0.6 mM biotin-azide, 0.75 mM TBTA ligand, 6 mM TCEP, and 6 mM copper sulfate for 2 hrs at room temperature. Reactions were quenched by adding EDTA (10 mM final concentration) and BHA (0.1 mM final concentration) and click chemistry reagents removed by addition of 6.7 volumes of ice cold methanol to precipitate proteins. Protein pellets were resuspended in 0.2% SDS in 100 mM ammonium bicarbonate pH 8.

2.4 Quantitation of sodium channel labeled with biotin via click chemistry

Streptavidin beads (GE Healthcare) were washed consecutively with water, 5 N NaCl, and 100 mM ammonium bicarbonate solution (pH 8). Resuspended protein was then added to 100 μ l of washed streptavidin beads and incubated overnight at 4°C with gentle rocking. After centrifugation, beads were washed consecutively with 1% SDS, 4 M Urea, 1 M NaCl, PBS, and water. Captured protein was eluted from the beads by boiling in LDS sample loading buffer with reducing agent at 70°C for 10 min, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The extent of modified Nav1.5 captured by this method, and thus representing modification by arachidonic acid peroxidation products, was determined by immunoblotting replicate membranes with anti-GFP antibody (1:1,000 dilution, Roche) and anti-Nav1.5 antibody (1:500 dilution, Upstate). Oxidants increased the average band density in the anti-sodium channel immunoblots about four-fold (Figure 1C). Band density was determined from three independent experiments using the gel analysis function of ImageJ Software from NIH.

2.5 Heterologous expression and cell culture

The Nav1.5 cDNA was subcloned into the pCGI vector for bicistronic expression with GFP as described previously [14]. HEK293 or HEK-cell derived tsA-201 cells growing on 6 cm-plates were transiently transfected with 1 μ g of Nav1.5 cDNA using lipofectamine (Invitrogen Co., Carlsbad, CA), and maintained in MEM medium supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin in a 5 % CO₂ incubator at 37°C for 24–36 hrs before current recordings. Cells exhibiting green fluorescence were chosen for current recordings. The atrial tumor myocyte cell line, HL-1, was a kind gift from Dr. W.C. Claycomb (Louisiana State University) and was cultured as described previously [30].

2.6 Electrophysiology

Membrane currents were recorded using whole-cell patch-clamp techniques at room temperature (22–24°C). The bath solution for recording membrane currents contained 145 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES (pH 7.35 with NaOH), and the one for HL-1 cells contained 145 NaCl, 4.5 CsCl, 1.5 MgCl₂, 1 CaCl₂, 5 HEPES, 5 glucose, 0.1 CdCl₂ (pH 7.35 with CsOH). The pipette solution contained (in mM) 10 NaF, 110 CsF, 20 CsCl, 10 EGTA, 10 HEPES (pH 7.35 with CsOH). Electrode resistance ranged 1.0 to 2.5

MΩ. Data acquisition was carried out using an Axopatch 200B amplifier and pCLAMP8.0 software (Axon Instruments Inc., Foster City, CA). Currents were acquired at 20–50 kHz (Digidata 1200 A/D converter, Axon Instruments Inc.), and low pass-filtered at 5 kHz. In all recordings, 80 % of the series resistance was compensated, yielding a maximum voltage error ~1 mV. To avoid the known time-dependent shift of activation and inactivation curves, all pulse protocols were applied at 8 mins after membrane rupture, except for Fig. 2 D, where the time point was set at 5 mins after rupture. It was initially determined that the time dependent shift became negligible at those time points. Recordings from cells exhibiting peak current amplitudes less than 0.5 nA were excluded from analysis to avoid potential endogenous current contamination. Cells exhibiting large currents (peak current > 10 nA) were also excluded if voltage control was compromised. All pulse protocols are described in the figure or figure legends. *t*-BHP or IsoK was dissolved directly in the bath (extracellular) solution, and cells were incubated for indicated intervals. When indicated, cells were preincubated with the indicated amounts of SA or 2 μM PPM in cell culture medium for at least 30 mins before application of *t*-BHP or IsoK.

2.7 Data analysis

Activation and steady-state availability curves were fitted with Boltzmann functions of the following forms: $y=1/\{1+\exp[(V_{1/2}-V_m)/K]\}$ or $y=1/\{1+\exp[(V_m-V_{1/2})/K]\}$ respectively, where y is the relative current, V_m is the membrane potential, $V_{1/2}$ is the voltage at which half of the channels are available to open, and K is the slope factor. All data are expressed as mean $\pm$ SEM, and statistical comparisons were made using one-way ANOVA (Microcal Origin, Northampton, MA) with $p<0.05$ indicating significance.

3. Results

3.1 Na_v1.5 is lipoxidatively modified during oxidant stress

We previously reported that *t*-BHP modifies the function of Na_v1.5 expressed in heterologous cells [14]. To determine if exposure of Na_v1.5-expressing cells to oxidants resulted in direct modification of the sodium channel by arachidonic acid metabolites such as IsoKs, we utilized arachidonic acid modified with a terminal alkyne for our experiments and then labeled this product with biotin *ex vivo* using click chemistry (schematically outlined in Fig. 1A). HEK-Na_v1.5-GFP cells were incubated with or without alkynyl-arachidonic acid (al-AA) overnight to allow incorporation into membrane phospholipids. Cells were then exposed to vehicle, iron/adenosine/ascorbate, 10 mM *t*-BHP or 50 mM *t*-BHP for one hour. Cells were then harvested, lysed, and alkynyl moieties biotinylated using click chemistry. Biotinylated proteins were captured on streptavidin beads and Na_v1.5 was identified among the captured proteins by immunoblot analysis. Immunoblot analysis with anti-GFP and anti-sodium channel antibodies demonstrated that Na_v1.5-GFP became modified by al-AA when the cells were exposed to any of the three oxidizing conditions (Fig. 1B and 1C). In contrast, very little or no Na_v1.5-GFP was captured in the absence of oxidants or if al-AA was not present in the cells. These results are consistent with the notion that Na_v1.5 is lipoxidatively modified when cells are exposed to oxidative stress.

3.2 Salicylamine protects against *t*-BHP-induced hyperpolarizing shift in voltage-dependence of inactivation of Na_v1.5 in a heterologous expression system

The voltage-dependent availability curves reflect the fraction of channels available to open and thus the proportion of channels *not* inactivated at a given membrane potential. A right (depolarizing) shift in the curve indicates a larger number of channels available to open at any given voltage, whereas a left (hyperpolarizing) shift indicates the opposite. To characterize the time course for effect of *t*-BHP on activation and steady-state inactivation, we incubated Na_v1.5 transfected HEK293 cells with 1 mM *t*-BHP for 5 to 30 min and recorded I_{Na} using

the pulse protocol shown in the insets of Fig. 2. During the periods measured, *t*-BHP did not significantly affect either amplitude, or activation kinetics of $I_{NaV1.5}$ (Figure 2A and Table 1). In contrast, and as we observed previously, *t*-BHP affected the availability curve of $I_{NaV1.5}$ upon exposure for 20 or 30 min by significantly shifting the $V_{1/2}$ values to hyperpolarizing potentials (Figure 2B, Table 1). The effects of *t*-BHP on the availability of $I_{NaV1.5}$ appeared to be saturated after 30 mins of incubation, and in subsequent experiments, we evaluated the $V_{1/2}$ of channel availability at this time point. The 30 min *t*-BHP incubation increased IsoK protein adducts from 22.5 ± 1.6 pg adduct/mg protein in untreated cells to 58.7 ± 6.0 pg adduct/mg protein ($P = 0.004$, untreated vs treated).

Our previous studies showed that the effects of *t*-BHP on I_{Na} can be mimicked by IsoK, but not other lipid peroxidation products such as F_2 -isoprostanes and 4-hydroxynonenal, suggesting that *t*-BHP-induced modification of I_{Na} is predominantly mediated through IsoK [14]. We therefore examined an IsoK scavenger, SA, for its ability to protect against *t*-BHP-induced changes in $I_{NaV1.5}$ availability. We found that pretreatment of the cells with 1 μ M SA protected $I_{NaV1.5}$ from the expected shift in channel availability induced by *t*-BHP (Figure 2C, Table 2). Incubation of the cells with SA alone (<100 μ M) did not affect channel availability of $I_{NaV1.5}$ (data not shown). These results indicate that *t*-BHP-induced shift in channel availability of $I_{NaV1.5}$ can be rescued by the IsoK scavenger, salicylamine. These findings were supported when we tested the effects of a second IsoK scavenger: Fig. 2D and table 2 show that pretreatment with 2 μ M PPM similarly protected the availability curve from the hyperpolarizing shift induced by *t*-BHP. However, in this case, incubation with PPM alone (2 μ M, for 30 mins) resulted in a depolarizing shift in channel availability of $I_{NaV1.5}$ by 5 mV, suggesting that the channel may be adducted by IsoK at a background level and that this can be prevented by PPM. This confirms that SA acts as IsoK scavenger and prevents IsoK from decreasing $I_{NaV1.5}$ channel availability.

To determine the effectiveness of SA on sodium channels treated with IsoK directly, we first incubated $I_{NaV1.5}$ -expressing HEK293 cells with varying concentrations of IsoK and then assessed the channel availability. Consistent with our previous study, treatment with 1, 3 or 10 μ M IsoK progressively and dose-dependently shifted the availability curves towards hyperpolarizing potentials (Fig. 3A). We then tested the effects of the IsoK scavenger, SA, on the shift in steady-state availability evoked by 2 μ M IsoK following a 30 mins treatment (Fig. 3B, Table 2). The results showed that treatment with 100 μ M SA significantly reduced the IsoK-induced left-shift in the availability curve (Fig. 3B, Table 2).

3.3 SA protects against *t*-BHP-induced decrease in I_{Na} amplitude and reduced channel availability in HL-1 cells

We next sought to determine whether the same effects could be observed in a cardiac-derived cell line that expresses endogenous I_{Na} . Preliminary evaluation of I_{Na} current profiles in the atrial tumor-derived myocyte cell line, HL-1 [30] revealed that exposure to 2 mM *t*-BHP for one hour suppressed current amplitude (Figure 4A, Table 3), although the kinetics of current activation remained unchanged (Figure 4B, Table 3). As we previously reported [14], exposure of HL-1 cells to 2 mM *t*-BHP for an hour shifted the availability curve of sodium channels in HL-1 cells toward hyperpolarizing potentials (Figure 4D, Table 3). The one hour exposure to 2 mM *t*-BHP significantly increased IsoK protein adducts from 44.4 ± 5.9 pg adduct/mg protein in untreated HL-1 cells to 157.5 ± 5.4 pg adduct/mg protein in treated cells ($P = 0.0001$ treated vs . untreated, student's *t*-test).

We next examined whether SA was able to protect endogenous I_{Na} against this *t*-BHP-induced functional modification. Pretreatment of the cells with 1 to 100 μ M SA dose-dependently blocked the shift in channel availability induced by *t*-BHP application (Figure 4D, Table 3).

The effects of SA pretreatment on I_{Na} amplitude were also evaluated. Although pretreatment of the cells with 1 μ M SA or 10 μ M SA did not affect *t*-BHP-induced suppression in I_{Na} amplitude, 100 μ M SA protected against *t*-BHP-induced suppression of I_{Na} (Figure 4A, Table 3).

4. Discussion

Although changes in I_{Na} have been well-established sequelae of myocardial infarction, the mechanisms underlying these changes have not been fully characterized. Because extensive oxidative injury accompanies infarction and ischemia, oxidative modification of Nav1.5 has been suggested as a potential mechanism for these changes. While oxidative stress may potentially alter protein function in a variety of ways, one of the most probable is via modification by the highly reactive IsoKs. These γ -ketoaldehydes react far more rapidly than other lipoxidation products like 4-hydroxynonenal, and significantly alter I_{Na} when added exogenously to cultured cells. Moreover, levels of IsoK protein adducts increase markedly in the epicardial border zone after experimentally induced myocardial infarction. Thus, modification of Nav1.5 in this arrhythmogenic zone seems highly probable.

Identification of IsoK-modified proteins in ischemic myocardium by standard immunoaffinity techniques has proven difficult, probably because of the lack of antibodies with sufficiently high affinity. We therefore utilized “click chemistry”, which allows *ex vivo* labeling of lipids possessing a terminal alkyne with high affinity tags such as biotin, to determine if Nav1.5 was modified by lipid peroxidation products during treatment of cultured cells expressing Nav1.5 with oxidants. Using alkynyl-arachidonic acid to enable us to follow the peroxidation products generated when cells were treated with two distinct ROS initiators (iron and *t*-BHP), we found that Nav1.5 was covalently modified by arachidonic acid peroxidation products during this oxidative stress. Although treatment with *t*-BHP has been widely used as a model to study the effects of oxidation on I_{Na} , to the best of our knowledge, this is the first demonstration that Nav1.5 is actually modified by a lipoxidation product in response to oxidants.

While identification of the specific residues of Nav1.5 that are modified by lipoxidation will be necessary to elucidate the precise mechanism whereby lipoxidation causes I_{Na} alteration, this result provided the rationale to examine whether treatment with IsoK scavengers could prevent changes in I_{Na} amplitude and channel availability in response to oxidants. Nav1.5 could potentially be modified by several lipoxidation products; however, the extreme reactivity of the IsoKs and their ability to inactivate Nav1.5 when added exogenously suggested that IsoKs were the peroxidation products most likely to contribute to I_{Na} alternations. Pretreatment of the cells with SA or PPM protected against the *t*-BHP-induced shift in availability of Nav1.5 expressed in heterologous systems. Importantly, while both SA and PPM are excellent IsoK scavengers because of their phenolic amine moiety, they are otherwise quite chemically distinct, so that the effect of the two compounds is likely to be mediated through their ability to scavenge IsoK rather than some other mechanisms. We also found that SA dose dependently protected against *t*-BHP induced alterations in channel availability and I_{Na} amplitude in HL-1 cells expressing Nav1.5 endogenously. A higher concentration of SA was required in HL-1 cells, which may be due to differences in steady-state parameters, the 2.7-fold greater level of IsoK adducts generated in HL-1 cells by the oxidation conditions utilized, and/or the activity levels of endogenous scavengers. Since HL-1 cells are myocyte-derived, they contain other ion channels, as well as sodium channel β subunits (Kupersmidt, unpublished data). This probably explains the slightly different biophysical characteristics between heterologously expressed channels and those in HL-1 cells. In addition to the *t*-BHP-induced suppression of endogenous HL-1 cell I_{Na} amplitude, a leftward shift in the availability curve was found to be the most prominent kinetic change. Because the availability curve is steep at voltages around the resting membrane potential of the ventricular myocardium (-85 mV), even small shifts in

the curve will have significant physiological consequences. Moreover, in ischemic ventricular myocardium, the resting membrane potential is secondarily depolarized [34], which further reduces the number of Na⁺ channels available to open. Thus, a slight leftward shift in availability curve potentially translates into a dramatic reduction of I_{Na} in the ischemic heart. It has been reported that alterations in I_{Na} in ischemic myocardium play a crucial role in the genesis of reentrant tachyarrhythmias [35–37]. Reduction of I_{Na} by oxidative stress might lead to slowed conduction and prolonged refractoriness in ventricular myocardium, which facilitate the formation of the substrate for reentry [36,37]. Furthermore, excessive reduction of I_{Na} may also contribute to transmural dispersion of repolarization in the surviving myocardial layer, another substrate for reentry [38–40]. Thus, current evidence indicates that restoration of reduced I_{Na} that manifests as a leftward shift in availability curve, and that is caused by oxidative stress, presents an attractive target for preventing the occurrence of re-entrant arrhythmias in ischemic heart.

In summary, our data provide the first evidence that the cardiac sodium channel is lipoxidatively modified during oxidant injury and that scavenging of IsoKs is sufficient to prevent the sodium channel dysfunction caused by oxidative injury in cultured cells. Therefore, IsoK scavengers may represent a new therapeutic approach to preventing arrhythmias in the setting of myocardial infarction.

Acknowledgments

This study was supported with funds from National Institute of Health grants HL079365 (to LJR) and GM56307 (to JRB.) We thank Dr. Svetlana Stepanovic for expert technical assistance. The stably expressing Nav1.5-GFP cell line was a kind gift from Dr. Kathy Murray, VUMC.

References

1. Wang Q, Shen J, Li Z, et al. Cardiac sodium channel mutations in patients with long QT syndrome, an inherited cardiac arrhythmia. *Hum Mol Genet* 1995;4:1603–1677. [PubMed: 8541846]
2. Bennett PB, Yazawa K, Makita N, George AL Jr. Molecular mechanism for an inherited cardiac arrhythmia. *Nature* 1995;376:683–685. [PubMed: 7651517]
3. Chen Q, Kirsch GE, Zhang D, et al. Genetic basis and molecular mechanism for idiopathic ventricular fibrillation. *Nature* 1998;392:293–296. [PubMed: 9521325]
4. Tan HL, Bink-Boelkens MT, Bezzina CR, et al. A sodium-channel mutation causes isolated cardiac conduction disease. *Nature* 2001;409:1043–1047. [PubMed: 11234013]
5. Wiecha J, Hombach V. Cellular electrophysiological properties in myocardial infarction. *Eur Heart J* 1993;14:9–19. [PubMed: 8223762]
6. Pu J, Boyden PA. Alterations of Na⁺ Currents in Myocytes From Epicardial Border Zone of the Infarcted Heart : A Possible Ionic Mechanism for Reduced Excitability and Postrepolarization Refractoriness. *Circ Res* 1997;81:110–119. [PubMed: 9201034]
7. CAST Investigators. Preliminary report: effect of encainide and flecainide on mortality in a randomized trial of arrhythmia suppression after myocardial infarction. *New Engl J Med* 1989;321:406–412. [PubMed: 2473403]
8. Delanty N, Reilly MP, Pratico D, et al. 8-epi PGF2 alpha generation during coronary reperfusion. A potential quantitative marker of oxidant stress in vivo. *Circulation* 1997;95:2492–2499. [PubMed: 9184579]
9. Schwedhelm E, Bartling A, Lenzen H, et al. Urinary 8-iso-prostaglandin F2alpha as a risk marker in patients with coronary heart disease: a matched case-control study. *Circulation* 2004;109:843–848. [PubMed: 14757688]
10. Woodward B, Zakaria MN. Effect of some free radical scavengers on reperfusion induced arrhythmias in the isolated rat heart. *J Mol Cell Cardiol* 1985;17:485–493. [PubMed: 3928898]

11. Bernier M, Hearse DJ, Manning AS. Reperfusion-induced arrhythmias and oxygen-derived free radicals. Studies with "anti-free radical" interventions and a free radical-generating system in the isolated perfused rat heart. *Circ Res* 1986;58:331–340. [PubMed: 3087653]
12. Bhatnagar A, Srivastava SK, Szabo G. Oxidative stress alters specific membrane currents in isolated cardiac myocytes. *Circ Res* 1990;67:535–549. [PubMed: 2168816]
13. Tarr M, Valenzano DP. Modification of cardiac ionic currents by photosensitizer-generated reactive oxygen. *J Mol Cell Cardiol* 1991;23:639–649. [PubMed: 1886141]
14. Fukuda K, Davies SS, Nakajima T, et al. Oxidative mediated lipid peroxidation recapitulates proarrhythmic effects on cardiac sodium channels. *Circ Res* 2005;97:1262–1269. [PubMed: 16284182]
15. Bhatnagar A. Electrophysiological effects of 4-hydroxynonenal, an aldehydic product of lipid peroxidation, on isolated rat ventricular myocytes. *Circ Res* 1995;76:293–304. [PubMed: 7834841]
16. Vila A, Tallman KA, Jacobs AT, Liebler DC, Porter NA, Marnett LJ. Identification of Protein Targets of 4-Hydroxynonenal Using Click Chemistry for ex Vivo Biotinylation of Azido and Alkynyl Derivatives. *Chem Res Toxicol* 2008;21:432–444. [PubMed: 18232660]
17. Speers AE, Cravatt BF. A Tandem Orthogonal Proteolysis Strategy for High-Content Chemical Proteomics. *J Am Chem Soc* 2005;127:10018–10019. [PubMed: 16011363]
18. Speers AE, Cravatt BF. Profiling Enzyme Activities In Vivo Using Click Chemistry. *Meth Chem & Biol* 2004;11:535–546.
19. Wang Q, Chan TR, Hilgraf R, Fokin VV, Sharpless KB, Finn MG. Bioconjugation by Copper(I)-Catalyzed Azide-Alkyne [3 + 2] Cycloaddition. *J Am Chem Soc* 2003;125:3192–3193. [PubMed: 12630856]
20. Krasinski A, Radic Z, Manetsch R, et al. In Situ Selection of Lead Compounds by Click Chemistry: Target-Guided Optimization of Acetylcholinesterase Inhibitors. *J Am Chem Soc* 2005;127(18): 6686–6692. [PubMed: 15869290]
21. Kolb HC, Finn MG, Sharpless KB. Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew Chem Int Ed Engl* 2001;2004–2021. [PubMed: 11433435]
22. Kolb HC, Sharpless KB. The growing impact of click chemistry on drug discovery. *Drug Disc Today* 2003;8:1128–1137.
23. Milne SB, Tallman KA, Serwa R, et al. Alkyne-Derivatized Glycerophospholipids and Their Dicobalthexacarbonyl Complexes: A Highly Sensitive Analytical Approach for Determining Cellular Lipid Changes. submitted *Nature Chem Biol*.
24. Brame CJ, Salomon RG, Morrow JD, Roberts LJ 2. Identification of extremely reactive gamma-ketoaldehydes (isolevuglandins) as products of the isoprostane pathway and characterization of their lysyl protein adducts. *J Biol Chem* 1999;274:13139–13146. [PubMed: 10224068]
25. Davies SS, Amarnath V, Roberts LJ 2. Isoketals: highly reactive gamma-ketoaldehydes formed from the H2-isoprostane pathway. *Chem Phys Lipids* 2004;128:85–99. [PubMed: 15037155]
26. Brame CJ, Boutaud O, Davies SS, et al. Modification of proteins by isoketal-containing oxidized phospholipids. *J Biol Chem* 2004;279:13447–13451. [PubMed: 14715668]
27. Amarnath V, Amarnath K, Davies S, Roberts LJ 2. Pyridoxamine: an extremely potent scavenger of 1,4-dicarbonyls. *Chem Res Toxicol* 2004;17:410–415. [PubMed: 15025512]
28. Davies SS, Brantley EJ, Vozian PA, et al. Pyridoxamine Analogues Scavenge Lipid-Derived gamma-Ketoaldehydes and Protect against H2O2-Mediated Cytotoxicity. *Biochemistry* 2006;45:15756–15767. [PubMed: 17176098]
29. Vozian PA, Metz TO, Baynes JW, Hudson BG. A post-Amadori inhibitor pyridoxamine also inhibits chemical modification of proteins by scavenging carbonyl intermediates of carbohydrate and lipid degradation. *J Biol Chem* 2002;277:3397–3403. [PubMed: 11729198]
30. Claycomb WC, Lanson NA Jr, Stallworth BS, et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci USA* 1998;95:2979–2984. [PubMed: 9501201]
31. Chan TR, Hilgraf R, Sharpless KB, Fokin VV. Polytriazoles as Copper(I)-Stabilizing Ligands in Catalysis. *Organic Lett* 2004;6:2853–2855.
32. Amarnath V, Amarnath K, Matherson T, Davies S, Roberts LJ. Simplified Synthesis of Diastereomers of Levuglandin E2. *Synth Comm* 2005;35:397–408.

33. Hallaq H, Yang Z, Viswanathan PC, et al. Quantitation of protein kinase A-mediated trafficking of cardiac sodium channels in living cells. *Cardiovasc Res* 2006;72:250–261. [PubMed: 16973141]
34. Priori SG, Mantica M, Napolitano C, Schwartz PJ. Early afterdepolarizations induced in vivo by reperfusion of ischemic myocardium. A possible mechanism for reperfusion arrhythmias. *Circulation* 1990;81:1911–1920. [PubMed: 2344683]
35. Ursell PC, Gardner PI, Albala A, Fenoglio JJ Jr, Wit AL. Structural and electrophysiological changes in the epicardial border zone of canine myocardial infarcts during infarct healing. *Circ Res* 1985;56:436–451. [PubMed: 3971515]
36. Lue WM, Boyden PA. Abnormal electrical properties of myocytes from chronically infarcted canine heart. Alterations in V_{max} and the transient outward current. *Circulation* 1992;85:1175–1188. [PubMed: 1371431]
37. Pu J, Boyden PA. Alterations of Na^+ currents in myocytes from epicardial border zone of the infarcted heart. A possible ionic mechanism for reduced excitability and postrepolarization refractoriness. *Circ Res* 1997;81:110–119. [PubMed: 9201034]
38. Krishnan SC, Antzelevitch C. Flecainide-induced arrhythmia in canine ventricular epicardium. Phase 2 reentry? *Circulation* 1993;87:562–572. [PubMed: 8425300]
39. Veldkamp MW, Viswanathan PC, Bezzina C, Baartscheer A, Wilde AA, Balser JR. Two distinct congenital arrhythmias evoked by a multidysfunctional Na^+ channel. *Circ Res* 2000;86:91–97.
40. Wang DW, Makita N, Kitabatake A, Balser JR, George AL Jr. Enhanced Na^+ channel intermediate inactivation in Brugada syndrome. *Circ Res* 2000;87:37–43.

Fig. 1.

(A) Schematic outline depicting the identification of proteins modified by peroxidation products of arachidonic acid in cells treated with ROS initiators. Arachidonic acid modified to have a terminal alkyne (al-AA) is incubated with cells to allow its incorporation into membrane phospholipids. Cells are then treated with ROS initiators (iron/adenosine/ascorbate or *t*-BHP) which peroxidize polyunsaturated fatty acids including al-AA to generate lipoxidation products (including al-IsoK.) The al-IsoK then reacts rapidly with cellular proteins, potentially including Na_v1.5. After the end of the treatment period, adducted proteins are captured from cellular lysates using "click chemistry". In the presence of copper catalyst, biotin-linked azide (biotin-N₃) selectively reacts with terminal alkynes to form a triazole linkage between the adducted protein and the biotin. Adducted proteins can then be captured on streptavidin beads, the captured proteins run on SDS-PAGE and immunoblotted for candidate proteins including Na_v1.5. (B) Exposure of HEK Na_v1.5-GFP cells to oxidants results in channel modification by arachidonic acid peroxidation products. HEK Na_v1.5-GFP cells were treated with (lanes 2, 4, 6, and 8) and without (lanes 1, 3, 5, and 7) al-AA overnight and then exposed for 1hr either to vehicle (veh-lanes 1 and 2), iron/ADP/ascorbate (Fe) (lanes 3 and 4), 10 mM *t*-BHP (lanes 5 and 6) or 50 mM *t*-BHP (lanes 7 and 8). Cellular proteins modified by al-AA peroxidation products were biotinylated by the click chemistry reaction and captured on streptavidin beads. The extent of Na_v1.5-GFP captured (and thus modified by lipoxidation) was determined by Western blot analysis using anti-GFP (top panel) or anti-sodium channel

(lower panel) antibodies. Lysate from Na_v1.5-GFP loaded directly on SDS-PAGE was used as a positive control (lane 9). (C) Relative band density of three replicate anti-sodium channel immunoblotting experiments for HEK Na_v1.5-GFP cells preincubated with al-AA and then treated with vehicle (veh), iron/ADP/ascorbate (Fe), 10 mM *t*-BHP or 50 mM *t*-BHP (Mean ± SEM).

Fig. 2.

Isoketal scavengers protects against *t*-BHP-induced shifts in steady-state availability of Na_v1.5. (A). Voltage-dependence of activation of Na_v1.5 expressed in HEK293 cells for untreated controls (filled squares, *n* = 11), or Na_v1.5 plus 1 mM *t*-BHP for 5 mins (open squares, *n* = 7), 10 mins (open triangles, *n* = 9), 20 mins (filled diamonds, *n* = 9), or 30 mins (open circles, *n* = 8). The pulse protocol is illustrated in the inset. Depolarizing test pulses were applied from a holding potential of -120 mV to various potentials between -90 mV and +60 mV in 10 mV increments for 20 milliseconds (ms). Currents were divided by the electrochemical driving force for Na⁺ ions and normalized to the maximum Na⁺ conductance. Activation curves were fitted to a Boltzmann function as described in Methods. The *V*_{1/2} values and slope factors are shown in Table 1. (B). Voltage-dependent steady-state availability of Na_v1.5 for untreated controls (*n* = 11), Na_v1.5 plus 1mM *t*-BHP for 5 mins (*n* = 9), 10 mins (*n* = 10), 20 mins (*n* = 11), or 30 mins (open circles, *n* = 15). The pulse protocol is illustrated in the inset. 500 ms prepulse potentials were applied from a holding potential of -120 mV to various potentials between -140 mV and -50 mV in 10 mV increments followed by a test pulse of -20 mV. Availability curves were fitted to a Boltzmann function as described in Methods. The *V*_{1/2} values and slope factors are shown in Table 1. (C). Voltage-dependent steady-state availability of Na_v1.5 for untreated control (filled squares, *n* = 11), Na_v1.5 plus 1 mM *t*-BHP (open circles, *n* = 12), or 1mM *t*-BHP plus 1 μM SA (open triangles, *n* = 12). The pulse protocol is the same as in (B). The *V*_{1/2} values and slope factors are shown in Table 2. Inset: Representative current tracings for untreated control (left), Na_v1.5 plus *t*-BHP (middle), or Na_v1.5 plus *t*-BHP plus

1 μ M SA (right). The red lines indicate the currents obtained at prepulse potentials of -90 mV. (D). Availability curves for untreated $\text{Na}_v1.5$ (filled squares, $n = 6$), plus 1 mM *t*-BHP (open circles, $n = 5$), plus 1 mM *t*-BHP and 2 μ M PPM (inverse open triangles, $n = 8$), and $\text{Na}_v1.5$ plus 2 μ M PPM (open diamonds, $n = 5$).

Fig. 3.

SA protects against IsoK-induced shift in steady-state availability of Na_V1.5. (A). Voltage-dependent steady-state availability of untreated Na_V1.5 (filled squares, $n = 6$), Na_V1.5 plus 1 μM IsoK (open circles, $n = 6$), 3 μM IsoK (filled triangles, $n = 6$), and 10 μM IsoK (inverse open triangles, $n = 5$). IsoK pretreatment lasted for at least 30 min. The pulse protocol is illustrated in the inset. The $V_{1/2}$ and slope factors are listed in Table 2. (B). Voltage-dependent steady-state availability of Na_V1.5 for controls (filled squares, $n=11$), or cells treated with 2 μM IsoK for 30 mins (open triangles, $n = 12$), or with 2 μM IsoK plus 100 μM SA (open diamonds, $n = 12$). The $V_{1/2}$ values and slope factors are shown in Table 2. Representative current traces for control (left), Na_V1.5 plus 2 μM IsoK (middle), or Na_V1.5 plus 2 μM IsoK together with 100 μM SA (right) are shown in the inset. The red lines indicate the currents obtained at prepulse potentials of -90 mV.

Fig. 4.

SA protects against t -BHP-induced suppression of I_{Na} and shift in steady-state availability of Na^+ channels in a cardiac-derived cell line (HL-1).

(A). Current-voltage relationship of Na^+ channels in HL-1 cells for untreated controls (filled squares, $n = 17$), $Na_V1.5$ plus 2 mM t -BHP for 1 hr (filled circles, $n = 13$), and $Na_V1.5$ plus 2 mM t -BHP (1 hr) plus 1 μ M SA (open triangles, $n = 5$), 10 μ M SA (reverse filled triangles, $n = 13$), or 100 μ M SA (reverse open triangles, $n = 10$). Peak currents obtained by the pulse protocol in the inset were normalized to cell capacitances. Peak current amplitudes measured at -20 mV are listed in Table 3. (B). Voltage-dependence of activation of untreated controls (filled squares, $n = 17$), $Na_V1.5$ plus 2 mM t -BHP (filled circles, $n = 13$), 2 mM t -BHP plus 1

μM SA (open triangles, $n = 5$), $10 \mu\text{M}$ SA (reverse filled triangles, $n=13$), or $100 \mu\text{M}$ SA (inverse open triangles, $n = 10$). The $V_{1/2}$ values and slope factors are listed in Table 3. (C). Representative current tracings for untreated control (left), $\text{Na}_V 1.5$ plus $2 \text{ mM } t\text{-BHP}$ (middle) and $2 \text{ mM } t\text{-BHP}$ and $100 \mu\text{M}$ SA (right). The pulse protocol is also shown. (D). Voltage-dependent steady-state availability of I_{Na} in untreated HL-1 cells (filled squares, $n = 19$), or in cells treated with $2 \text{ mM } t\text{-BHP}$ (filled circles, $n = 17$), or with $2 \text{ mM } t\text{-BHP}$ plus $1 \mu\text{M}$ SA (open triangles, $n = 5$), $10 \mu\text{M}$ SA (inverse filled triangles, $n = 13$), or $100 \mu\text{M}$ SA (inverse open triangles, $n = 13$). The pulse protocol is illustrated in the inset. The $V_{1/2}$ value and slope factor are shown in Table 3. (E). Representative current traces in untreated cells (left), or with $2 \text{ mM } t\text{-BHP}$ (middle), or $2 \text{ mM } t\text{-BHP}$ plus $100 \mu\text{M}$ SA (right). Pulse protocols are in the inset. The red lines show the currents obtained at prepulse potentials of -80 mV .

Table 1

Activation and steady-state inactivation properties of heterologously expressed NaV1.5 before and after application of *t*-BHP.

	Activation			Steady-state inactivation	
	Amplitude (pA/pF) at -20 mV	V1/2 (mV)	k (mV)	V1/2 (mV)	k (mV)
Control	-664 ± 81	-41.7 ± 0.9	6.5 ± 0.2 (n=11)	-89.7 ± 1.1	5.9 ± 0.1 (n=11)
<i>t</i> -BHP (5mins)	-656 ± 160	-42.3 ± 1.1	5.9 ± 0.2 (n=7)	-87.4 ± 1.0	6.3 ± 0.2 (n=9)
<i>t</i> -BHP (10mins)	-671 ± 34	-40.4 ± 0.8	7.2 ± 0.3 (n=9)	-89.1 ± 1.6	6.8 ± 0.2 ^{**} (n=10)
<i>t</i> -BHP (20mins)	-688 ± 65	-39.9 ± 1.2	7.4 ± 0.2 ^{**} (n=9)	-94.5 ± 1.7 [*]	7.3 ± 0.2 ^{**} (n=11)
<i>t</i> -BHP (30mins)	-577 ± 89	-40.7 ± 1.3	7.8 ± 0.4 ^{**} (n=8)	-96.0 ± 1.7 ^{**}	7.1 ± 0.2 ^{**} (n=15)

* p<0.05,

** p<0.01 vs. control.

Table 2

Steady-state inactivation properties for control, *t*-BHP-treated, *t*-BHP plus SA-treated and control, IsoK, and IsoK plus SA.

	Steady-state inactivation			Steady-state inactivation	
	V1/2 (mV)	k (mV)		V1/2 (mV)	k (mV)
Control	-87.7 ± 1.0	5.4 ± 0.2 (n=11)	Control	-87.5 ± 1.3	5.9 ± 0.2 (n=6)
<i>t</i> -BHP	-95.0 ± 2.0**	7.2 ± 0.3** (n=12)	1 μM IsoK	-91.6 ± 1.7	6.3 ± 0.2 (n=6)
<i>t</i> -BHP plus SA	-89.4 ± 0.8§	6.8 ± 0.2** (n=12)	3 μM IsoK	-99.0 ± 2.5**	6.7 ± 0.3 (n=6)
			10 μM IsoK	-108.9 ± 1.5**	7.6 ± 0.4 (n=5)
Control	-86.6 ± 1.0	5.7 ± 0.2 (n=6)	Control	-87.6 ± 1.9	5.9 ± 0.1 (n=11)
<i>t</i> -BHP	-102.1 ± 1.7**	8.2 ± 0.2** (n=5)	2 μM IsoK	-95.7 ± 1.6**	6.4 ± 0.3 (n=12)
<i>t</i> -BHP plus PPM	-88.1 ± 0.4**	6.2 ± 0.3 (n=8)	2 μM IsoK plus 100 μM SA	-89.8 ± 1.5§	6.4 ± 0.2 (n=12)

** p<0.01 (vs. control),

§ p<0.05 (vs. *t*-BHP or IsoK)

Table 3

Activation and steady-state inactivation properties measured in HL-1 cells for untreated, *t*-BHP treated, and *t*-BHP plus SA treated samples.

	Activation		Steady-state inactivation	
	Amplitude (pA/pF) at -20 mV	V1/2 (mV)	V1/2 (mV)	k (mV)
Control	-161 ± 15 (n=17)	-34.3 ± 0.6	-73.8 ± 0.9 (n=19)	5.5 ± 0.2
<i>t</i> -BHP	-92 ± 9 ^{**} (n=13)	-34.5 ± 1.6	-83.9 ± 1.6 ^{**} (n=17)	6.1 ± 0.2
<i>t</i> -BHP + 1 μM SA	-60 ± 22 ^{**} (n=5)	-32.6 ± 1.1	-82.8 ± 1.5 ^{**} (n=5)	6.3 ± 0.4
<i>t</i> -BHP + 10 μM SA	-81 ± 7 ^{**} (n=13)	-33.3 ± 2.2	-78.5 ± 1.2 ^{*§} (n=13)	5.8 ± 0.2
<i>t</i> -BHP + 100 μM SA	-135 ± 16 [§] (n=10)	-34.7 ± 1.2	-74.6 ± 1.7 ^{§§} (n=13)	5.6 ± 0.4

* p<0.05,

** p<0.01 (vs. control),

§ p<0.05,

§§ p<0.01 (vs. *t*-BHP)