LIMITING OXIDATION IN POTASSIUM CHANNEL KV2.1 USING

CYSTEINE-ALANINE MUTATION

By

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ABSTRACT OF THE THESIS

Limiting Oxidation in Potassium Channel kv2.1 Using Cysteine-Alanine Mutation

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Dr. Federico Sesti

The Kv2.1 (KCNB1) channel is expressed in the cortex and hippocampus. Interaction between cysteine residues of the kv2.1 channel plays a role in the formation of disulfide bonds. Disulfide bond formation following oxidative stress suggests that cysteine interaction in voltage-gated K^+ channel kv2.1 plays a key role in the oxidation of kv2.1. Previous research has shown that oxidation of potassium (K^+) channels by reactive oxygen species (ROS) is a major factor in the loss of neuronal function [6]. The purpose of this study was to use cysteine-alanine mutations to prevent oxidation of K^+ channel kv2.1. In this thesis, the anti-oxidant properties of the double mutant C73AC29A were investigated. The affects were observed using site-directed mutagenesis and the polymerase chain reaction (PCR). PCR was utilized to form a double mutant between C73A and C29A. SDS-Page and Western Blot analysis were used to analyze whether there was more or less oxidation in the double mutant C73AC29A compared to that of the kv2.1 control. The double mutant C73AC29A showed protective properties, showing less oxidation than the kv2.1 control when placed under oxidative stress. Findings suggest that C73AC29A could provide protection from oxidation-induced loss of function in the kv2.1 channel.

Acknowledgement

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CHAPTER 1

Background

1.1 Mechanisms and Consequences of Cellular Oxidation in Aging

Reactive oxygen species (ROS) are oxygen containing free radicals generated during oxygen metabolism, a normal cellular activity. The production of free radicals by mitochondria is a normal reaction that occurs during the oxidative phosphorylation process. Oxidative phosphorylation is the process in which enzymes in the mitochondria synthesize ATP during the oxidation of NADH by molecular oxygen. ROS can lead to oxidative damage by targeting DNA, RNA, proteins, and lipids. Increased levels of ROS may result in damage to cell structures. Mitochondria have multiple electron carriers with the ability to produce ROS and a defense system of antioxidants. Antioxidants play an important role in preventing ROS damage by eliminating these free radicals.

Mitochondria are the primary consumers of oxygen, using redox enzymes to transfer single electrons to oxygen to generate the ROS superoxide (O_2^-) [8]. Free radicals like O_2^- diffuse poorly through lipid membranes; unlike its product hydrogen peroxide, which has the ability to diffuse freely [8]. Hydrogen peroxide can lead to cellular damage as a superoxide and also generate hydroxyl radicals in the presence of redox-active metals. Mitochondria promote oxidative damage by producing excess hydrogen peroxide which leads to mitochondrial and lysosomal degradation, leading to increased ROS production and continuing cycles of oxidative damage. Oxidative damage to DNA, RNA, and proteins initiated by ROS is thought to contribute to the physiology of aging, a concept known as the free-radical theory. The oxidation of RNA is less understood, but

oxidative damaged nucleic acids may interfere with the actions of transcription and translation factors.

Based on the link between ROS production and aging, there is evidence to support that by enhancing mitochondrial antioxidant defenses, longevity may be improved [10]. A recent study of gene expression in the brain suggests that oxidative damage plays a significant role in cognitive decline associated with aging [10]. A study using transcriptional profiling of postmortem frontal cortex samples from individuals aged from 26 to 106 found that after age 40 there was a decrease in the expression of genes involved synaptic plasticity, vesicular transport, and mitochondrial function [11]. The study also found that there was an increase in the expression of genes involved in stress-response, antioxidant defense, and DNA-repair [11]. Age-downregulated genes had noticeably increased oxidative damage compared with the age-stable or age-upregulated genes [11].

1.2 Ion Channels: Potassium Channels in Physiology and Pathophysiology

1.2.1 Biological Activity

Ion channels are pore-forming proteins that allow the flow of ions down their electrochemical gradient to establish a voltage gradient across the plasma membrane of cells. Voltage-gated ion channels open and close in response to membrane potential, unlike ligand-gated ion channels which open and close in response to the binding of a chemical messenger. Voltage-gated K^+ channels (Kv) are made up of four pore-forming subunits known as alpha subunits. An established model of a single alpha subunit is depicted in Figure 1.1. Kv channels are transmembrane channels allow the flow of potassium ions across the membrane, but block the flow of other ions such as sodium

ions. The selective pore allows one sodium ion to pass for every ten thousand potassium ions. The N- and C-termini are important regions that play a role in inactivation, channel assembly, targeting, and interactions with accessory proteins. Neurons have a variety of voltage-dependent ion channels, most importantly are the Kv channels that control membrane electrical excitability.

Kv are diverse in that they control different characteristics of neuronal function, including subcellular localization and changes in neuronal function. Kv channels can be localized to synaptic terminals, axons, soma, or dendrites. Studies using cortical and hippocampal pyramidal neurons show findings of active electrical processing of synaptic input by somatodendritic voltage-dependent ion channels [15]. Studies using mutant genes to show altered properties of Kv channel proteins have helped to reveal the functional roles of K^+ channel protein domains as well as individual amino acids within these domains [15]. Among important recent findings are a number of mutations in potassium channels that cause channelopathies, meaning disease due to dysfunction of ion channels [15]. These include channelopathies affecting the muscle, kidney, and heart. KCNQ2 and KCNQ3 are two closely related potassium channels discovered during a search for mutations underlying a rare epilepsy disorder, which has led to advances in understanding neuronal signaling mechanisms [15].

1.2.2 Potassium channel kv2.1

Voltage-dependent kv2.1 K^+ channels mediate delayed rectifier Kv currents and are expressed in somata and dendrites of principal pyramidal neurons in the cortex and hippocampus [18]. In mammalian neurons, the kv2.1 channel may play a role in translating synaptic input to the output function of a neuron. The kv2.1 channel protein

core domain consists of approximately 300 amino acids. The N- and C-termini are cytoplasmic; the former of the two contains the tetramerization (T1) domain consisting of the molecular determinant for subfamily assembly of alpha subunits into functional tetrameric channels [18]. These cytoplasmic domains are involved in protein-protein interactions that regulate channel trafficking, localization and function. Many Kv genes are expressed in mammals and most notable is the high level of expression of kv2.1 in mammalian brain [18].

1.3 The Aging Process in the Mammalian brain

### 1.3.1 Normal aging mammalian brain

The aging process occurs over a lifespan and can be defined as a loss of physiological ability in some major organs resulting in senescence. This is not to say that all age-related biological changes cause dysfunction or disease. The normal mammalian brain will undergo critical biological changes as we age [7]. We can only consider the proposed mechanisms of differential brain aging such as neurotransmitter systems, stress and corticosteroids, microvascular changes, calcium homeostasis, and demyelination as we consider the age-related differences in the brain [7]. There is evidence that supports the idea that regions of the brain decrease in size matter as we age, and there is loss of neurons contributing to loss in function and overall cognitive decline [7].

1.3.2 Oxidation in the aging mammalian brain

Several studies have established an association between neuronal oxidative stress and the normal aging process. Aging and many age-related diseases are linked to mechanisms that maintain cellular function by reducing the nonselective oxidative stress

that can lead to protein aggregation and amyloid formation [7]. Age-related increases of incorrectly folded proteins can lead to protein damage, a process associated with the formation of protein aggregation and amyloid formation. Amyloid fibrils are protease resistant structures, meaning their formation cannot be reversed, which will only lead to further aggregation [7]. Further investigation is necessary to determine whether oxidative stress plays a primary role in neurodegenerative diseases or a secondary role to the pathological changes associated with diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD).

Protein oxidation is important in aging and age-related neurodegenerative diseases. Oxidation can cause modification of proteins and lead to diminished protein functioning [1]. Strong correlations have been observed between the accumulation of oxidized proteins and life-span where cellular concentrations of antioxidant enzymes have been up-regulated, suggesting a contributory role of ROS in aging [7]. Furthermore, the administration of free radical scavengers to aging animals reversed protein oxidation in aged brain [7]. It is important to investigate the relationships between oxidative stress and protein oxidation in aging brain.
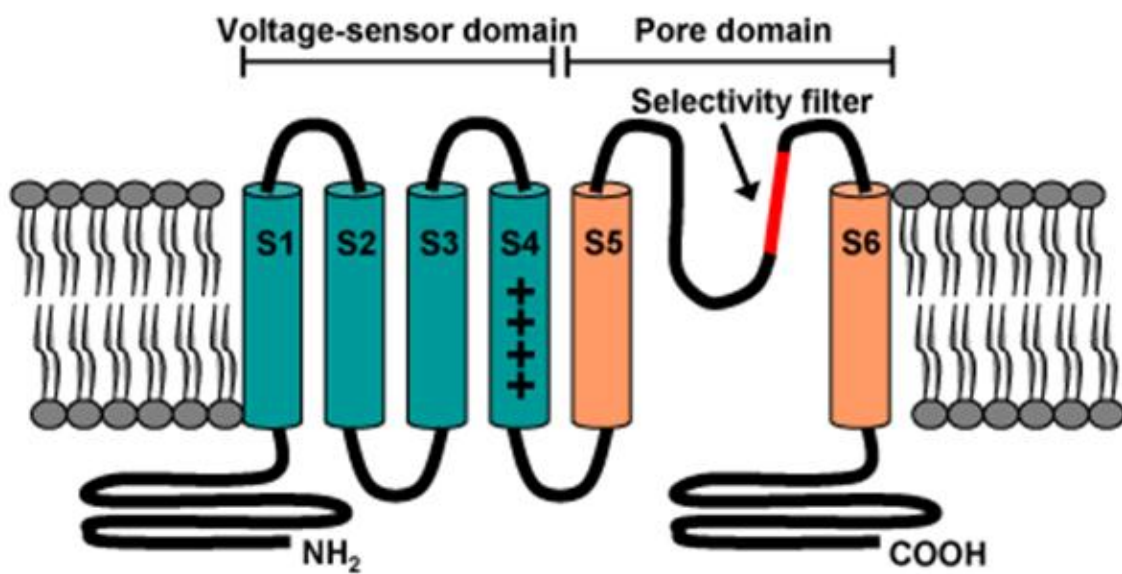
Figure 1.1 Illustrative representation of a single kv2.1 α subunit. Ref [18]
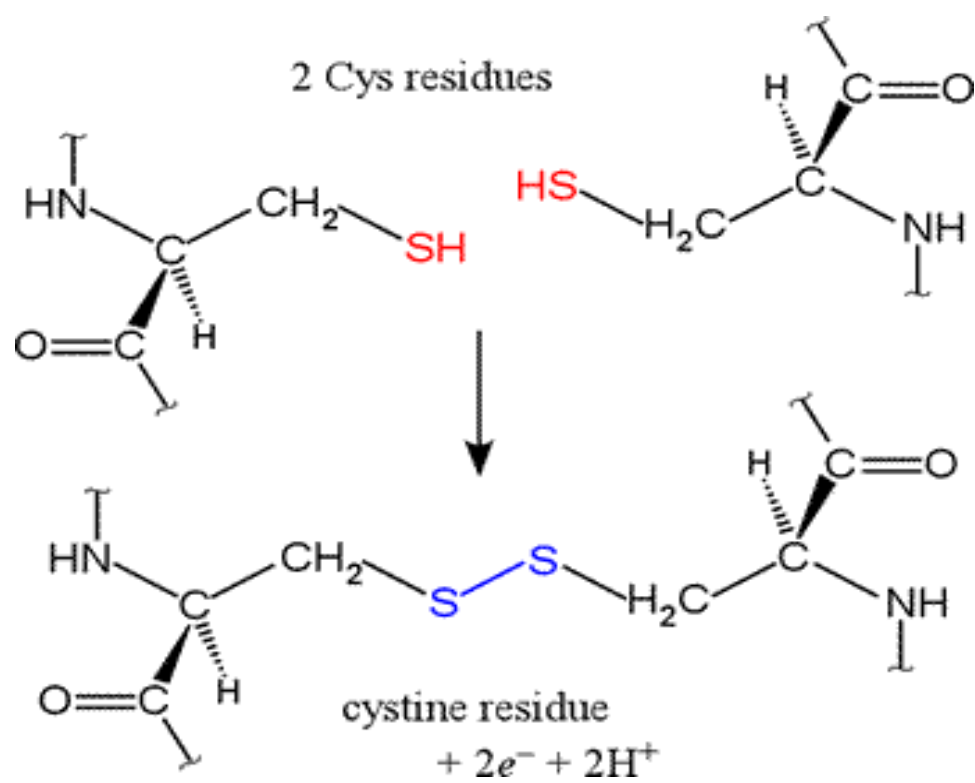
Figure 1.2 The formation of a disulfide bond from cysteine-cysteine interaction.
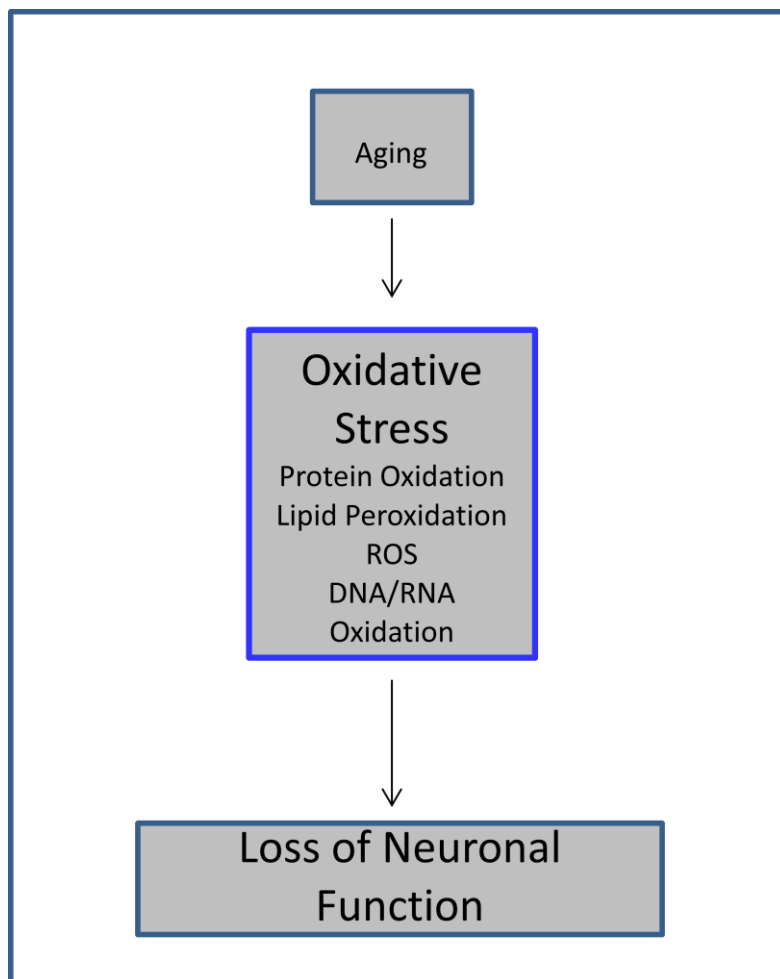
Figure 1.3 Proposed model of the role of oxidative stress in AD. Modified from Ref [1].

CHAPTER 2

Limiting oxidation in potassium channel kv2.1 using mutant C73A C29A

2.1 Introduction

Potassium (K^+) channels are ion channels that allow rapid and selective flow of potassium ions across the membrane. This flow of ions generates electrical signals in cells. Voltage-gated K^+ channels operate based upon changes in the transmembrane potential, generating electrical impulses in the nervous system. Conditions resulting in mutation or loss of function in K^+ channels, such as the oxidation of K^+ channels by reactive oxygen species (ROS), could potentially cause loss of neuronal function and/or lead to neurological disorders. During oxygen metabolism the transfer of single electrons to oxygen has a tendency to form ROS that can go on to initiate free radical reactions that lead to cellular damage.

ROS interacts with and modifies many ion channels including the oxidation of ion channels Kv1.4 and Kv1.7, which slows down inactivation and accelerates inactivation, respectively [5] Oxidation of channel SK upregulates channel activity, modifying how SK performs its normal physiological role of modulating mitochondrial ROS production in neutrophils [5]. Discussed further, hippocampal neurons responsible for long-term memory and spatial orientation have periods of activity associated with a rise in intracellular calcium leading to cellular after-hyperpolarization (AHP). AHP can be divided into fast, medium, and slow AHP (fAHP, mAHP, sAHP respectively). Any change in activity among one or more affecting excitability. Previous studies have shown that small conductance calcium-activated potassium (SK) channels generate mAHP. Most

relevant was the finding that upregulation of an SK isoform causes age-related memory loss in mice [5]. The fact that over expression of superoxide dismutase in aging rodents improves hippocampal-related learning and that ROS in *C.elegans* indirectly modifies neuronal excitability via voltage-gated K^+ channels is evidence of a relevant link between K^+ channels and ROS in the aging mammalian brain [5].

KVS-1 is a *Caenorhabditis elegans* voltage-gated K^+ channel associated with sensory function. The oxidation of the K^+ channel KVS-1 during aging causes sensory function loss in *C. elegans*, so it would stand that limiting the oxidation of KVS-1 could maintain neuronal function [4]. Data from a study assessing oxidation of KVS-1 focusing on chemotaxis to water-soluble attractants, a function controlled by KVS-1, indicated that mutation of a single cysteine (C113S) made KVS-1 channels significantly resistant to oxidation. The effects of H_2O_2 and CHT, both oxidants, produced a significant loss of function in WT-KVS-1 compared with that of C113S-KVS-1. Aging worms progressively lost the ability to perform chemotaxis, which is consistent with the theory that ROS levels increase during aging [4]. Further experimentation showed that the C113S mutation was less progressive in this decline. Superoxide dismutase activity was higher in aging worms, also consistent with increased levels of ROS during aging. The data shows C113S plays a significant role by which changes in oxidation slow KVS-1 inactivation, presenting the question whether oxidation of this residue leads to formation of disulfide-bonded cysteines [4]. KVS-1 being a homolog of the kv2.1 channel, these data provides further evidence that changes in K^+ channel homeostasis are associated with aging in the mammalian brain.

C. *elegans*, an established model for studying the biological aging process, were used to determine whether ROS interactions in K$^+$ channels play a role in mechanisms underlying age-related neurodegeneration [4]. In an invertebrate AD model using C. *elegans*, the C73A-KCNB1 homolog C113S-KVS-1 proved resistant to oxidation and protected specific neurons [4]. Located in the N terminus, cys113 corresponds to cys73 in humans and is protected in the kv2.1 channel [6]. Evidence that the C113S mutation is significantly resistant to oxidation suggests that cys73 would produce similar results.

Cys73 was mutated to alanine (C73A) and to serine (C73S) and treated with oxidants [6]. Treatment with oxidizing agents led to the formation of oligomers, but oligomer formation was reduced in C73A, as shown in Figure 2.1. The results of both mutants showed no oligomerization when the proteins were oxidized [6]. We define the oxidation of kv2.1 with the formation of oligomers upon treatment with oxidizing agents and quantify the results by measuring the intensity of the results bands (oligomer/monomer bands) to obtain the oxidation ratio, as shown in Figure 2.2. The kv2.1 channel produces multiple oligomers indicating that cys73 cannot oligomerize alone [6]. Mutant C73A proved most useful for these studies as the channel formed was most similar to WT kv2.1 channels, in contrast to the nonconductive channels formed by C73S. These findings led to the current study in which we used alanine scanning mutagenesis to identify other cysteines that fail to oligomerize when oxidized, as shown in Figure 2.4.
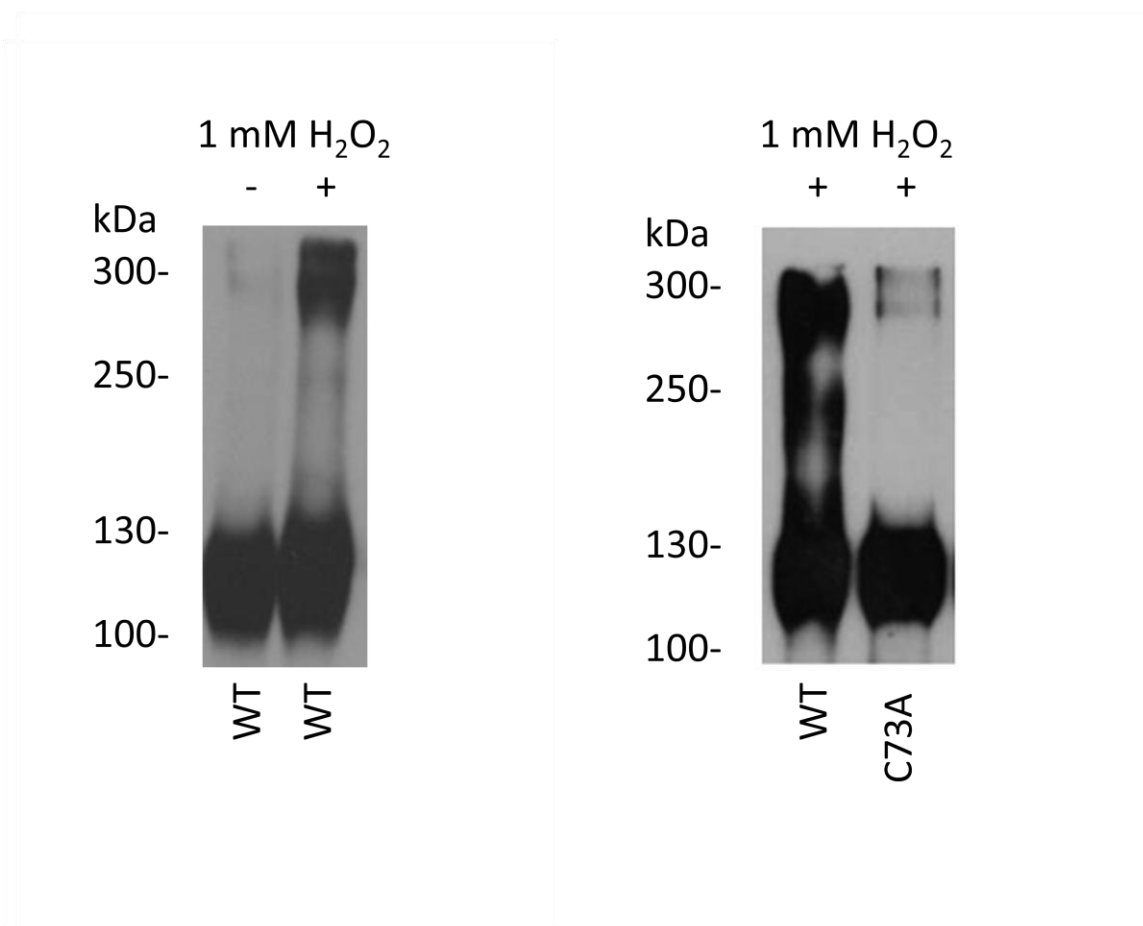
Figure 2.1 Western visualization of wild-type kv2.1 and C73A with and without 1mM H_2O_2 for 5-10 min before lysis (with oligomers running between ~250 kDa and ~350 kDa). WT kv2.1 and C73A were expressed in CHO cells as described in "Material and Methods". Mutant C73A showed less oligomerization than WT kv2.1. Modified from Ref [6].
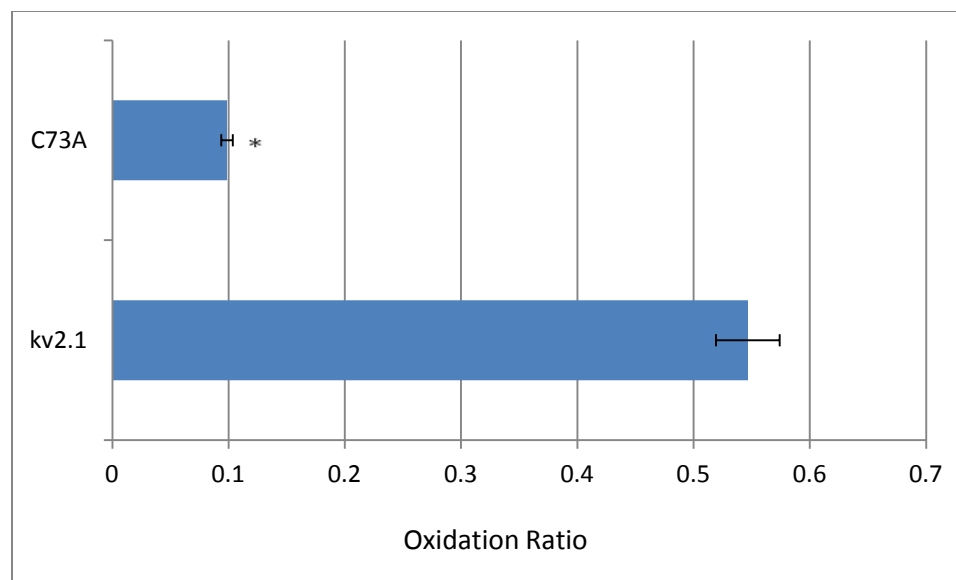
Figure 2.2 Bar graph showing the oxidation ratio difference between WT kv2.1

and C73A from Fig 2.1. Bands were measured and the difference between

oligomer bands and monomer bands used to obtain the oxidation ratio

(oligomer/monomer = oxidation ratio) (students *t*-test *0.01, p>0.05) [Data from

Ref 6].

2.2 Material and Method

Materials

All cell culture products were obtained from Invitrogen (Carlsbad, CA). The primers used for RT-PCR are from Integrated DNA Technologies (Coralville, IA).

Cell Culture

The Chinese hamster ovary (CHO) cells were grown in Gibco F12 Nutrient Medium with 10% Fetal Bovine Serum (FBS) and kept in a humidified incubator of 5% CO_2 at 37°C.

Molecular Biology

Cysteine to alanine mutants (C29A, C111A, C132A, C133A, C167A, C168A, C237A, C397A, C398A, C831A) were constructed by polymerase chain reaction (PCR) using pfu polymerase and the kv2.1 HA plasmid. Double mutants (C73A-C29A and C237A-C29A) were later constructed using similar methods. Primers and sequences are listed in Table 2.1. Each amplification cycle consisted of 1 min at 95°C for denaturation, 0.5 min at 63°C for primer annealing, and 9 min at 72°C for extension. After PCR amplification, the products were analyzed by agarose gel electrophoresis and visualized using ethidium bromide staining.

Western Blotting

Cells were plated to grow using 12-well plates twenty-four hours before transfection. Cells were transfected at 90% confluence using Lipofectamine 2000. Twenty-four hours after transfection, cells were washed with phosphate buffered saline (PBS) and exposed to 0.5 mM H_2O_2 (diluted in PBS) for 3 minutes. After cells were washed with PBS twice for 2 minutes, the cells were lysed with 0.05 mL of RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1%

SDS, 10 mM iodoacetamide, phosphatase, and protease inhibitors) and 0.05 mL 2x

sample buffer (2M Tris, pH 6.8, 20% SDS, 20% glycerol, dd H_2O, and traces of

bromophenol blue). The cells were sonicated for 5 seconds, followed by boiling for 5

minutes and centrifugation. Proteins were run on a resolving gel of 8% SDS-PAGE (non-

denaturing) and transferred to a polyvinylidene fluoride (PDVF) membrane. The

membrane was incubated in a solution of nonfat milk and PBS with Tween 20 (PBST)

for 1 hour before incubation in a solution of 2% albumin and TBST for 2 hours. After

washing the membrane with PBST for 15 minutes (three washes for 5 minutes each

wash), the membrane was incubated with POD chemiluminescence substrates and

exposed.

Statistics Analysis

The results of each western blot were quantified by the densiometric measuring of the

bands (oligomer/monomer bands) to obtain the oxidation ratio, using ImageJ software.

All values are presented as the mean ± standard deviation (SD) of the mean. All

experiments were repeated at least three times. The statistical significance was evaluated

using the paired Student's *t*-test for comparison between two means. P values < 0.05 were

considered to be statistically significant.

2.3 Results

2.3.1 Oxidizing the kv2.1 channel

Fifteen cysteines were identified in the kv2.1 channel, as shown in Figure 2.3. Using the polymerase chain reaction (PCR) ten cysteine-alanine mutants were constructed: C29A, C111A, C132A, C133A, C167A, C168A, C237A, C397A, C398A, and C831A. Previously constructed mutants were: C73A, C710A, C813A, and C594A. The mutant C63A could not be obtained successfully through PCR reaction. Oligos were constructed using primer design software, substituting each cysteine for an alanine, and ordered from Integrated DNA Technologies. Plasmids were obtained for each mutant and protein quantification of each mutant was observed using the western blotting technique. The oxidant H_2O_2 was used to induce oxidation in each mutant after expressing each mutant in Chinese Hamster Ovary (CHO) cells. It was determined that the concentration of 0.5 mM of H_2O_2 produced optimal results as initial tests of a 1 mM concentration of H_2O_2 resulted in excessive oxidation in both the WT and mutants constructs (with a WT kv2.1 oxidation ratio of 2.04). The amount of oxidation was measured for a control construct (WT kv2.1) and for all ten mutants (C29A, C111A, C132A, C133A, C167A, C168A, C237A, C397A, C398A, and C831A). Previous research has found that the mutated kv2.1 channel C73A is functionally indistinguishable from WT kv2.1 channel, but does not oligomerize after treatment with oxidizing agents [6].

Based on the results of the alanine scanning mutagenesis, as shown in the graph of Figure 2.4, C29A and C831A showed less oligomerization when placed under oxidative conditions (C29A with an oxidation ratio of 0.44 and C831A with an oxidation ratio of 0.64 compared to the WT kv2.1 ratio of 1.06). Both the C29A and C831A

mutants showed less oligomerization, but C29A was used for further studies on limiting oxidation in the kv2.1 channel. C29A and C73A are both located in the N terminal of the kv2.1 channel. C29A and C73A have the potential to form a disulfide bridge due to the oxidation of the cysteine residues and play a role in the crosslinking of proteins. We examined the amount of oligomerization between WT kv2.1 and mutant C29A, as shown in Figure 2.5. There was a significant difference in the amount of oxidation between WT kv2.1 and mutant C29A (0.04, $p>0.05$) as indicated using the student's *t* test.

Both free cysteine residues and cysteine residues covalently bonded to other cysteine residues to form disulfide bonds can be found in native proteins. Cysteine residues play an important role by crosslinking proteins to increase the rigidity of proteins. Disulfide bonds are formed by oxidation of the thiol groups of cysteine residues and play an important role in the folding of proteins. Thus, disulfide bridges between cysteine residues within a polypeptide support the protein's tertiary structure. Disulfide bond formation can lock a channel in a non-conducting state and prevent the conformational transitions required to open the channel. Results from previous research concluded that oligomer formation is the result of crosslinking kv2.1 channel subunits to each other [6]. The experiments presented here identified C73A, C29A, and C831A as having the potential to form a crosslinked dimer or trimer arrangement, as shown in Figure 2.6.

```
  1  MPAGMTKHGSRSTSSLPPEPMEIVRSKACSRRVRLNVGGLAHEVLWRTLDRLPRTRLGKLRDCNTHDSLL
 71  EVCDDYSLDDNEYFFDRHPGAFTSILNFYRTGRLHMMEEMCALSFSQELDYWGIDEIYLESCCQARYHQK
141  KEQMNEELKREAETLREREGEEFDNTCCAEKRKKLWDLLEKPNSSVAAKILAIISIMFIVLSTIALSLNT
211  LPELQSLDEFGQSTDNPQLAHVEAVCIAWFTMEYLLRFLSSPKKWKFFKGPLNAIDLLAILPYYVTIFLT
281  ESNKSVLQFQNVRRVVQIFRIMRILRILKLARHSTGLQSLGFTLRRSYNELGLLILFLAMGIMIFSSLVF
351  FAEKDEDDTKFKSIPASFWWATITMTTVGYGDIYPKTLLGKIVGGLCCIAGVLVIALPIPIIVNNFSEFY
421  KEQKRQEKAIKRREALERAKRNGSIVSMNMKDAFARSIEMMDIVVEKNGENMGKKDKVQDNHLSPNKWKW
491   TKRTLSETSSSKSFETKEQGSPEKARSSSSPQHLNVQQLEDMYNKMAKTQSQPILNTKESAAQSKPKEEL
561   EMESIPSPVAPLPTRTEGVIDMRSMSSIDSFISCATDFPEATRFSHSPLTSLPSKTGGSTAPEVGWRGAL
631   GASGGRFVEANPSPDASQHSSFFIESPKSSMKTNNPLKLRALKVNFMEGDPSPLLPVLGMYHDPLRNRGS
701   AAAAVAGLECATLLDKAVLSPESSIYTTASAKTPPRSPEKHTAIAFNFEAGVHQYIDADTDDEGQLLYSV
771   DSSPPKSLPGSTSPKFSTGTRSEKNHFESSPLPTSPKFLRQNCIYSTEALTGKGPSGQEKCKLENHISPD
841   VRVLPGGGAHGSTRDQSI
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Figure 2.3 Human kv2.1 protein translation map highlighting all 15 cysteines.
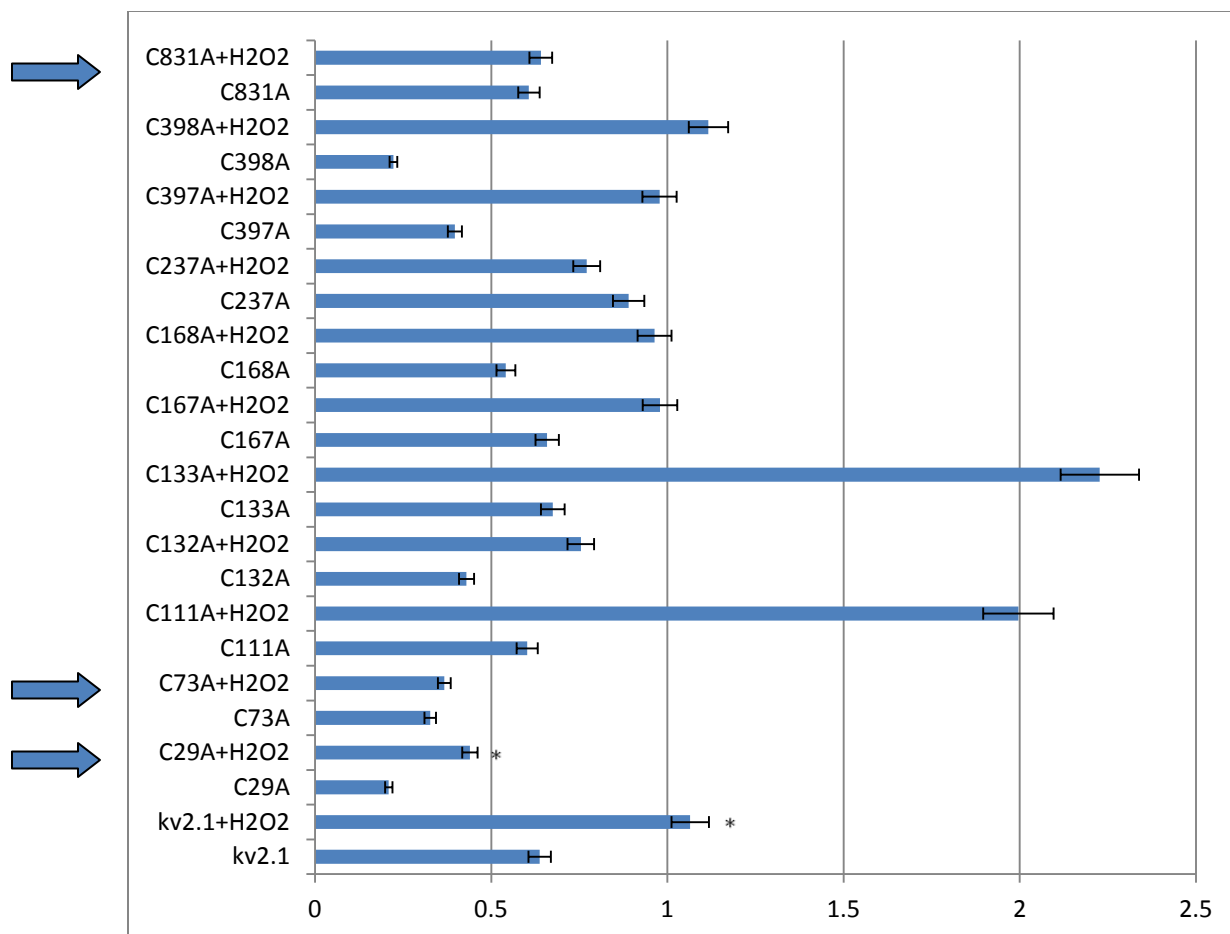
Figure 2.4 Bar graph showing the results of screening ten cysteine to alanine

mutants and C73A based on the kv2.1 protein translation map shown in Fig 2.3

(C29A, C111A, C132A, C133A, C167A, C168A, C237A, C397A, C398A, and C831A

(students *t*-test *0.04, p>0.05).
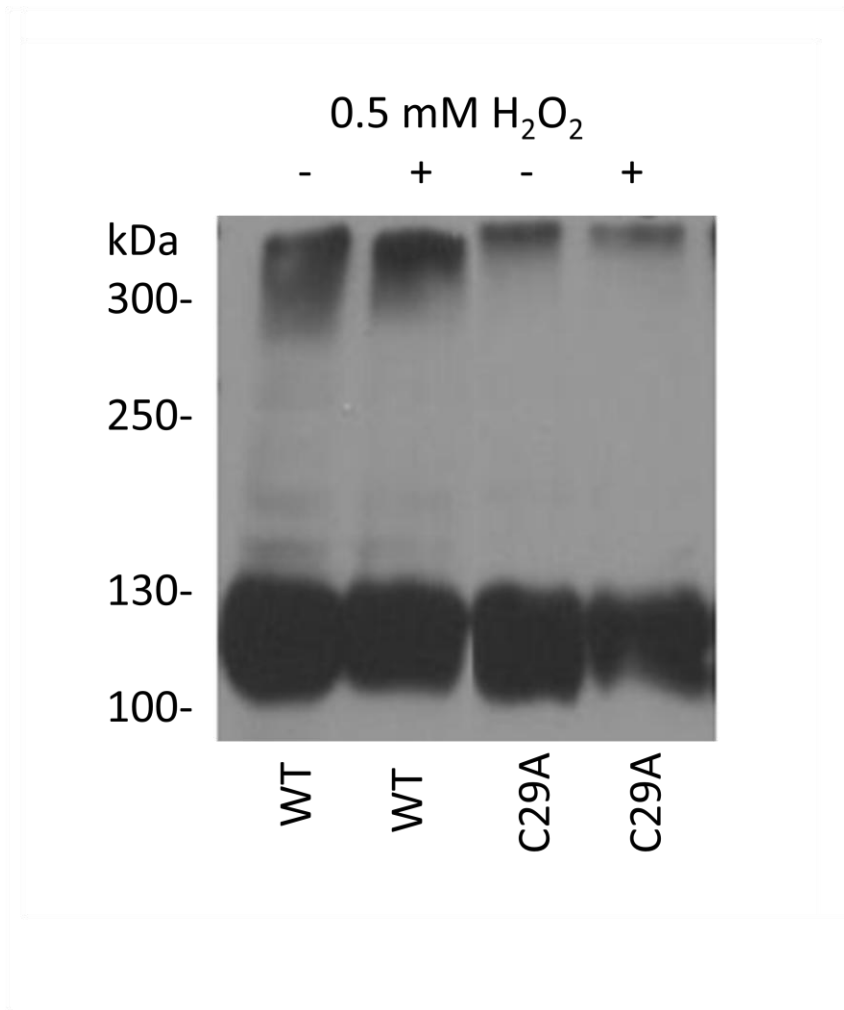
Figure 2.5 Western visualization of wild-type kv2.1 and C29A with and without 0.5 mM H_2O_2 for 3 min before lysis. WT kv2.1 and C29A were expressed in CHO cells as described in "Material and Methods". Mutant C29A showed less oligomerization than WT kv2.1
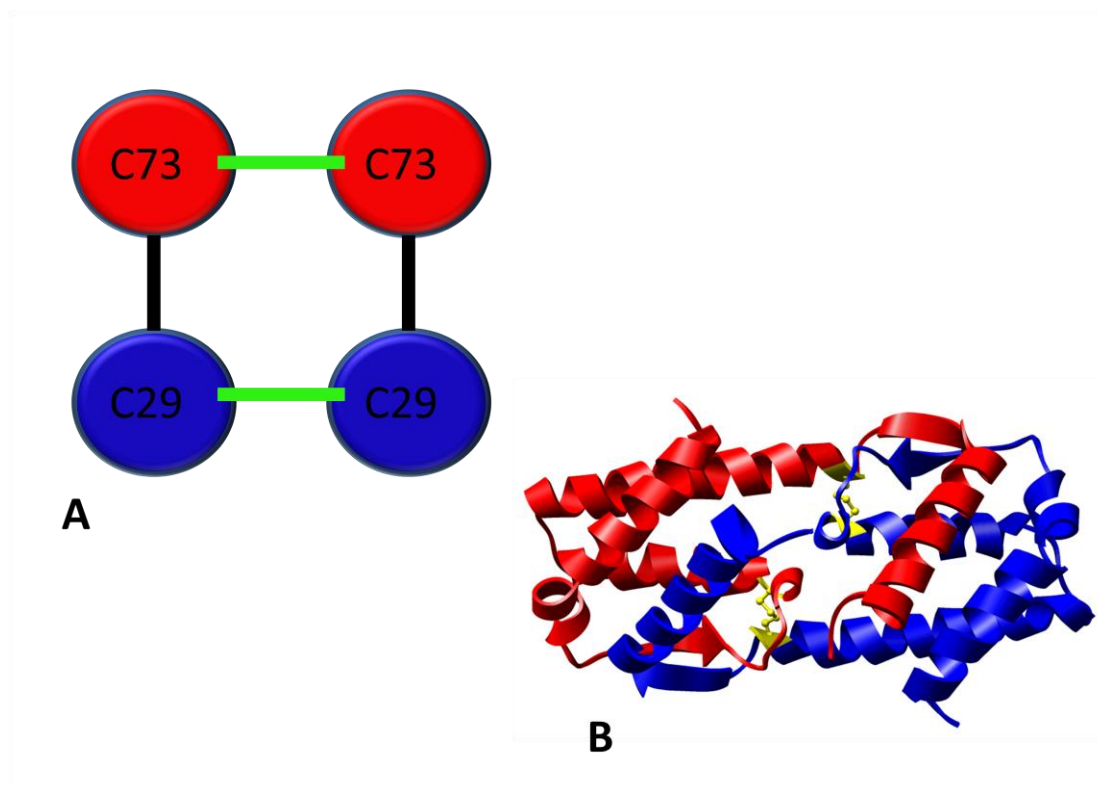
Figure 2.6 A) Cysteines C29 and C73 are indicated with blue and red spheres,

respectively. The two green bars represent the inter-subunit disulfide bonds.

**B)** Ribbon model of the dimeric structure of C29A and C73A.

2.3.2 Similar properties between C73A and C29A

Oligomerization results of the cysteine to alanine (C73A) mutation of cys73 showed that one or more cysteines may be involved in the process. The oxidation of C29A showed less oligomerization than that of WT kv2.1 when placed under oxidative conditions. Using the student's *t* test, there was a significant difference between WT kv2.1 and C73A (0.01, p>0.05) and between WT kv2.1 and C29A (0.04, p>0.05). Since there was a significant difference in the amount of oxidation between WT kv2.1 and mutant C29A, similar to the results of C73A [6], both mutants were combined to produce a double mutant (DM) construct (C73AC29A).

2.3.3 Limiting oxidation using the C73AC29A double mutant

We examined the amount of oligomerization between WT kv2.1 and mutants C29A, C73A, and C73AC29A as shown in Figure 2.7. C73AC29A showed less oligomerization when placed under oxidative conditions (with an oxidation ratio of 0.13 compared to kv2.1 with 1.11). There was a significant difference between WT kv2.1 and mutant C73AC29A (0.005, p>0.05). C73AC29A showed less oligomerization compared to the single mutants C29A (0.18, p>0.05) and C73A (0.13, p>0.05) when placed under oxidative conditions, but neither comparisons were significantly different. The DM C237AC29A was constructed and observed as a negative control. We examined the amount of oligomerization between WT kv2.1 and mutants C29A, C237A, and C237AC29A, as shown in Figure 2.9. C237AC29A showed similar oligomerization results to kv2.1 when placed under oxidative conditions (with an oxidation ratio of 0.35 compared to kv2.1 with 0.36). C237AC29A showed more oligomerization compared to

the DM C73AC29A (0.02, p>0.05) when placed under oxidative conditions (Figure

2.12), there was a significant difference between the double mutants.
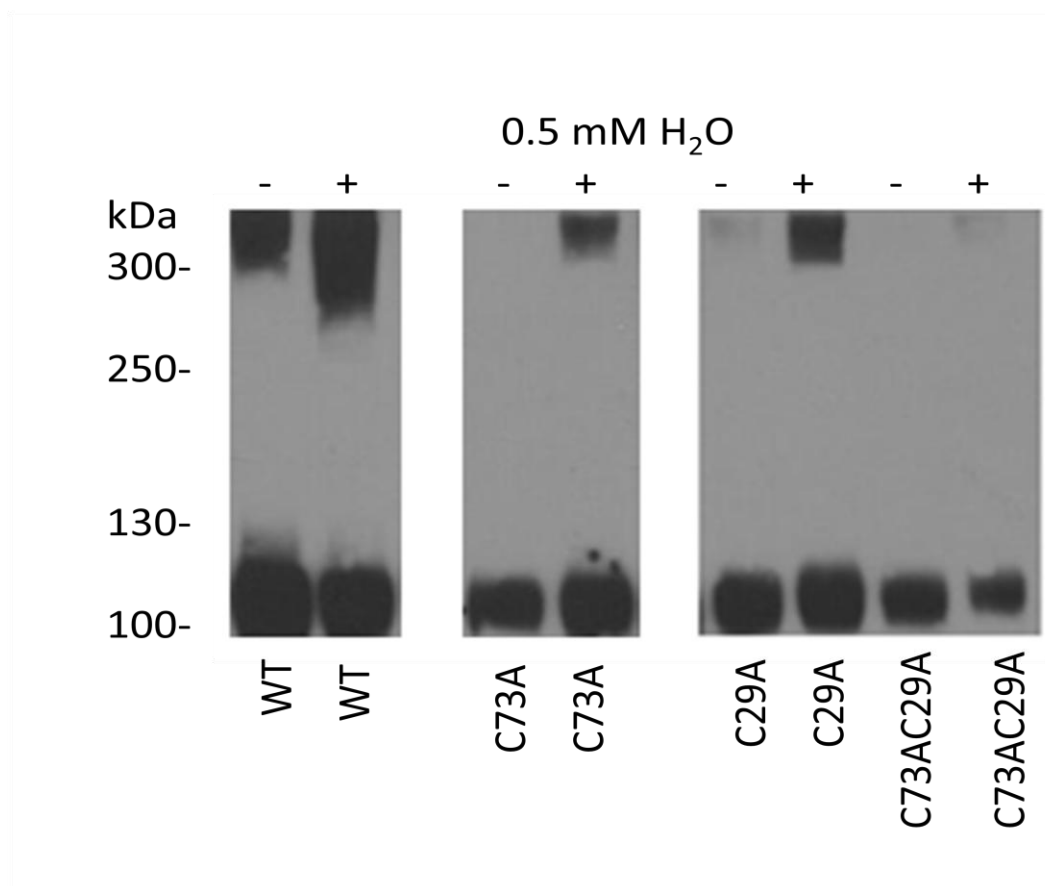
Figure 2.7 Western visualization of wild-type kv2.1 and double mutant C73AC29A with and without 0.5 mM H_2O_2 for 3 min before lysis. WT kv2.1 and double mutant C73AC29A were expressed in CHO cells as described in "Material and Methods". The result of the Western Blot analysis showed less oligomerization in the double mutant C73AC29A, C73A, and C29A.
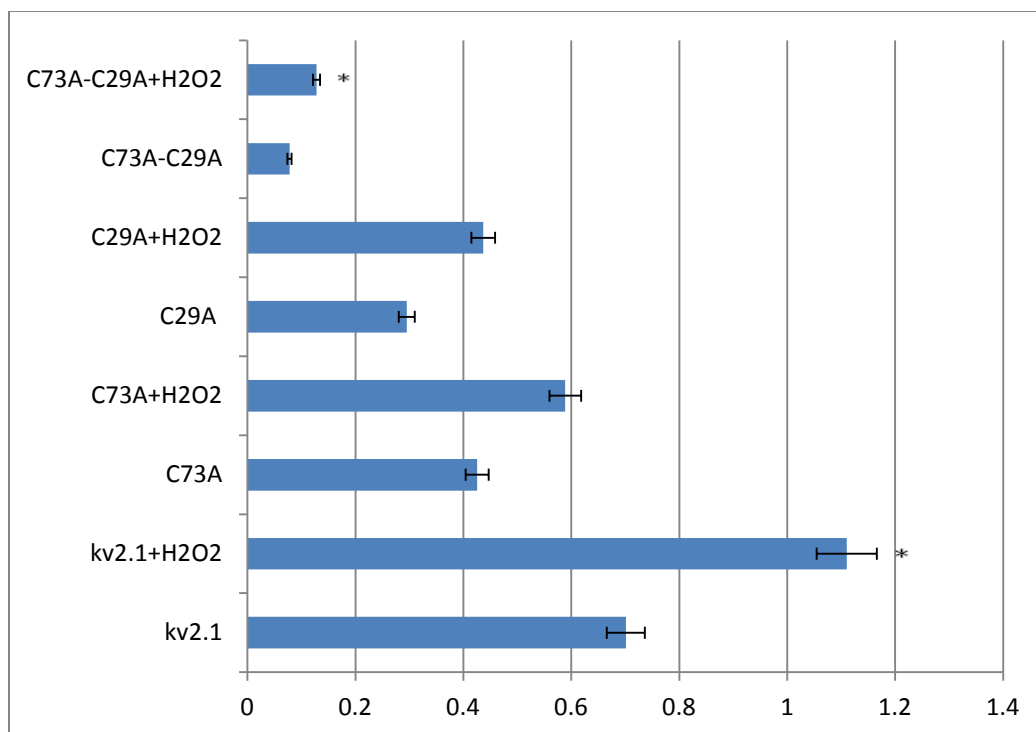
Figure 2.8 Bar graph showing the oxidation ratio difference between WT kv2.1 and DM C73AC29A. The results of the Western Blot Analysis in Fig 2.7 were quantified using the oxidation ratio (oligomer bands/monomer bands) (students *t*-test *0.005, p>0.05).

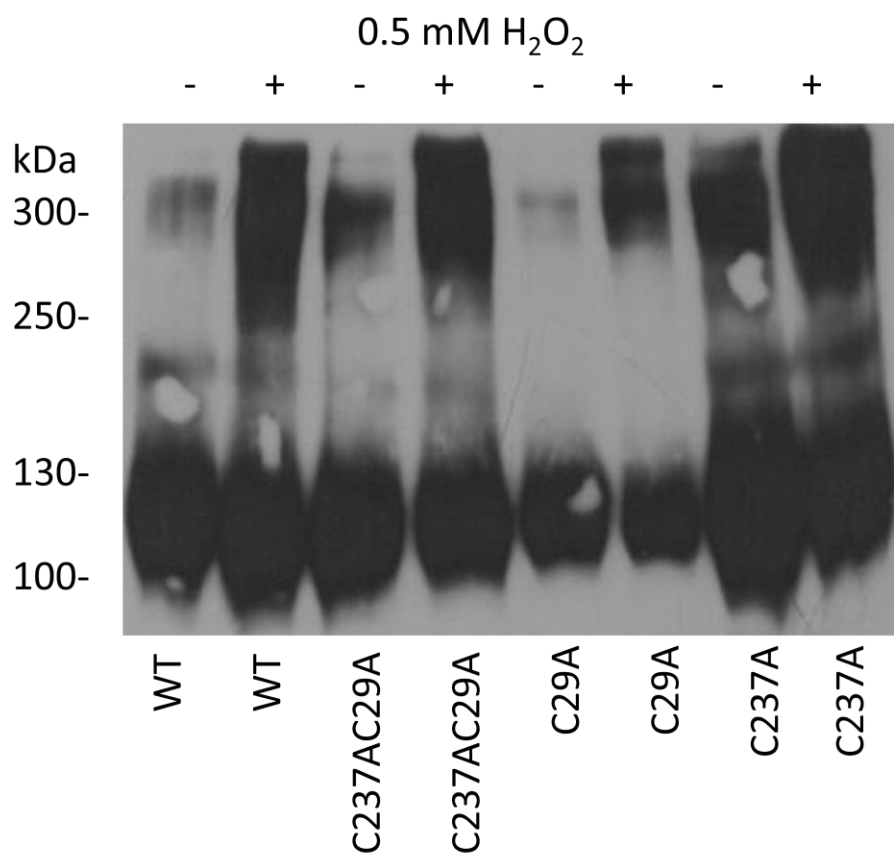**Figure 2.9** Western visualization of wild-type kv2.1 and double mutant C237AC29A with and without 0.5 mM H_2O_2 for 3 min before lysis. WT kv2.1, C237A, and DM C237AC29A were expressed in CHO cells as described in "Material and Methods".
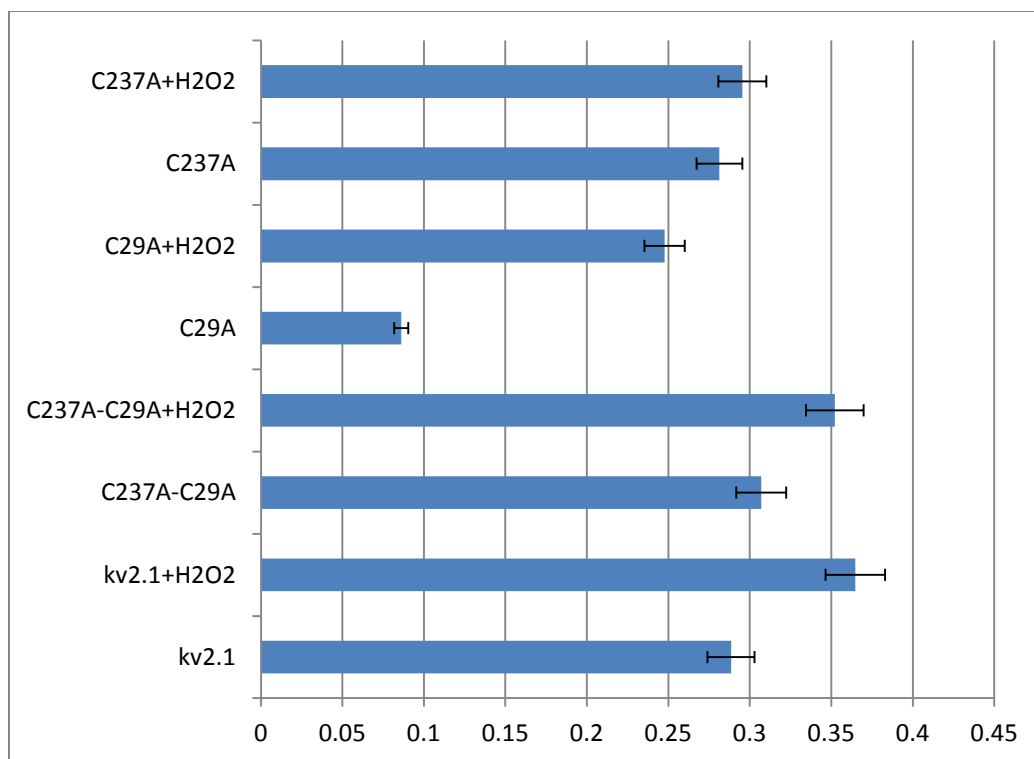
Figure 2.10 Bar graph showing the oxidation ratio difference between WT kv2.1

and DM C237AC29A. The results of the Western Blot Analysis in Fig 2.9 were

quantified using the oxidation ratio (oligomer bands/monomer bands).

Figure 2.11 Western visualization of wild-type kv2.1 and both DM C237AC29A and C73AC29A with and without 0.5 mM H_2O_2 for 3 min before lysis. DM C237AC29A was used as a negative control.

Figure 2.12 Bar graph showing the oxidation ratio difference between WT kv2.1 and both DM C73AC29A and C237AC29A. The results of the Western Blot Analysis in Fig 2.11 were quantified using the oxidation ratio (oligomer bands/monomer bands) (students *t*-test *0.02, p>0.05).
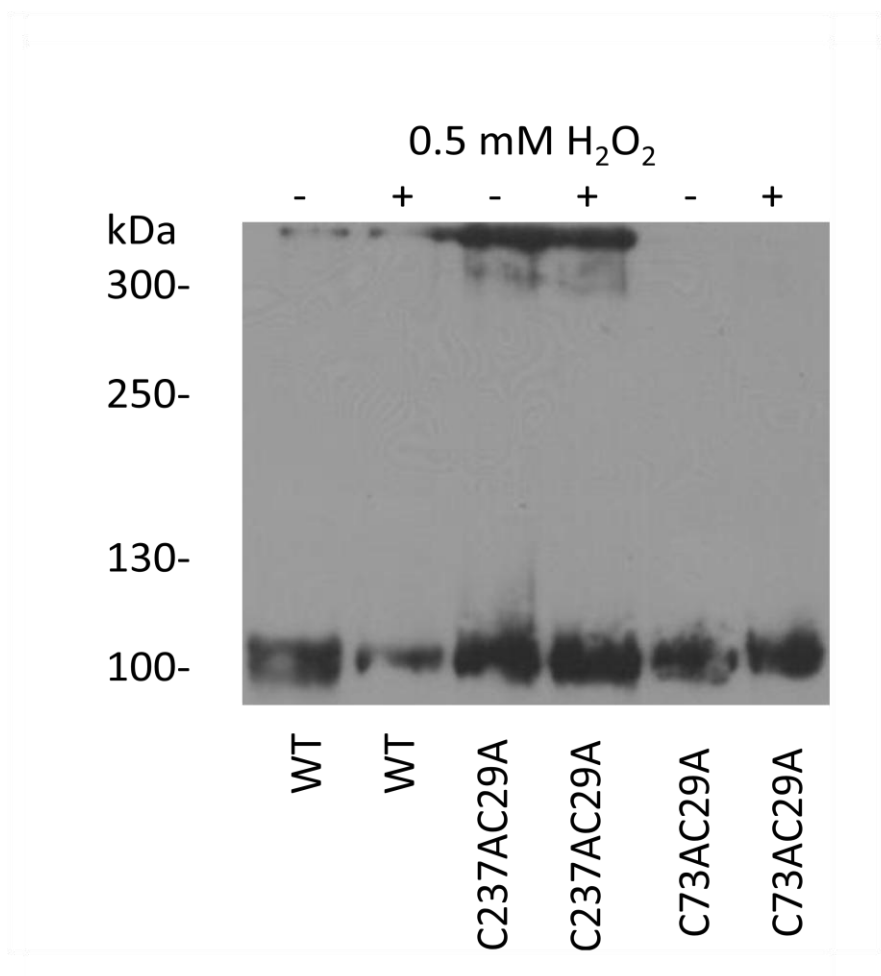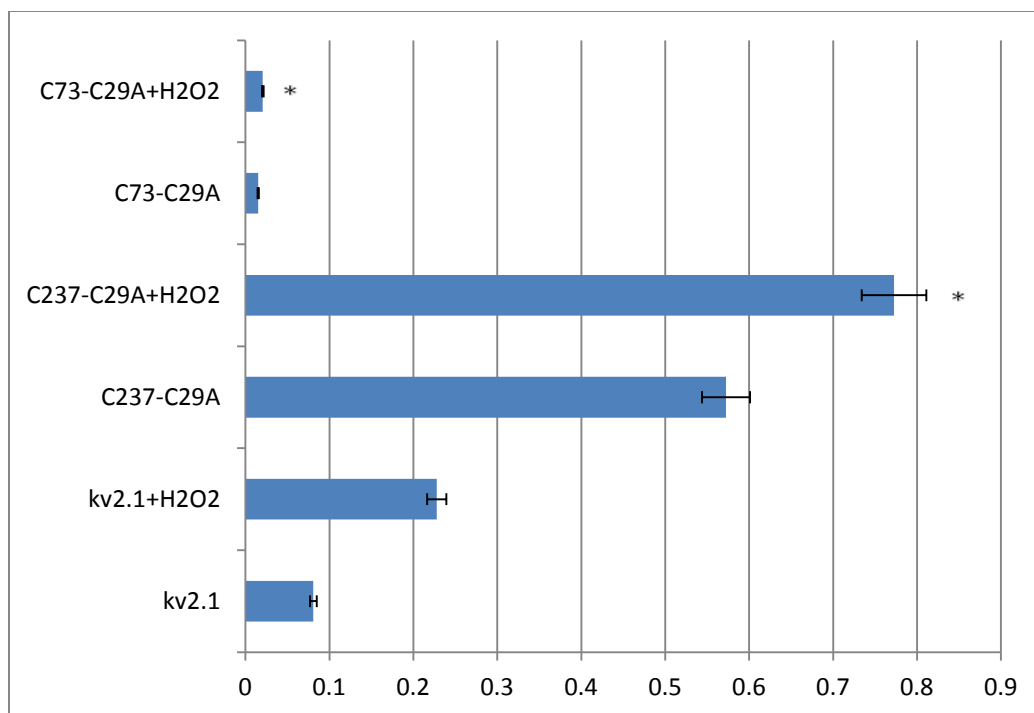
2.4 Discussion

2.4.1 Similar properties between C73A and C29A

Oxidation of K^+ channels by ROS leads to altered function of these channels. Previous research has suggested that the kv2.1 channel is susceptible to oxidative stress [6]. Oxidation of kv2.1 can lead to loss of channel function and neuronal damage. The formation of disulfide bonds between cysteine residues in proteins following oxidative stress suggests that cysteine interaction in voltage-gated K^+ channel kv2.1 plays a key role in the oxidation of kv2.1. The complexity of potential interactions between ROS, phosphorylation, membrane localization, and other protein interactions that regulate kv2.1 make it difficult to target this pathway as a specific factor affecting oxidation.

Expressing the kv2.1 channel in CHO cells with the oxidizing agent, H_2O_2, led to the formation of oligomers (Figure 2.1). Oligomer formation is the result of cross-linking kv2.1 subunits with one another [6]. Mutants C29A, C73A, and C831A have the potential to form disulfide bridges, allowing crosslinking of channel subunits. Both C29A and C73A are located in the N- terminus. Mutant C831A is located in the C-terminus. Residues in both domains influence the physical association between the N and C-termini. Interactions between the N- and C-termini are involved in channel assembly and subunit specificity. Previous research has shown that a domain in the N-terminus can interact with the C-terminus [6]. The double mutant C73A-C710A did not oligomerize upon treatment with H_2O_2, suggesting that other cysteine-cysteine interactions are possible [6].

2.4.2 Limiting oxidation using the C73AC29A double mutant

Previous research has shown that C73A does not oligomerize upon treatment with oxidizing agent H_2O_2 [6]. Findings from this study suggest that C29A shares similar properties to C73A. C29A shows less oligomerization than WT kv2.1, providing evidence that using both C73A and C29A variants could provide effective protection against oxidative damage to the kv2.1 channel. The data suggests that the double mutant C73AC29A would fail to oligomerize under oxidative conditions. The results show that C73AC29A does not oligomerize upon treatment with H_2O_2. There was a significant difference between C73AC29A and the WT kv2.1 channel. There was not a significant difference between C73AC29A and single mutants C73A and C29A. The double mutant C237AC29A was used as the negative control to observe any differences that may have been seen in a double mutant using the C29A mutant. These results are in good agreement with existing knowledge that oxidative conditions induce oligomerization and that more than one cysteine may be involved in this process.

2.5 Conclusions

Previous research suggests that oxidation of K^+ channels is a general mechanism contributing to neurotoxicity [6]. This paper supports the idea that the oligomerization of kv2.1 due to oxidative stress leads to a continuous cycle of increased oxidative stress and results in neurotoxicity. C73AC29A has been proven to show less oligomerization when exposed to oxidative stress, suggesting it may have protective properties against oxidation of K^+ channel kv2.1. Oxidative stress occurs early in the physiological process of aging and protein oxidation is important in aging and age-related neurodegenerative diseases. The K^+ channel kv2.1 plays a significant role in neuronal excitability. Preserving the function of the channel by limiting the extent of oxidation could prove to

be an effective intervention against further oxidative damage, as we continue to establish

an association between neuronal oxidative stress and the normal aging process.

Primer	Orientation	Sequence
C29A	Sense	CGCAGCAAGGCGgcCTCTCGGCGGGTC
	Antisense	GACCCGCCGAGAGgcCGCCTTGCTGCG
C73A	Sense	CGCTGCTCGAGGTGgcCGATGACTA
	Antisense	GCTGTAGTCATCGgcCACCTCGAGCA
C237A	Sense	GTGGAGGCCGTGgcCATCGCATGGTTC
	Antisense	GAACCATGCGATGgcCACGGCCTCCAC

Table 2.1 The primers used for amplification in RT-PCR.

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