Oxidative Stress Suppresses The Heteromeric Kir4.1-Kir5.1 Channel Via S-Glutathionylation

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OXIDATIVE STRESS SUPPRESSES THE HETEROMERIC KIR4.1-KIR5.1 CHANNEL VIA S-GLUTATHIONYLATION

by

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Under the Direction of Chun Jiang

ABSTRACT
Potassium transport channels regulate cellular excitability and membrane potentials. Dysfunction of potassium channels may happen under certain pathophysiological conditions via post-translational modification (PTM), inducing the S-glutathionylation, in oxidative stress. Here, we try to demonstrate the effect of ROS on Kir4.1 and Kir5.1 channel. It is found that the target subunit of ROS is Kir5.1 instead of Kir4.1 subunit. Kir4.1-Kir5.1 heteromeric channel is inhibited by different kinds of oxidants. The patch clamp study confirmed that these oxidants work on the intracellular side of the Kir5.1 subunit. Further experiments demonstrate that the inhibition of Kir4.1-Kir5.1 channels is through S-glutathionylation of the Kir5.1 subunit on Cys158. The information we find may help to better understand the Kir4.1 and Kir5.1 channel modulation mechanism in various pathophysiological conditions, such as oxidative stress.

INDEX WORDS: Kir4.1, Kir5.1, ROS, Oxidative stress, S-Glutathionylation, H₂O₂, Diamide, PDSs, GSH, GSSG
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1 INTRODUCTION

Inwardly rectifying potassium (Kir) channels are distinct from the conventional voltage-gated potassium (Kv) channels. There are classical Kir channels, G protein-gated Kir channels, K⁺ transport channels and ATP-sensitive K⁺ channels in this family. The third group of Kir channels, such as Kir1.1, and Kir4.1, play a role as K⁺ transporters (Butt-Kalsi, 2006). The Kir4.1 is composed of homomorphic channels or heteromorphic channels with Kir5.1 in tetrameric forms. The heteromorphic Kir4.1-Kir5.1 channel endues extended functional range of Kir channel [1-3]. Kir4.1-Kir5.1 channel is regulated by different G protein coupled receptors of neurotransmitters [4], as well as post-translational modification (PTM), such as phosphorylation via PKC [5]. Therefore, like other ion channels [6-9], Kir4.1 and Kir4.1-Kir5.1 channel are possibly regulated by PTMs (e.g. S-nitrosylation, S-glutathionylation and etc.) in pathological conditions, especially oxidative stress.

Oxidative stress occurs when the production of reactive oxygen species (ROS) overwhelms the anti-oxidant defense system of the cell. Production of ROS has several major sources, in which the pathway of the mitochondrial electron transport chain takes over 90% responsibility of ROS formation [10]. It has been demonstrated that ROS is the outstanding contributor to the disruption of protein function in many diseases, such as ischemia, inflammation and neurodegenerative diseases [11-13].

1.1 ROS production, regulation and physiological roles

Typical reactive oxygen species (ROS) contain superoxide (\(^{\cdot}O_2^-\)), hydrogen peroxide (H₂O₂) and hydroxyl radicals (\(^{\cdot}OH\)). More broadly conceived, singlet oxygen (\(^{1}O_2\)), nitric oxide (NO), and peroxynitrite anion also belong to this group. Superoxide anion (\(^{\cdot}O_2^-\)) is the precursor
of most ROS, which is produced from a one-electron reduced reaction of oxygen. Superoxide dismutases catalyzing the dismutation of \( \cdot O_2^- \) produce hydrogen peroxide (H\(_2\)O\(_2\)), which in turn may be partially reduced by catalase to hydroxyl radical (\( \cdot \)OH), one of the strongest oxidant in nature, or be completely reduced to water. Major sources of ROS include enzymatic and non-enzymatic pathways from cell membrane of various cells and some cellular organelles, as peroxisomes, endoplasmic reticulum and mitochondrion. Enzymatic sources implicate NADPH oxidases (NOX) on the cell membrane of macrophages, endothelial cells and white blood cells and cytochrome P\(_{450}\)-dependent oxygenases \[14\]. In vivo, the non-enzymatic production of ROS may occur when a single electron is directly transferred to \( O_2 \) by reduced forms of coenzymes in mitochondrial electron transport chain (ETC), or by reduced xenobiotics. The mitochondrial electron transport chain is considered as the major source of ROS \[10, 15\].

ROS is regulated on several levels to keep the balance of its generation and scavenging. First, several antioxidant enzymes built up the antioxidant defenses, such as superoxide dismutases (SOD) (Fridovich, 1995), catalase (Radi et al. 1991), glutathione peroxidases (GPXs) (Chance et al. 1979) and peroxiredoxin (PRXs). Superoxide (\( \cdot O_2^- \)) is converted to hydrogen peroxide (H\(_2\)O\(_2\)) by SOD. Also, the dismutation can happen spontaneously. Additionally, the precursor of \( \cdot \)OH and the product of \( \cdot O_2^- \), H\(_2\)O\(_2\), is mostly diminished by GPXs. Catalase is another major antioxidant enzyme, decomposing the powerful and damaging H\(_2\)O\(_2\). Except these antioxidant enzymes, vitamin E, also known as ascorbic acid or \( \alpha \)-tocophenol, reduced GSH, as antioxidant buffer, cytochrome c, a scavenger of \( \cdot O_2^- \), and nuclear factor-erythroid 2-related factor 2 (Nrf2) are also the determinants for ROS clearance. Nrf2 is a redox stress-sensitive transcription factor which is activated by ROS, working as a detoxification and antioxidant gene regulator.
These protective systems work together to prevent the deleterious effects of the excessive production of ROS.

The appropriate level of ROS performs significant physiological functions to the body. There are evidences suggesting that ROS play physiological roles in regulating signaling pathways in various cellular processes, including immune cell activation, adaptation to hypoxia, cellular survival, cell differentiation and aging regulation [10]. Therefore ROS is beneficial to physiological processes in a tightly-controlled manner.

### 1.2 Oxidative stress and diseases

When the production of ROS overwhelms the ameliorative ability of catalase, superoxide dismutase and other anti-oxidant factors, ROS causes damage via oxidative stress. In other words, oxidative stress is a deleterious consequence of the excessive generation of ROS and relatively antioxidant deficiency. The imbalance of ROS production and antioxidant defense makes proteins, lipids and DNA to be the targets of these numerous free radicals. The disrupted balance between oxidants and its defenses will result in tissue damage and cell death. There are accumulating studies revealing the relations between oxidative stress and diseases, including inflammation disorders, diabetes mellitus, cancer, neurodegenerative diseases, cardiovascular diseases and atherosclerosis, plus some physiological conditions, such as tissue hypoxia, starvation, radiation and increasing age.

In inflammatory system, oxidative stress may initiate and augment inflammation, and may also result from inflammation. ROS is known for the role of directly killing pathogens through the oxidative burst by NADPH oxidases (NOXs) and the mitochondrial electron transport chain (Mt-ETC) of the phagocytes as well as non-phagocytic cells. The oxidative burst, which requires the activation of superoxide, represents one of the first lines of defense against
the infected pathogens. Even at the early stage of inflammation, the marked amount of ROS could be released. For example, in the microvascular system, the injured endothelial and smooth muscle cells release a lot of ROS at the early stage of tissue injury [16]. And more ROS is release from the phagocytic cells at later stage. The activated phagocytic cells generating ROS utilize NADPH as the major electron donor to convert $O_2$ to $`O_2^-$ and $H_2O_2$. Besides working as a direct killer, the soaring ROS also induces pro-inflammatory genes coding inflammatory mediators, such as IL-1, IL-6 and TNF-α, which are regulated by a group of redox-sensitive transcription factors, including activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) [15, 17-19]. In the process of infection and inflammation, over generation of ROS initiates the tissue injury and potential organ failure.

Hypoxic conditions (hypoxia, 0.3% - 3% $O_2$) are well known as a favorable situation of ROS generation. It has been reported that the moderately hypoxic conditions could lead to a dramatic increase in oxidative stress [20]. Brain is the top one organ extreme sensitive to the change of oxygen level. By using a standard detection probe of ROS, 2’,7’-dichlorodihydrofluorescein diacetate (H2DCFDA), researches show that hypoxia increases fluorescence in cells (Chandel et al. 2001). Further studies elucidate that the mitochondria is partially the actual determinant for the soaring ROS [21, 22]. Still the mechanism that how the ROS is formed during hypoxia is unclear, more studies are needed to reveal the truth.

1.3 ROS toxicity on ion channels

The harmful effects of ROS are extensive, including damage of DNA, oxidation of lipids, inactivation of specific enzymes, and oxidations of amino acids of proteins. Exposure to excess ROS may result in post-translational modification, including S-glutathionylation, S-nitrosylation, acetylation and phosphorylation, which regulate the cellular function. In point of ion channel,
previous studies show that S-glutathionylation occurring on ion channels suppresses their functions in oxidative stress.

S-glutathionylation is a PTM by adding a glutathione (GSH) moiety on the cysteine residues. GSH, a tripeptide, containing a glycine, a glutamate and a cysteine, is a major donor of S-glutathionylation during oxidative stress. GSH is the reduced form of oxidized glutathione (GSSG), working as an antioxidant factor. The concentration of GSH is varied in different types of cells, from 1mM to 5mM. The ratio of reduced glutathione to oxidized glutathione is usually considered indicative of oxidative stress, as GSH is over consumed during the process of the elimination of ROS. In the presence of H$_2$O$_2$, GSH promotes the reaction of S-glutathionylation, while GSSG alone also can perform the PTM of the proteins in experimental studies.

Ion channels play critical roles in various aspects of the regulation of physiological function. The studies on the post-translational modifications of ion channels receive more and more attention. And S-glutathionylation is one that cannot be ignored. Ryanodine receptor (RyR) is the first ion channel identified to be S-glutathionylated [6]. Continued researches demonstrated that $K_{ATP}$ channels [8, 9] and Cystic Fibrosis transmembrane conductance regulator (CFTR) [7] are also targets of S-glutathionylation. S-glutathionylation exclusively happens on cysteine residues by adding a glutathione moiety on thiol group [8]. RyR expressed on the endoplasmic reticulum is a Ca$^{2+}$ channel, which is highly sensitive to redox and has multiple potential cysteine residues under the regulation of S-glutathionylation. The chloride channel, CFTR, regulates the salt and water equilibrium between lumens and cytoplasmic sides of the epithelial cells in lung and gut. Cys344 of CFTR is identified as the primary site under the control of S-glutathionylation. The molecular weight of GSH is about 305 Da. The tripeptide structure endues itself relatively big
size, which could interrupt the conformational change in open stage of the channel, thus blocking
the channels.

1.4 The properties of Kir4.1 and Kir5.1 channels, and their modifications

The Kir4.1 and Kir4.1-Kir5.1 heteromeric channels are expressed in various tissues, in-
cluding the kidney, retina and brain, responding to the changes in pH and pCO₂ within physio-
logical context, playing an important role in pH sensitivity. The basic block of Kir channels is
two transmembrane helixes; four blocks compose one functional channel. The ion conduction
pore is composed of three zones: selectivity filter, central cavity and the internal part of the tun-
nel formed by intracellular face of TM2. The transmembrane domains guard the ion selectivity
and gating, while the cytoplasmic domain functions as a gating regulator. Kir4.1 is predominant-
ly expressed in glial cells of the CNS [23] as a form of homotetrameric functional channel on the
cell membrane, while Kir5.1 expressed alone as a homomeric channel is nonfunctional, and exists
in the cytoplasm. Kir4.1 and Kir5.1 also coexpress in various tissues, such as the kidney, retina
and brain. An inside-out patch experiment reveals the pH sensitivity differences between Kir4.1
homomeric channel and Kir4.1-Kir5.1 heteromeric channel [24]. Kir4.1 current is only slightly
inhibited (at pH=6.2) during physiological range of the changes of pH. While pH of 6.5 almost
completely abolished the currents of the coexpressed Kir4.1-Kir5.1 channel. At pH of 7.5, the
baseline currents of the hetermeric channels are inhibited to 50%. The high sensitivity of Kir4.1-
Kir5.1 channel to the pH extends the physiological function of Kir channels.

Some studies also suggest that abnormality of Kir4.1 and/or Kir4.1-Kir5.1 channels are
involved in several pathophysiological conditions, due to their functions in pH sensing [3, 25],
“spatial buffering of K⁺” [26], and maintaining the activity of other ionic transporters [27-30].
The functional impairment of Kir4.1 and Kir4.1-Kir5.1 channels could happen in various patho-
physiological conditions, such as oxidative stress. Oxidative stress is known to induce injurious PTMs to various channels, such as S-glutathionylation [8]. Furthermore, CNS, cochlea, kidney and retina are highly sensitive to redox changes, and are usually the targets in these disease conditions. Thus, the specific expression feature of Kir4.1 and Kir4.1-Kir5.1 channels increases their chance for them of being targets of reactive oxygen species (ROS) and other oxidants, leading to the S-glutathionylation on critical residue followed by functional blockage of these channels.
2 HYPOTHESIS AND SPECIFIC AIM

Hypothesis: Oxidative stress modifies the transport isoform of the inward rectifying potassium channels by causing the inhibition of K4.1-Kir5.1 channels via S-glutathionylation of Kir5.1 subunits.

Specific aim: To demonstrate that oxidative reagents modify the transport Kir4.1-Kir5.1 channels via S-glutathionylation of Kir5.1 subunit, and to identify specific amino acid residue in the channel protein which is responsible for the S-glutathionylation.

To address this aim, different tetrameric structures, including Kir4.1-Kir5.1 tandem dimer, Kir4.1-Kir5.1 C158A tandem dimer, and a chimera of tandem-tetrameric Kir4.1-Kir5.1 C158A-Kir4.1-Kir5.1, were constructed. The constructed channels were expressed in mammalian HEK system, and the activities of the channels were tested with different oxidants in patch clamp studies. Also the involvement of S-glutathionylation was confirmed by immunoprecipitation experiments.
3 MATERIALS AND METHODS

3.1 Chemicals

Chemicals were purchased from Sigma unless otherwise stated in this study. Powder chemicals were dissolved in double-distilled water or DMSO. In the working solution, the concentration of DMSO is less than 0.1% (v/v). H₂O₂ solution, BioGEE solution and glutathione solution were prepared freshly before the experiments.

3.2 Cell preparation

The cells used in all the studies were human embryonic kidney (HEK) cells (CRL-1573, Batch no. 2187595, ATCC, Rockville, MD, USA). Cells were revived from the stocks, and were cultured in Dulbecco’s Modified Eagle’s medium (DMEM)-F12 with 10% fetal bovine serum (FBS) at 37°C and a 5% CO₂ atmosphere. Cells were split with 0.25% trypsin and changed with fresh culture medium every three days.

3.3 cDNA construction

Rat Kir4.1 cDNA (GenBank # X83585) and rat Kir5.1cDNA (GenBank #X83581) were gifts from Dr. John Adelman (Oregon Health Science University, Portland, OR, USA). Commercial vector PcDNA3.1(+) (INVITROGEN, Carlsbad, CA, USA) was used to carry Kir4.1 and Kir5.1 cDNA. Tandem dimer were cloned into mammalian expression vector PcNDA3.1(+) by polymerase chain reaction (PCR) using Pfu DNA polymerase. The fragment of Kir5.1subunit was generated with a stop codon followed by EcoR I recognition sequence on the 3’end, and with a Mfe I recognition sequence on the 5’ end. The stop codon (TGA) of Kir4.1 subunit in Tandem dimer was mutated to tryptophan (TGG) on the 3’ end. Two sides of the Kir4.1 fragment were flanked with EcoR I sequence on the 3’ end and Mfe I sequence on the 5’ end sepa-
rately. This design facilitated the mutual DNA ligation, and simplified the screening procedure, as both restriction sites were lost after EcoR I- Mfe I ligation. The constructed Kir4.1-Kir5.1 tandem dimer was confirmed by DNA sequencing. Site-specific mutations of Kir5.1 C158A and C158T were performed using a kit of a site-directed mutagenesis (Stratagene). The tandem tetramer of Kir4.1-Kir5.1C158A-Kir4.1-Kir5.1* (* stands for stop codon) was constructed via the same strategy stated previously. The stop codon (TGA) of the Kir5.1 C158A fragment in tandem tetramer was mutated to leucine (TTA). The primers used are listed in table 1. All the constructions were tested by DNA sequencing.

**Table 1: List of primers for plasmid constructions**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence</th>
</tr>
</thead>
</table>
| Kir5.1*     | Fw: AATCCATGAGCTATTACCGGAAG  
              | Re: ATCCAATTGGCTACAGCTGCTCACCATG |
| Kir5.1      | Fw: AATCCATGAGCTATTACCGGAAG  
              | Re: ATCCAATTGGCTACAGCTGCTCACCATG |
| Kir5.1C158A | Fw: GTCCATCCTGAGCGCCATCATAAACAC  
              | Re: CAGGTAGGACTCGCGGTAGTATTTGTG |
| Kir5.1C158T | Fw: GTCCATCCTGAGCGACCACATCATAAACAC  
              | Re: CAGGTAGGACTCGTGGTAGTATTTGTG |
| Kir4.1      | Fw: CGGAATTCCATGACATCGTGGCC  
              | Re: ATCCAATTGGCCAGACGTTACTAATG |

Note: * stands for stop codon (TGA). EcoR I sequence and Mfe I sequence are underlined.

### 3.4 Gene expression

Target genes were transfected using Lipofectamine\textsuperscript{2000} (Invitrogen, Carlsbad, CA, USA) following the instruction. Briefly, 4µg cDNA and 0.5 µg green fluorescence protein (GFP) cDNA were incubated with 10µl Lipofectamine\textsuperscript{2000}, the mixture was transferred to the 35mm dishes with cell density of 60% ~ 90%. After 4 to 6 hrs, solution is changed, and the cells were incubated 18 to 36 hrs further with cell culture medium before experiments.
3.5 Electro physiology

Whole cell currents were recorded with single-electrode whole-cell voltage clamp technique as described previously (Shi et al. 2007; Yang et al. 2010; Zhang et al. 2011). 1.2 mm borosilicate capillary glass (Sutter Instruments, Novato, CA, USA) is used to make fire-polished patch pipettes. The resistance of the patch electrodes is about 2 to 5 MΩ. Signal was amplified using amplifier Axopatch 200B (Molecular Devices, Sunnyvale, CA, USA). Current records were low-pass filtered, digitized and recorded for further data analysis using the pCLAMP 9 software (Molecular Devices). Patch solution contains (in mM): KCl 40, K gluconate 90, KF 5, Na vanadate 0.1, K pyrophosphate 10, ADP 0.2, PIPES 10, glucose 10, spermine 0.1 and EGTA 1 (pH was adjusted to 7.4). Inside-out patch clamp was performed on HEK cells. Bath and pipette solution was prepared as stated previously with same potassium concentration (in mM): KCl 40, K gluconate 90, KF 5, Na vanadate 0.1, K pyrophosphate 10, EGTA 1, ADP 0.2, PIPES 10 and glucose 10 (pH was adjusted to 7.4). Additional 0.1 mM spermine was added into the bath solution to elicit inward rectifying current. Single channel currents were recorded with a holding potential of -60 mV. Slope command potentials of -100 mV to 100 mV were performed to test the channel conductance. Data was analyzed later with Clampfit 10 software (Axon Instruments).

3.6 Western blotting and immunoprecipitation

HEK cells expressing wide-type (WT) Kir4.1-Kir5.1 tandem dimer or mutant Kir4.1-Kir5.1C158A tandem dimer channels were used to do western blotting. Cells were grown in the 35 mm dishes, and incubated with fresh medium without FBS for 2 hrs before treatment. Biotinylated glutathione ethyl ester (BioGEE; 250 μM) (Invitrogen) then was added to the medium for one hr followed by a challenge of 15 min 750 μM H₂O₂. Excess free BioGEE was removed
by three washes with PBS. Radioimmune precipitation assay buffer (Sigma) was used to lyse the cells. Protein concentration was measured via BCA assay (Thermo Scientific, Waltham, MA). Protein lysate was adjusted to 1 mg/ml. For the Western blot, 15μl samples of both WT and mutant lysates were loaded into a 10% SDS-polyacrylamide non-reducing gel and then transferred to a PVDF membrane (Bio-Rad). Rabbit primary antibodies against Kir5.1 (1:1000) (Sigma), rabbit primary antibody against GAPDH (1:1,000) and goat-anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) (1:10,000) (Invitrogen) were used to detect target Kir5.1 and endogenous GAPDH bands. Super Signal West Pico chemiluminescent substrate (Thermo Scientific) was used for signal visualization. Experiments were repeated at least four times.

For the pull-down experiments, 100μl cell lysate was used. Before the incubation, the Dynabeads were washed three times with washing buffer to remove preservatives. Protein lysates were incubated with Dynabeads®M-280 Streptavidin (Invitrogen) at room temperature for 30 mins with gentle rotation to purify the biotin-GSH-conjugated proteins. The protein and Dynabeads complex was separated from the free proteins by a magnet. The coated beads were resuspended and washed three times with washing buffer. The pellets were mixed with 20 μl protein loading buffer which contains 0.1% SDS, then the mixture was boiling in a hot water bath to release the glutathionylated proteins. 15μl solvent was loaded to the gel for Western blot. Kir5.1 antibody and GAPDH antibody were used as described above.

3.7 Data analysis

Data were presented as the mean value ± SEM. T test was adopted to compare the differences in means for paired data. If P ≤ 0.05, the differences were considered as significant.
4 RESULTS

4.1 Kir5.1 subunit is the target of reactive oxygen species (ROS)

Kir4.1 monomer and Kir4.1-Kir5.1 tandem dimer were cloned into mammalian expression vector PcNDA3.1(+) by PCR, and transfected into mammalian expression system, HEK cells. DNA sequencing test confirmed the correct sequence of the channels. The transfected Kir4.1 cDNA alone automatically expressed homotetrameric Kir4.1 channels. The constructed K4.1-Kir5.1 tandem dimer secured accurate expression of the heterologous system. Co-transfected green fluorescent protein (GFP) nicely indicated the HEK cells which expressed functional channels. A series of -100 mV to 100 mV voltage commands were applied to the GFP-positive cell with each pulse of an increment of 20 mV and duration of 400 ms. A holding potential of 0 mV was given to the cell in equal 145 mM potassium concentration of both pipette and bath solution. The transfected cells showed typical inward rectifying currents. A series of H$_2$O$_2$ concentration from 100 µM to 1mM we applied to the sealed and ruptured cells after several-minutes balance. The currents of Kir4.1-Kir5.1 channels were inhibited in a concentration-dependent manner of H$_2$O$_2$ (Fig.1.1A). 100 µM H$_2$O$_2$ gave 15 ± 5% inhibitory effect to the heteromeric channels. When the concentration of H$_2$O$_2$ reached 1mM, a maximum effect was recorded as the Kir4.1-Kir5.1 currents decreasing by 55 ± 5% (n=5) (Fig.1.1B).
Figure 1.1. Kir5.1 subunit is the target of reactive oxygen species (ROS). A. Kir4.1-Kir5.1 channels were expressed on HEK cells, whole-cell currents were studied on single HEK cell. A series of -100 mV to 100 mV voltage commands were applied to the cell with an increment of 20 mV and duration of 400 ms each. A holding potential of 0 mV was given to the cell with even potassium concentration of 145 mM in both pipette and bath solution. B. The current of different concentration of H$_2$O$_2$ was summarized and normalized (n=5). C, D. Exposure of Kir4.1-Kir5.1 channels to oxidant diamide with concentration of 30 and 300 mM (n=5). E, F. The effect of diamide on homomeric Kir4.1 channel (n=4). Data is shown as means ± SEM.
Other oxidant also showed inhibitory effect to the heteromeric channels. Diamide, reported to be used as a thiol oxidizing agent and specific glutathione oxidant, was used to get the similar inhibitory effect on Kir4.1-Kir5.1 channel in a concentration-dependent manner. The currents of Kir4.1-Kir5.1 channels were inhibited by 25 ± 5% with a challenge of 30 µM diamide, and by 67 ± 5% with a challenge of 100 µM diamide (n=5) (Fig. 1.1 C, D). Another oxidant, 2, 2’-Dithiodipytidine (2-DTP), which is sulfhydryl group specific oxidant of cysteine residue, was applied to Kir4.1-Kir5.1 channels. The whole-cell record showed a significant decrease of channel current by 56 ± 5% (n=5) with a single dose of 50µM 2-DTP (Fig. 1.2 A, B). As we previously mentioned, 2-DTP, a membrane-permeable oxidant, belongs to pyridine disulfides (PDSs) which selectively oxidize sulfhydryl group or thiol group of cysteine residue (Yang et al. 2010; Yoshida et al. 2006).

However, the treatment of diamide had little effect on inward rectifying currents of Kir4.1. High concentration of H₂O₂ (1 mM) showed only 8 ± 5% inhibitory effect to Kir4.1 currents (data did not show here). And the Kir4.1 channel was only inhibited slightly by 7 ± 5% with 100 µM (n=4) (Fig. E, F). 50 µM 2-DTP inhibited the homomeric Kir4.1 channels by only 7 ± 5% (n=6) (Fig. 1.2 C, D).

Therefore, these results suggest that different kinds of oxidants, including H₂O₂, diamide and 2-DTP, target on the hetotetrameric Kir4.1-Kir5.1 channel, instead of the homotetrameric Kir4.1 channel. The sensitive subunit to oxidants may be Kir5.1 via mediation of oxidation on thiol groups.
Figure 1.2. Pyridine disulfide inhibits the Kir4.1-Kir5.1 channel but not Kir4.1 channel. A. A membrane permeable 2, 2'-Dithiodipytidine (2-DTP, 50µM), which is sulfhydryl group specific oxidant of cysteine residues, was applied on Kir4.1-Kir5.1 channels. Currents of whole-cell recording were studied with −100 to 100mV voltage commands. The heteromeric channels were inhibited by the treatment of 50µM 2-DTP. B. Normalized results were compared (n=5; ***, P < 0.001). C, D. Application of cysteine specific oxidant has little effect on the homomeric Kir4.1 channels.

4.2 The critical protein domain is located on the intracellular side

The oxidants used in our experiments have similarities. First, they perform an ability to oxidize the reduced glutathione (GSH) to disulfides. In other words, they all have the possibility to exert the reaction of S-glutathionylation. Additionally, these reagents are membrane-permeable. The common features of these oxidants raise another question: whether the location of oxidation site of Kir5.1 is on the intracellular or extracellular side.
Figure 2.1. Critical protein domain of Kir5.1 subunit is on the intracellular side. A. The membrane-permeable pyridine disulfide, 2, 2'-dithiobis-5-nitropyridine (DTNP, 50 μM) inhibited whole-cell currents of Kir4.1-Kir5.1 channels strongly. B. The membrane-impermeable cysteine specific oxidant, 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB, 200 μM) did not inhibit the whole-cell currents of Kir4.1-Kir5.1. C. Summary of the effects of membrane-permeable and membrane-impermeable PDSs on Kir4.1-Kir5.1 currents (n=5; **, P < 0.005). D, E. Current studies were performed in inside-out patch experiments. The reagents were added in the bath solution (intracellular side). Membrane potential was held at 0mV, and a ramp voltage from -100 mV to 100mV was exerted to the electrode. Currents of Kir4.1-Kir5.1 were inhibited by both DTNP and DTNB markedly. F. Currents with different agents were compared (n=4-6; **, P < 0.005).

In the family of pyridine disulfides, two other pyridine disulfides (PDSs), a membrane-permeable pyridine disulfide, 2, 2'-dithiobis-5-nitropyridine (DTNP), and a membrane-impermeable cysteine specific oxidant, 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB), were used to
identify the critical domain of Kir5.1 subunit. Similar to 2-DTP, 50 µM DTNP inhibited the whole-cell currents of Kir4.1-Kir5.1 channels by 49 ± 5% (n=5) (Fig. 2.1 A, C). However, whole-cell recording of the heteromeric channels indicated a very little inhibitory effect by only 1 ± 5% (n=5) (Fig. 2.1 B, C) with the challenge of membrane-impermeable DTNB (high concentration, 200 µM). While the inside-out patch currents with both DTNP and DTNB were markedly inhibited by 52 ± 7% and 45 ± 8% (n=4-6) respectively at equal concentration of 50µM (Fig. 2.1 D, E, F). These results suggest that the critical oxidized domain of the Kir4.1-Kir5.1 channel by different oxidants is located on the intracellular side.

4.3 S-Glutathionylation of Kir5.1 inhibits the functional channel

Protein were expressed in HEK293 cells in DMEM with 10% fetal bovine serum at 37°C with a 5% CO2 explosion. The groups of blank HEK293 cells (B) and empty vector PcDNA3.1(+) transfected cells (P) were set as control groups. And the Kir4.1-Kir5.1 channel transfected groups are considered as experimental group. The western blot results did not show any band in the two blank control groups (B and P) in Kir5.1 detection study (Fig. 4.2 A). Cells were incubated with BioGEE (250 µM) (Invitrogen) for 1hr and further incubated with 750µM H2O2 for 15 mins at 37°C to induce S-glutathionylation. Biotinylated protein was purified by using Dynabeads®M-280 Streptavidin (Invitrogen), and detected using Kir5.1 specific antibody. A dark band of Kir5.1 was shown in the pull-down experiment (Fig. 4.1 B), confirming the involvement of S-glutathionylation on Kir5.1 peptide.
Figure 3.1. Oxidized glutathione inhibits the Kir4.1-Kir5.1 channel. A, B. 300 µM reduced glutathione combined with 300 µM H₂O₂ (H₂O₂/GSH), or 300 µM reduced glutathione combined with 30 µM diamide (DIA/GSH) markedly inhibited the current of Kir4.1-Kir5.1 channels. C, D, E. Oxidants (H₂O₂ or DIA) or glutathione (GSH) alone had limited effect on the current of channels. F. Summary of the effects of different reagents is shown (n=5-6; ***, P < 0.001).

The introduction of the oxidants suggested the existence of the oxidation of the thiol group specifically on cysteine residues. What’s more, the effects of the diamide and PDSs also provided the possibility of an occurrence of the modification of S-glutathionylation rather than
simple formation of disulfide bonds. Therefore, we tested the potential formation of the special modification, S-glutathionylation, by inside-out patch studies with glutathione reagents. 300 μM reduced glutathione combined with 300 μM H_{2}O_{2} (H_{2}O_{2}/GSH), or 300 μM reduced glutathione combined with 30 μM diamide (DIA/GSH) were used to introduce S-glutathionylation. A combination of H_{2}O_{2}/GSH and DIA/GSH markedly inhibited the current of Kir4.1-Kir5.1 channels by 46 ± 4% (n=5) and 49 ± 5% separately (n=6) (Fig. 3.1 A, B, F). However, the application of H_{2}O_{2} or diamide alone did not induce the obvious inhibitory effect on Kir4.1-Kir5.1 heteromeric channels, neither did reduced glutathione (GSH) (Fig. 3.1. C, D, E, F). In the presence of H_{2}O_{2}, GSH is switched to an oxidized form, GSSG. Both GSH/H_{2}O_{2} and GSSG have the possibility to modify cysteine residues of certain proteins. Inside-out patch studies showed the Kir4.1-Kir5.1 channels were inhibited by 16 ± 4% (n=5) and 58 ± 7% (n=5) respectively with the exposure of 2.5 mM and 5.0 mM GSSG on the intracellular side of the membrane (Fig. 3.2). The inhibitory effect on heteromeric channels is in a concentration-dependent manner. These results strongly suggest the involvement of S-glutathionylation of Kir5.1.
Figure 3.2. S-Glutathionylation of Kir5.1 inhibits the function of Kir4.1-Kir5.1 channel. A. Inside-out patch studies were done with the exposure of 2.5 mM and 5.0 mM oxidized flutathione (GSSG) on the intracellular side of the membrane. The inhibitory effect on Kir4.1-Kir5.1 channels is in a concentration-dependent manner. B. The effects of GSSG was summarized (n=5; *, P < 0.05; C).

4.4 The primary target of S-glutathionylation of Kir5.1 is Cys158

After screening of the sequence of Kir5.1 cDNA, we found that there are five cysteine residues in the inside domain of Kir5.1 peptide, in which there is only one cysteine, Cys158, located in the second transmembrane domain (TM2) of Kir5.1. Previous studies of another Kir channel demonstrate that the TM2 is the critical domain of S-glutathionylation (Yang et al. 2010, 2011). Therefore, this Cys158 is highly possible of the critical residue in S-glutathionylation.
The site-specific mutation study was performed on the only cysteine of Kir5.1 peptide to reveal the S-glutathionylation mechanism of Kir4.1-Kir5.1 channel. Related Cys158 (TGC) was replaced by alanine (A, GCC) or threonine (T, ACC) via PCR reaction by designed primers. Mutated Kir channels were expressed in HEK293 cells. The mutation of Kir5.1 C158A or C158T eliminated the inhibitory effects with the exposure of 5.0mM GSSG and 50µM DTNP in inside-out patch study (Fig. 4.1).

The S-glutathionylated proteins were purified and detected in immunoprecipitation experiments. Tandem dimer Kir4.1-Kir5.1 C158A protein is expressed in mammalian HEK cell system, and S-glutathionylated Kir5.1 peptide was detected with the specific antibody via western blot experiments. The mutation of Kir5.1 did not change the peptide density at the expected size (monomeric Kir5.1 is about 50 KDa) (Fig. 4.2 A). After the treatment of BioGEE followed by challenge of H_2O_2, the S-glutathioylated protein was purified with Dynabeads system. In the pull-down experiments, the S-glutathioylated Kir5.1 peptide is markedly reduced, comparing to the wide type one (Fig. 4.2 B). The normalized results show the relative density of glutathionylated Kir5.1 is diminished by 74 ± 12% (Fig. 4.2 C). These results suggested the critical cysteine residue of Kir5.1 peptide is Cys158.
Figure 4.1. Cyst158 is the critical S-glutathionylation site. A, B. Mutation of Cys158 to other amino acids prohibit the inhibitory effect of GSSG in inside-out patch studies. C, D. Mutation eliminated the effect of membrane-permeable DTNP. E. Currents of channels were significantly diminished by the mutation of Cys158 (n=6, **, P < 0.01; ***, P < 0.001).
Figure 4.2. Mutation of Cys158 decreased S-glutathionylated Kir5.1 markedly. A. Western blot showed the C158A mutation had the same density of the band as wide-type Kir4.1-Kir5.1 peptide. GAPDH was the endogenous control. The size of monomeric Kir5.1 is about 50 KDa (B, blank HEK cells; P, PcDNA 3.1(+) vector; WT, wide-type Kir4.1-Kir5.1 tandem dimer; C158A, mutant protein with mutation of C158A on Kir5.1). B. The S-glutathionylated proteins were purified, and S-glutathionylated Kir5.1 peptide was indicated as the arrow. The density of the C158A Kir5.1 band is markedly reduced, compared to the wide type one. C. The density of wide-type Kir5.1 and C158A Kir5.1 peptide was analyzed and normalized with ImageJ system (n=7; ***, P < 0.001).
4.5 One glutathione is sufficient to inhibit the channel

Figure 5.1. The responses of channel with single mutation to different oxidants are intact. 
A, C. In whole-cell studies, heteromeric channels with single mutation of Cys158 on Kir5.1 still had inhibitory responses to both diamide and DTNP. B, D. The responses to diamide and DTNP of wide-type channel and mutant with single C158A on Kir5.1 were compared, indicating that the
inhibitory level of channels with single mutation of Kir5.1 were similar to the wide-type one in the challenge of 30µM diamide and 50µM DTNP (n=4-6; **, P < 0.01; ***, P < 0.001). E, F. Similar effects were observed in inside-out experiments with the challenge of GSSG (n=4-6; **, P < 0.01; ***, P < 0.001).

The channels generated by transfection of Kir4.1-Kir5.1 C158A cDNA have two mutated Cys158 based on the tetrameric structure of Kir channel. Therefore, it is necessary to reveal how many cysteine residue is involved in modifying the function of the Kir4.1-Kir5.1 channel. The chimeric tetramer was generated in a sequential series of monomer, tandem dimer, tandem trimer and tandem tetramer to get the structure with only one mutated Cys158. Functional channels were expressed in HEK system. The current of the Kir4.1-Kir5.1C158A-Kir4.1-Kir5.1* tandem tetramer was inhibited by the similar level of the current of wide-type Kir4.1-Kir5.1 tandem dimer with the exposure of 30µM diamide or 50µM DTNP (Fig. 5.1 A, B) in whole cell recording experiments. The inside-out studies also showed that the inhibitory effect of the tandem tetramer carrying one mutation was also remarkable as the wide-type one with 5mM GSSG exposure (Fig. 5.1 C). In view of these studies, one glutathione is sufficient to hinder the function of the Kir4.1-Kir5.1 channel.
5 DISCUSSION

The imbalance between the production of ROS and protective reducing environment can cause toxic effects through the overproduced free radicals and peroxides, which may damage the cellular components, such as lipids, DNA and proteins. When the production of the reactive oxygen species (ROS) exceeds the cleaning ability of the cellular antioxidant system, the oxidative stress occurs. The disturbance, oxidative stress, is involved a number of disease conditions, including inflammation disorders, diabetes mellitus, cancer, neurodegenerative diseases, cardiovascular diseases, tissue hypoxia, radiation and increasing age. Some of the disorders are related to post-translational modification, such as acetylation [31], S-nitrosylation [32-34], S-glutathionylation [6-9, 35-37] by the addition of small chemical groups. Within these post-translational modifications, S-glutathionylation is what we are most concerned. Potassium transport channels of functional Kir4.1 channel and nonfunctional Kir5.1 channel as potential target of ROS in different pathophysiological conditions expressed on the cell membrane and in the cytoplasm respectively. Co-expression of Kir4.1 and Kir5.1 exists in various tissues, such as the kidney, retina and brain which are susceptible targets of oxidative stress. The disorder of the potassium channels by oxidative stress can severely threaten the normal operation of the function of the cells.

Indeed, our studies show that the Kir4.1 and Kir5.1 channel is the target of several oxidants, as the heteromeric channel is inhibited by H$_2$O$_2$, diamide and PDSs by different degree. While, these oxidants barely have influence on the homomeric Kir4.1 channel. As small molecule, addition of the external H$_2$O$_2$ generated a concentration gradient through cell membrane, building up the oxidative environment to mimic oxidative stress. Diamide was reported as a reagent for the intracellular oxidation of glutathione to the disulfide [38]. PDSs have the propensity
of cysteine residues within a peptide or protein with the consequence of reversible disulfide bond. All these features suggest the generation of S-glutathionylation on available Cys-SH groups in oxidative stress. Additionally, the effects of various compounds of PDSs in whole cell and inside-out studies reveal that the certain domain responding to oxidants is located in the inner side of cellular membrane. To sum up, the Kir5.1 is the major subunit which responds to oxidative stress in Kir4.1-Kir5.1 channel, and the major domain responsible for redox reactions is located intracellularly.

To demonstrate the redox mechanisms of the Kir4.1-Kir5.1 channel, we studied the effects of specific S-glutathionylation donors, including a combination of GSH and H$_2$O$_2$, GSH and diamide, and GSSG. GSH, as reported as non-enzymatical antioxidant, reacts directly with oxidants, such as $\cdot$O$_2^-$, NO, H$_2$O$_2$ and $\cdot$OH, to inhibit oxidative stress in the cell. What’s more, GSH reacts with redox enzymes, including GSH-S-transferase (GST) and GSH peroxidase (GPx), which perform the cytoprotection against neurodegeneration [39]. In the process of stress disposal, oxidative stress leads to the accumulation of GSSG from the reaction between GSH and H$_2$O$_2$. The excessive production of GSSG facilitates the S-glutathionylation of proteins, making them as cellular targets of oxidative stress [40-42]. The S-glutathionylation triggered by the potent inducers markedly impaired the function of the Kir4.1-Kir5.1 channel. Therefore, the reversible S-glutathionylation may be a common factor in the regulation of the redox sensitive Kir channels in oxidative stress.

The cysteine residue Cys158 on the intracellular side of Kir5.1 peptide is demonstrated as the major residue responsible for S-glutathionylation based on the patch clamps studies and immunoprecipitation results. Previous studies on one member of K$_{ATP}$ family provide some suggestions about the blocking mechanism of Kir channel by S-glutathionylation (Yang et al. 2010,
2011). $K_{\text{ATP}}$ channels share the similar structure that four pore forming Kir subunits surrounded by four sulphonylurea receptors (SUR) of the ATP sensitive cassette [43]. Systematic mutational analysis revealed Cys176 on inner part of Kir6.1 transmembrane domain was the prominent site contributing to more than 80% of the channel inhibition. And the mechanism is due to that the conjugated glutathione moiety occupied the margin between two transmembrane helixes and the slide helix, preventing the conformational changes of channel gating [8, 9]. Through systematic mutational analysis, four cysteine residues are suspected as the target of ROS in the inside domain of Kir5.1, in which Cys158 is located in the inner side of second transmembrane domain (TM2) of Kir5.1 (Fig. 6.1). A membrane-impermeable PDS, DTNB, did not have the inhibitory effect on the activity of the Kir4.1-Kir5.1 channel when DTNB was only placed in the extracellular solution. A glutathione moiety which is applied intracellularly possibly encroaches on the crucial Cys158, preventing the channel from opening. Of course, it is possibility there are other cysteine residues responsible to S-glutathionylation. Totally, nigh cysteine residues are found in the sequence of Kir5.1, in which five of them is located intracellularly. However the modification of other cysteines by ROS is only modest, as suggested by the immunoprecipitation assay. To summarize, the critical residue occupied by the glutathione moiety, which inhibits the activity of the channel, is residue Cys158.
The crystallographic structure of Kir4.1-Kir5.1 channel indicates there are two Cys158 residues in the TM2 of pore-forming domain which nears or is in the central cavity of the conductive tunnel (Fig. 6.1). However, the inner diameter of cavity (about 10 Å) which involves Cys158 is only slightly larger than the size of the glutathione moiety (about 8 Å). In addition, our results suggest that only single glutathione moiety is needed for the blockage of the channel. Thus, we have reasons to conjecture that the free space of the inner cavity surrounded by domains containing Cys158 could only accommodates one glutathione, and the incorporated glutathione moiety facing the inner cavity may prevent the channel from a fully opening stage by hin-
dering the conformational changes. The occupation of the space by the glutathione moiety may also prevent the flow of the ions, therefore impairing the channel activity.

Oxidative stress is a hallmark of many diseases, including diabetic retinopathy, immunodisorder, cardiovascular diseases, neurodegenerative diseases, cancer, lung diseases, etc. The changes of S-glutathionylation of specific proteins are related to these diseases. Kir channels have been identified as substrate of reversible S-glutathionylation, but few study demonstrated the S-glutathionylation dependent changes in functional Kir4.1 and Kir5.1 channels in physiological and pathophysiological contexts. Our studies of S-glutathionylation on Kir channels make a step closer to reveal the molecular mechanisms of redox related diseases.
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