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GLUTATHIONE DISULFIDE REGULATION OF δ-ENAC SUBUNIT PLAYS AN IMPORTANT ROLE IN RENAL INJURY

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ABSTRACT

Renal handling of Na^+ and water is significantly compromised in renal injury and disease. Epithelial Na^+ Channel (ENaC) dysfunction has been implicated in the pathogenesis of renal injury/disease because it is responsible for generating the osmotic gradient needed for net solute transport across the renal epithelium. ENaC is a trimeric structure made up of α or δ, β, and γ subunits. Recent studies have suggested a link between ENaC dysfunction and oxidative stress in renal injury/disease, however, the precise mechanisms responsible for progression of renal disorders remain unclear. Since δ-ENaC subunit is primate specific, we investigated the response of αβγ- and δβγ-ENaC under prooxidizing conditions as it may occur in renal patients (and not easily appreciated in pre-clinical animal models of renal disorder). Because δβγ-ENaC generate large conducting channels, we hypothesized that the δ-ENaC subunit plays a significant role in renal disorders. In order to test our hypothesis, we measured whole cell current (2 electrode voltage clamp) of heterologously expressed αβγ, δβγ, and αβγδ-ENaC cRNA in a Xenopus laevis oocyte model system. We modeled redox stress by perfusing oxidized glutathione (GSSG; 400μM) across the cell membrane during recordings. GSSG significantly decreased ENaC activity in oocytes expressing αβγ-ENaC ($n=10$; $p<0.05$). Conversely, GSSG significantly increased whole cell current by 38% in oocytes expressing δβγ-ENaC ($n=12$; $p<0.05$). Although whole cell current in βγ (only) injected oocytes were low, GSSG did not alter βγ transport. This suggests that δ-ENaC confers GSSG sensitivity in δβγ channels. Interestingly, when αβγδ cRNA was injected at equal ratios, GSSG significantly increased whole cell current by 65% in all 13 independent observations from 2 distinct batches of oocytes. Together, our results indicate that αβγ- and δβγ- ENaC are regulated differently under redox stress, and that the δ-ENaC subunit may play an important role in renal injury.

A. *Introduction*

Amiloride-sensitive ENaC is a membrane bound ion channel with selective inward permeability to Na^+ under resting cell membrane potentials. To date, four ENaC subunits (α-, β-, γ-, and δ-ENaC) and splice variants have been cloned in mammalian species (1). Based on high sequence identity to the acid-sensing ion channel (ASIC) family of voltage insensitive channels, and heterologous expression studies, it is clear that trimeric assembly of αβγ and δβγ- ENaC subunits comprise functional sodium channels in the cell membrane. In mice, αβγ-ENaC subunits are largely responsible for net Na^+ transport across an epithelial monolayer. In humans the physiological role(s) of δ comprising sodium channels remain unclear. Herein, we evaluate heterologous expression of ENaC subunits in Xenopus oocyte membrane which allows for

distinctions between the functional regulations of αβγ versus δβγ to be drawn. The aim of this study is to better understand redox regulation of αβγ and δβγ ENaC under experimental conditions that model a pathophysiological scenario, where high levels of extracellular glutathione disulfide (GSSG) can accumulate in the lung epithelial lining fluid during oxidative stress. Furthermore, we tested the hypothesis that glutathione disulfide has divergent effects on αβγ vs. δβγ ENaC.

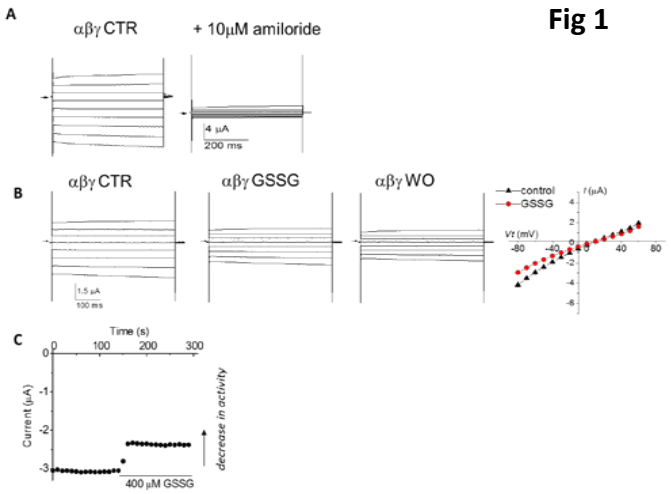
B. ***Materials and Methods***

Vectors: Human α, β, γ, and δ ENaC cDNA's were cloned in pTNT Vector (Promega).
Xenopus Oocytes: Defolliculated stage V Xenopus oocytes were purchased from Ecocyte Bioscience.
Reagents and Chemicals: All chemicals and reagents were purchased from Sigma-Aldrich. The extracellular solution used for voltage-clamp experiments contained: 98 NaCl, 2 KCl, 1 CaCl2, 1 MgCl2, 5 HEPES; pH was adjusted to 7.6 with NaOH. ENaC expressing oocytes were pre-treated with 10μM N-Ethylmaleimide (NEM) prepared in extracellular solution immediately prior to experimentation.
Electrophysiology: Whole cell ENaC currents were recorded with two-electrode voltage-clamp techniques. Oocytes were placed in a Plexiglas chamber and continuously perfused with extracellular solution at a flow rate of 75mL/hour. Recording electrode resistances were between 0.5-1.0 ΩM. Oocytes were voltage clamped at holding potentials between -80mV to +60 mV applied in 10 mV increments. GeneClamp 500 amplifier, Digidata 1322A data acquisition system and pCLAMP ver. 8.2 software were used. Single channel patch clamp analysis was conducted as previously described in (2).
Tissue Culture: Primary human epithelial cells (SAEC) were purchased from Lonza (Alpharetta, GA) and maintained in epithelial cell media (Lonza) supplemented with BPE, insulin, hydrocortisone, GA-1000, retinoic acid, BSA, transferrin, triiodothyronine, epinephrine, and hEGF per manufacturer suggestions.
Immunohistochemistry: ENaC subunits were individually labeled using rabbit anti- α–, β–, γ–, or δ–ENaC antibodies purchased from StressMarq (Victoria, BC, Canada). A 1:350 fold dilution of the primary antibody was applied to formaldehyde fixed cells overnight, followed by incubation with Alexa488-conjugated goat anti-rabbit secondary antibody diluted 1:250. Cells were fixed and mounted using DAPI anti-fade reagent. Cell images were captured using an Olympus IX inverted microscope with a digital camera mount and processed using NIH Fiji ImageJ imaging software.

C**. Results**

Divergent Redox Sensitivities of α- and δ- epithelial sodium channel (ENaC) subunits. Amiloride-sensitive whole cell αβγ -ENaC current is inhibited by glutathione disulfide (GSSG). We showed GSSG's inhibitory effect on Amiloride sensitive whole cell αβγ -ENaC current in an oocyte expression system (**Fig 1AC**). Additionally, voltage clamping oocytes shows that inward rectification of Na^+ current does not shift following 400μM GSSG perfusion across cell membrane, and that membrane voltages negative to the Na^+ equilibrium potential are significantly affected by GSSG. Since the resting

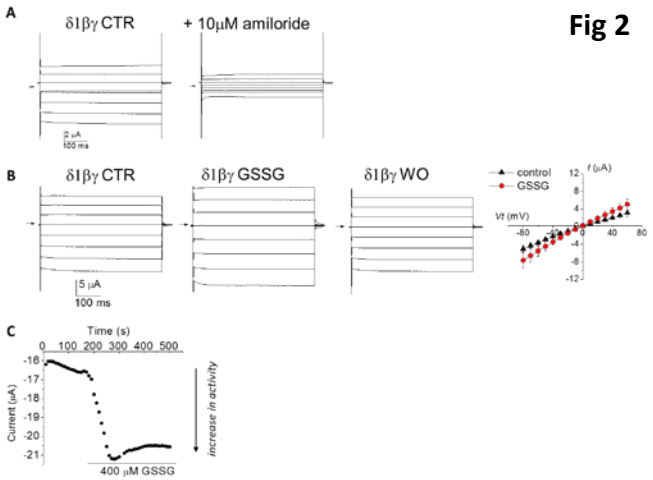
Fig 1



cell membrane potential of epithelial cells is near -60mV, this finding indicates that the GSSG induced decrease in αβγ -ENaC would be relevant at physiological potentials. The decrease in ENaC whole cell current is statistically significant (n=10, p<.0001). Washout of excess GSSG indicates stable current and that GSSG covalently modifies ENaC protein (**Fig 1B**). Real time study of whole cell current indicates that GSSG significantly decreases ENaC function within 10 seconds of perfusion (**Fig 1C**).

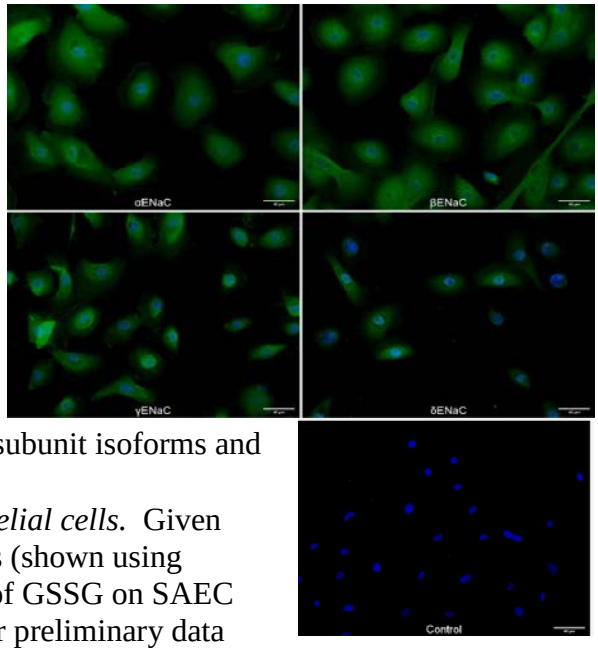
Amiloride-sensitive whole cell δβγ-ENaC current is activated by GSSG. We tested the effect of GSSG on whole cell current using both δ1 and δ2 slice variants alongside βγ-ENaC subunits. We found that δ1 or δ2 expression alongside βγ ENaC cRNA expression in oocytes resulted in Amiloride sensitive, nonvoltage-dependent sodium current (**Fig 2a**). GSSG significantly increases δβγ whole cell current (n=12, p<.05) (**Fig 2b)**. This immediate increase in ENaC activity (**Fig 2c**) is in stark contrast to the significant decrease in αβγ -ENaC current that we observed earlier. We also found that βγ -ENaC expression alone did not respond to GSSG (data not shown).

Fig 2



GSSG modulates δβγ-ENaC via direct thiol modification. Bioinformatics and predictive analysis of αβγ-ENaC strongly suggests that these subunits are post-translationally modified at conserved Cys thiol sites. We evaluated the mechanism by which GSSG regulates δ1βγ ENaC activity by pretreating cells with a thiol modifying agent termed N-Ethylmaleimide (NEM). NEM is an alkene agent reactive towards thiols and is commonly used to modify cysteine residues in proteins. Oocytes expressing δβγ-ENaC were pre-treated with 10μM NEM approximately 1 min before GSSG perfusion (400μM). In these groups of δ1βγ (n=8) ENaC pre-treated cells, GSSG failed to inhibit whole cell current. These outcomes show that Cys thiols play an important role in post-translational modification of all ENaC subunit isoforms and splice variants.

Fig 3



GSSG regulates net Na^+ transport in primary human epithelial cells. Given that human epithelial express each of the four ENaC subunits (shown using confocal microscopy in **Fig 3**), we next evaluated the effect of GSSG on SAEC Na^+ transport using single channel patch clamp analysis. Our preliminary data indicates that GSSG does not significantly change ENaC NPo in human epithelial cells (n=8; data not shown).

D. ***Discussion***

The physiological role of δ -ENaC subunit remains unclear. It is important to better understand δ-ENaC subunit because it is widely expressed in the human body. As such, addressing this gap in scientific knowledge can be directly applicable towards several diverse disease systems (hypertension, acute lung injury, psoriasis, and carcinomas to name a few). Our study shows that sodium channels comprised of αβγ -ENaC subunits respond to GSSG (which accumulates under

pro-oxidizing conditions) with net increase in Na^+ transport in oocytes. This is in stark contrast to the decrease in Na^+ transport in αβγ -ENaC expressing oocytes and mouse epithelia previously reported. Thus far, our preliminary single channel patch clamp analysis of primary human epithelial cells shows that GSSG fails to significantly alter ENaC activity (neither significant increase nor decrease was observed). Additional studies are needed to better understand and dissect out the different regulatory mechanisms influencing α- and δ-ENaC subunit function at the apical membrane.

E. ***References***

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