Glutathionylation of the Aquaporin-2 Water Channel

A NOVEL POST-TRANSLATIONAL MODIFICATION MODULATED BY THE OXIDATIVE STRESS*

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Background: The trafficking of the vasopressin-dependent water channel AQP2 is regulated by post-translational modifications as phosphorylations and ubiquitylation.

Results: AQP2 is subjected to S-glutathionylation, which is modulated by ROS production.

Conclusion: AQP2 is sensitive to oxidative stress.

Significance: Identifying this novel post-translational modification is crucial to understand renal diseases characterized by oxidative stress and AQP2-dependent water balance disturbance.

Aquaporin-2 (AQP2) is the vasopressin-regulated water channel that controls renal water reabsorption and urine concentration. AQP2 undergoes different regulated post-translational modifications, including phosphorylation and ubiquitylation, which are fundamental for controlling AQP2 cellular localization, stability, and function. The relationship between AQP2 and S-glutathionylation is of potential interest because reactive oxygen species (ROS), produced under renal failure or nephrotoxic drugs, may influence renal function as well as the expression and the activity of different transporters and channels, including aquaporins. Here, we show for the first time that AQP2 is subjected to S-glutathionylation in kidney and in HEK-293 cells stably expressing AQP2. S-Glutathionylation is a redox-dependent post-translational modification controlling several signal transduction pathways and displaying an acute effect on free cytosolic calcium concentration. Interestingly, we found that in fresh kidney slices, the increased AQP2 S-glutathionylation correlated with tert-buty! hydroperoxide-induced ROS generation. Moreover, we also found that cells expressing wild-type human calcium-sensing receptor (hCaSR-wt) and its gain of function (hCaSR-R990G; hCaSR-N124K) had a significant decrease in AQP2 S-glutathionylation secondary to reduced ROS levels and reduced basal intracellular calcium concentration compared with mock cells. Together, these new findings provide fundamental insight into cell biological aspects of AQP2 function and may be relevant to better understand and explain pathological states characterized by an oxidative stress and AQP2-dependent water reabsorption disturb.

The aquaporins (AQP)s are a family of integral membrane proteins that mediate water transport across membranes in response to osmotic gradients. In the kidney, AQP2 is the most characterized aquaporin controlling renal collecting duct water balance, being a selective target of the hormone vasopressin. Vasopressin binds its cognate V2R receptor at the basolateral membrane of collecting duct principal cells activating the cAMP/PKA signal transduction cascade, resulting in multiple phosphorylating events in the C terminus of AQP2. Specifically, phosphorylation of AQP2 at Ser256 is required for the vasopressin-regulated translocation of the AQP2 bearing vesicles toward the apical plasma membrane, increasing water luminal permeability (1, 2). This process is of physiopathological relevance because the failure to insert functional AQP2 into the luminal side of renal collecting ducts can be the cause of different acute and/or chronic renal injury characterized by water balance disorders and often accompanied by inflammation, fibrosis, and reactive oxygen species (ROS) production.

A proper balance between oxidation and reduction reactions is fundamental to control numerous signaling cascades (3), and oxidative post-translational modifications of cysteines, like glutathionylation, can directly influence these processes. Interestingly, recent evidence demonstrated that vasopressin stimulation increased S-glutathionylation of different proteins in mpkcd cells, likely suggesting the involvement of ROS into vasopressin-activated signal transduction pathway (4). In addition, under physiological conditions, vasopressin causes a significant increase in intracellular calcium level that is reduced by inhibiting NADPH oxidases, which is the major source of ROS in the kidney (5). In this respect, a novel study indicates NADPH oxidases as new actor regulating AQP2 transduction because NADPH oxidase deficiency results in a relevant decrease in AQP2 mRNA synthesis (6). AQP2 is subjected

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2 The abbreviations used are: AQP, aquaporin; ROS, reactive oxygen species; tBHP, tert-buty! hydroperoxide; hCaSR, human calcium-sensing receptor; PTM, post-translational modification; ER, endoplasmic reticulum.
**AQP2 Glutathionylation**

to different protein post-translational modifications (PTMs) such as phosphorylations (7, 8), short chain ubiquitylation (9), and polyubiquitylation (10), which play key roles in controlling the cellular localization and stability of AQP2.

We show here that AQP2 is subjected to S-glutathionylation both in kidney and in renal cell culture. S-Glutathionylation is an important post-translational modification, which allows protection of cysteine residues against irreversible oxidation during redox imbalance. We provide evidence that AQP2 glutathionylation is tightly modulated by changes in cellular ROS content both in renal tissue and in HEK cells stably expressing AQP2. The relationship that binds AQP2, S-glutathionylation, and oxidative stress signaling can be of physiological interest and might be of clinical relevance in the field of renal water balance disorders associated with abnormal ROS production.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—**All chemicals were purchased from Sigma-Aldrich. tert-Butyl hydroperoxide (tBHP) was kind gift from A. Signorile (University of Bari). To immunoprecipitate and detect the total amount of AQP2, we used antibodies (Pre-C-tail Ab) against the 20-amino acid residue segment just N-terminal from the polyphosphorylated region of AQP2 (CLKGLEPDTDWEEREVRRRQ) (11). Alternatively, AQP2 was detected using a specific antibody (C-tail Ab) raised against (Fig. 1A). AQP2 glutathionylation was evaluated under resting condition and after oxidant stimulation with tBHP (2 mM) for 30 min. The treated sections were subjected to immunoprecipitation studies or used for ROS detection.

Animals were housed according to local and international requirements.

**Immunoprecipitation—**Immunoprecipitation experiments were performed as described (14). Briefly, mice kidney medulla, fresh kidney slices, or HEK-293 cells were lysed with 1% Triton X-100, 150 mM NaCl, 25 mM HEPES (pH 7.4). The obtained supernatants were precleared with 50 μl of immobilized protein-A for 1 h and incubated overnight with anti-AQP2 antibodies coupled to protein A-Sepharose. Immunocomplexes were washed three times, resuspended in 50 μl of Laemmli’s buffer in not denaturating condition and subjected to immunoblotting using AQP2 (1:1000) and glutathione (1:100) antibodies.

**S-Glutathionylation Assay—**HEK-293 cells were seeded on Ø40-mm glass coverslips treated with poly-l-lysine hydrobromide and transfected as described above. 48 h after transfection, cells were incubated for 1 h with BioGEE reagent (250 μM) at 37 °C, 5%CO2. Cells were lysed in 1% Triton X-100, 150 mM NaCl, 25 mM HEPES (pH 7.4). Cellular debris were removed by centrifugation at 13,000 × g for 10 min at 4 °C. The clarified lysate supernatants were incubated with streptavidin-agarose-conjugated for 4 h at 4 °C and then washed three times in the lysis buffer. Glutathionylated proteins were resuspended in 50 μl of Laemmli’s buffer in non-denaturing conditions and subjected to immunoblotting using AQP2 (1:1000) and glutathione (1:100) antibodies.

**ROS Detection—**HEK-293 cells and kidney sections were incubated in serum- and antibiotic-free medium or in Ringer equilibration buffer for 1 h before assaying. Cells or kidney slices were then incubated with dihydrodihydarodamine-123 (10 μM) in PBS for 30 min at 37 °C with 5% CO2 and recovered in complete medium for 30 min. In the last 15 min of recovery, cells were treated with H2O2 (2 μM) and DTT (50 μM). Alternatively, kidney slices were treated with tBHP (2 mM) for 30 min. Cells and kidney slices were lysed in a buffer containing 1% Triton X-100 150 mM NaCl, 25 mM HEPES (pH 7.4). Lysates were analyzed by RF-5301PC fluorimeter (excitation wavelength, 512 nm; emission wavelength, 530 nm).

**Evaluation of NADPH Oxidase—**HEK-293 cells were grown and transfected as described above. The cells were washed with ice-cold PBS, scraped, and centrifuged at 750 × g for 10 min at 4 °C. The obtained pellet was resuspended in 100 μl of homogenizing buffer (20 mM KH2PO4, 1 mM EGTA, pH 7.0). Cells suspension was homogenized by sonication, and protein content was measured. The lucigenin-enhanced chemiluminescence assay was used to evaluate NADPH oxidase activity (15). The reaction mixture solution (150 μl) contains 50 mM phosphate buffer, 1 mM EGTA (pH 7.0), and 5 μM lucigenin. The reaction was started by the addition of cell homogenate (40 μg), and the light emission was recorded. NADPH (1 mM) was added to the mixture, and light emission was measured for the all indicated time points.

**RESULTS**

**AQP2 Is Glutathionylated in Kidney—**AQP2 glutathionylation was first analyzed from solubilized mice kidney medulla (Fig. 1A). AQP2 was immunoprecipitated and revealed using pre-C-tail antibodies. No specific bands were revealed after...
incubation of lysates isolated from native tissue, with unspecific IgG or with protein A beads only (Fig. 1A). Furthermore, immunoprecipitates were immunoblotted and revealed for glutathione. Obtained results indicate glutathionylated AQP2 band approximately at 29 kDa (Fig. 1A). As further control, AQP2 immunoprecipitated from renal tissue and kidney homogenate (15 μg) was immunoblotted with or without antibodies against the C-tail. The data in Fig. 1B showed the specificity of the immunoprecipitation because no specific bands were detected in the negative control. Together, these data indicate that AQP2 is modified by S-glutathionylation in native tissue.

To evaluate whether AQP2 S-glutathionylation is modulated by oxidative changes, ex vivo experiments were performed. Renal kidney slices were prepared and treated as described under methods. Oxidative stimulation was obtained with tBHP (2 mM) for 30 min. Compared with the total amount of AQP2, the relative immunoreactive signal of AQP2 S-glutathionylated significantly increased after tBHP treatment (Fig. 2A, tBHP: 1.66 ± 0.1364 versus control: 1.00, n = 3; *, p < 0.01). The redox state of renal sections was studied using a ROS indicator named dihydorhodamine-123, which is an uncharged and nonfluorescent probe that became highly fluorescent when oxidized by intracellular ROS and other peroxides. Relative to untreated kidney slices, incubation with tBHP resulted in a significant increase in ROS content (Fig. 2B; tBHP: 715.2 ± 147.0 versus control: 100 ± 23.0, n = 6; *, p < 0.01). Together, these findings likely indicate that changes of the redox state correlate with alterations of AQP2 S-glutathionylation in renal native tissue.

AQP2 Is Glutathionylated in HEK-293 Cells—AQP2 S-glutathionylation was further investigated in HEK-293 cells stably expressing human AQP2. Interestingly, we found that the level of S-glutathionylated AQP2 was significantly reduced in cells expressing hCaSR-wt, hCaSR-R990G, and hCaSR-N124K, suggesting an interplay between CaSR signaling and this post-transcriptional modification of AQP2 (Fig. 3: hCaSR-wt: 0.663 ± 0.102; hCaSR-R990G: 0.453 ± 0.144; hCaSR-N124K: 0.408 ± 0.098 versus mock: 1.00). No specific bands were revealed after incubation of lysates isolated from mock cells, with unspecific IgG or with protein A beads only. As a control, AQP2 immunoprecipitated from mock and hCaSR-wt expressing cells immunoblotted with or without glutathione antibodies (Fig. 3, right panels) showed the specificity of GSH antibodies because no selective bands were detected at 29 kDa (negative control).

Alternatively, AQP2 S-glutathionylation was analyzed with a different methodological procedure using BioGEE reagent, a cell-permeant biotinylated glutathione ethyl ester raised for the detection of protein glutathionylation. After affinity precipitation of cellular glutathionylated proteins with streptavidin-agarose beads, immunoblotting analysis, with pre-C-tail antibodies, confirmed AQP2 S-glutathionylation, which was found decreased in HEK-293 cells expressing hCaSR-wt and even more in CaSR gain of function variants (hCaSR-wt: 0.670 ± 0.115; hCaSR-R990G: 0.353 ± 0.107; hCaSR-N124K: 0.358 ± 0.108 versus mock: 1.00) (Fig. 4), confirming our previous findings obtained with immunoprecipitation experiments (Figs. 1–3).

ROS content was measured to evaluate whether changes in AQP2 S-glutathionylation in CaSR-wt and its gain of function variants are associated with alterations in intracellular ROS level. To this end, cells were incubated with the ROS indicator dihydorhodamine-123. Compared with cells transfected with the empty vector encoding for EGFP, HEK-293 cells expressing CaSR-wt and its gain of function variants (hCaSR-R990G and
hCaSR-N124K) showed a significant decrease of intracellular ROS (Fig. 5; hCaSR-wt: 64.13 ± 2.5%; hCaSR-R990G: 64.32 ± 4.55%; CaSR-N124K: 65.46 ± 5.22% versus mock: 100 ± 2.24%). Additionally, as internal experimental control, HEK-293 cells were incubated with H$_2$O$_2$ (2 μM for 15 min) or with DTT (50 μM for 15 min) or co-treated with H$_2$O$_2$ and DTT. H$_2$O$_2$ incubation increases, whereas DTT reduces ROS content, respectively (H$_2$O$_2$: 499.00 ± 87.5; DTT: 40.68 ± 12.90 versus mock: 100 ± 2.24%). Treatment with DTT partially abolished H$_2$O$_2$-induced increase of ROS content (H$_2$O$_2$ + DTT: 177.0 ± 32.33 versus mock: 100 ± 2.24%).

NADPH oxidase is known to be the major source for ROS production in several cell models including kidney (6, 16). Therefore, we next examined NADPH oxidase activity with a lucigenin-based assay. The time course of lucigenin chemiluminescence revealed that mock cells hold higher NADPH oxidase activity compared with cells expressing hCaSR wt or its gain of function variants (hCaSR-R990G and hCaSR-N124K) (Fig. 6) as indicated by the linear regression analysis within 5 min after NADPH addition (for slope, hCaSR-wt: 0.01705 ± 0.005629; hCaSR-R990G: 0.009804 ± 0.005220; CaSR-N124K: 0.01706 ± 0.006094 versus mock: 0.05157 ± 0.02061). Statistical analysis revealed that mock cells showed a significantly higher NADPH oxidase activity already 15 min following NADPH addition. Remarkably, with respect to mock cells, in hCaSR-wt and its gain of function variants, the difference in NADPH oxidase activity remained significantly higher until the end of the assay (Fig. 6).

**DISCUSSION**

We report here the first evidence that AQP2 is glutathionylated in native mammalian kidney and in renal cell culture and that this post-translational modification is modulated by the oxidative stress. Specifically, in ex vivo experiments, tBHP, a known oxidant inducer (17), causes a significant increase in AQP2 S-glutathionylation secondary to an increase in ROS content, indicating that this redox sensitive PTM is linked to the redox condition of the tissue. Moreover, functional expression of hCaSR and its gain of function variants (hCaSR-R990G and hCaSR-N124K) in renal HEK cells caused a reduction in ROS which in turn was accompanied by a reduction in AQP2 S-glutathionylation with respect to mock cells.

Protein PTMs play key roles in cellular signaling (18). Among them, AQP2 undergoes ubiquitylation (9, 19) and phosphorylation at different sites (8), two events known to regulate intracellular distribution and cellular stability of AQP2.

We show here that AQP2 is also glutathionylated, apparently as a consequence of modification of the redox cellular state, both in kidney and in renal cell culture. Redox PTMs have emerged as important molecular signals described in all organisms. Increasing evidence revealed that ROS and reactive nitrogen species function as signaling components transducing extracellular or intracellular information and elaborating highly selective responses by promoting PTMs of thiol residues on target proteins (3, 20). Glutathione can be considered one of the major cellular antioxidant molecules that are continuously converted into the reduced form of GSH. Glutathione can bind an accessible free thiol on a target protein through the thiol group of its cysteine promoting S-glutathionylation. Topological analysis of AQP2 suggests that Cys$^75$ and Cys$^79$ on cytosolic B-loop and highly conserved among species, might be target of S-glutathionylation. Analysis of the primary amino acid sequence, surrounding possible residues of S-glutathionylation, did not highlight any consensus site motif, suggesting that the nature of this PTM may be dependent on the microenvironment features of the cysteine within the three-dimensional structure of the protein (21). However, Chen et al. (22) have recently published a database of cysteine glutathionylation, suggesting that the GSH motifs contain a higher preference of negatively charged residues than non-GSH sites. S-Glutathionylation allows protection of specific cysteine residues from irreversible oxidation. In addition several data demonstrated that S-glutathionylation may also affect the activity and the cellular distribution of target proteins (23, 24). At steady state, whether or not S-glutathionylation affects the localization and the activity of AQP2 remains unclear. We found here, however, that the expression of hCaSR and its gain of function variants (hCaSR-R990G and hCaSR-N124K) reduced AQP2 S-glutathionylation. In vivo and in vitro studies demonstrated a
FIGURE 3. AQP2 was immunoprecipitated (I.P. AQP2) from HEK-293 cells stably expressing hAQP2. Lysates from HEK-293 cells were also subjected to precipitation with only protein A-coupled Sepharose (ProtA) or with nonspecific IgG. Throughout this study, nonequivalents (10 and 90%) of the immunoprecipitates were immunoblotted for AQP2 or GSH, respectively. The data (means ± S.E.) were analyzed by one-way analysis of variance followed by Newman-Keuls multiple comparison test with $p < 0.05$ (*) considered to be statistically different. AQP2 was immunoprecipitated from mock and hCaSR-wt expressing cells and immunoblotted with or without glutathione antibodies. W.B., Western blot.

FIGURE 4. S-Glutathionylation assay with BIOGEE reagent was performed in HEK-293 cells as described under “Experimental Procedures.” Glutathionylated proteins were separated on electrophoresis gel and immunoblotted for AQP2. Densitometry revealed that AQP2 S-glutathionylation decreased in HEK-293 cells expressing hCaSR-wt and its variants with respect to mock. The data (means ± S.E.) were analyzed by one-way analysis of variance followed by Newman-Keuls multiple comparison test with $p < 0.05$ (*) considered to be statistically different.
clear interplay between the vasopressin-stimulated signal transduction cascade activating AQP2 trafficking and CaSR (25). In particular, under physiological conditions, vasopressin caused a significant increase in AQP2 excretion accompanied by an increase in urinary osmolality, as expected. Hypercalciuric patients showed higher excretion of AQP2, which did not increase after vasopressin treatment. Consistent with these findings, in vitro experiments revealed that the cell surface abundance of AQP2 in cells exposed to CaSR agonists was higher than in control cells and did not increase in response to short term exposure to the cAMP-elevating agent forskolin (25). It is still unclear whether CaSR agonists control the cellular distribution of AQP2 by affecting PTMs, which are known to regulate AQP2 trafficking. However, we found that expression of hCaSR and its gain of function variants reduced intracellular calcium level (13) and significantly altered AQP2 phosphorylation (unpublished data). The role of calcium on AQP2 localization and phosphorylation has been debated for long time. In this respect, we have recently demonstrated that low cytosolic calcium level down-regulates the phosphatase PP2A increasing phosphorylation of AQP2 at serine 256 (14). Therefore, to avoid transmission failure, cells tightly control cytosolic and ER calcium concentration by modulating the expression and the relative activity of different calcium signaling proteins. In fact, the decrease in cytosolic calcium observed in cells expressing hCaSR and its gain of function variants is paralleled by calcium accumulation in the endoplasmic reticulum (ER) because of increased sarcoplasmic/ER calcium ATPase activity and expression and a reduced plasma membrane calcium ATPase abundance (13). Mitochondria are deeply involved in calcium homeostasis because they promote ER depletion by calcium buffering. Importantly, mitochondria calcium uptake stimulates ROS production, which plays a role in modulating ER and cytosolic calcium concentration (26) by affecting the activity of pivotal calcium signaling proteins via S-glutathionylation (20). We found that expression hCaSR-wt, hCaSR-R990G, and hCaSR-N124K not only reduced intracellular calcium level (13) but is also accompanied by a significant decrease of AQP2 S-glutathionylation secondary to a decrease of ROS content, which is associated to a relevant decrease of NADPH oxidase activity.

The finding that AQP2 is subjected to S-glutathionylation is itself important to better elucidate the involvement of AQP2 in a broad spectrum of acute and chronic renal diseases characterized by exacerbate oxidative stress. Together, these findings provide evidence that changes in oxidative cellular state correlate with glutathionylation of AQP2. On the other hand, these observations strengthen the importance of AQP2-CaSR signaling interplay involving, in addition to modulation of AQP2 phosphorylation, AQP2 alteration of its glutathionylation state, which emerges as a novel post-translational modi-
fication modulated by intracellular calcium and the oxidative stress.

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