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Discoveries

NEWS & VIEWS

# Tyrosine Kinase Signal Modulation: A Matter of $H_2O_2$ Membrane Permeability?

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**Abstract**

$H_2O_2$ produced by extracellular NADPH oxidases regulates tyrosine kinase signaling inhibiting phosphatases. How does it cross the membrane to reach its cytosolic targets? Silencing aquaporin-8 (*AQP8*), but not *AQP3* or *AQP4*, inhibited $H_2O_2$ entry into HeLa cells. Re-expression of AQP8 with silencing-resistant vectors rescued $H_2O_2$ transport, whereas a C173A-AQP8 mutant failed to do so. Lowering AQP8 levels affected $H_2O_2$ entry into the endoplasmic reticulum, but not into mitochondria. *AQP8* silencing also inhibited the $H_2O_2$ spikes and phosphorylation of downstream proteins induced by epidermal growth factor. These observations lead to the hypothesis that $H_2O_2$ does not freely diffuse across the plasma membrane and AQP8 and other $H_2O_2$ transporters are potential targets for manipulating key signaling pathways in cancer and degenerative diseases. *Antioxid. Redox Signal*. 19, 1447–1451.

## Introduction

REACTIVE OXYGEN SPECIES can be highly cytotoxic. At low concentrations, however, they are essential for cell function and survival. $H_2O_2$ inhibits phosphatases and can activate certain kinases (4). How it can diffuse in and between cells is, thus, a fundamental question in pathophysiology. Many pathways can generate $H_2O_2$: NADPH oxidases in the plasma membrane, oxidative phosphorylation in mitochondria, and oxidative protein folding in the endoplasmic reticulum (ER) (5). In all cases, $H_2O_2$ must cross a membrane to reach its cytosolic targets. $H_2O_2$ has long been thought to cross lipid bilayers freely. However, the slightly larger dipole moment of $H_2O_2$ makes its simple diffusion through membranes less likely than for water. Its size and electrochemical properties qualify $H_2O_2$ as a possible substrate of aquaporins (AQP). In fact, some members of the AQP family (8) have been shown to transport $H_2O_2$ (2, 6). In this study, we investigate the intracellular transport of $H_2O_2$ exploiting a panel of organelle-targeted ratiometric *HyPer* sensors (1, 3).

**Innovation**

Our observations that efficient transport across the plasma and endoplasmic reticulum membranes requires AQP8, or other membrane proteins, introduce a novel level of regulation in redox signaling. Indeed, in our cell model, AQP8 silencing inhibited not only epidermal growth factor -induced entry of $H_2O_2$, but also downstream tyrosine phosphorylation of target proteins. Thus, $H_2O_2$ transporters may be potential targets in cancer and degenerative diseases.

## Results

To investigate $H_2O_2$ transport across the plasma membrane, we expressed a cytosolic *HyPer* sensor (1) into HeLa cells. Addition of 50 $\mu M$ $H_2O_2$ clearly activated the sensor, as determined by the profound 488/405 nm shifts observed in live cell imaging (Fig. 1A). A C199S *HyPer* mutant that has lost redox sensitivity did not shift its fluorescence emission, excluding pH-dependent events. Pretreatment with $HgCl_2$,

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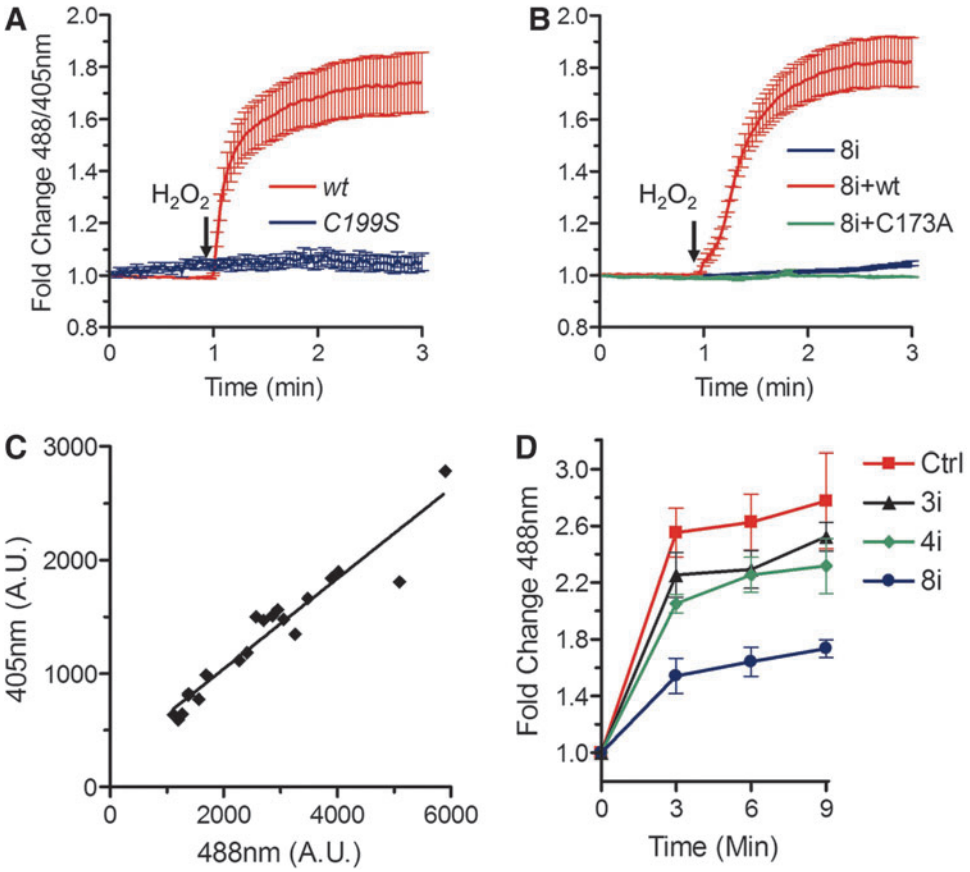


**FIG. 1. Entry of exogenous $H_2O_2$ into HeLa cells requires AQP8. (A)** Time course response of wt cytosolic *HyPer* (red line) or a redox-insensitive mutant (C199S, blue line) to 50 $\mu M$ $H_2O_2$. **(B)** Kinetics of $H_2O_2$-dependent *HyPerCyto* activation in HeLa cells silenced for AQP8 expression (8i), and then transfected with silencing-resistant constructs driving the expression of wt (8i + wt) or mutant AQP8 (8i + C173A, see Methods for details). Graphs in **A** and **B** show mean fold change ± SEM of the 488/405 nm ratio measured by confocal laser scanning. **(C)** The *HyPer* 488/405 nm ratio is not influenced by the expression levels of the probe. Transiently transfected HeLa cells were analyzed by confocal laser scanning. Despite cells being varied in the extent of probe expression, the ratio remained rather constant, as demonstrated by the linear correlation of 405 and 488 nm light excitation ($r = 0.92$). (A.U. stands for Arbitrary Units). **(D)** $H_2O_2$-dependent activation of *HyPerCyto* was analyzed in HeLa cells treated with specific silencing oligos for *AQP8, AQP4*, or *AQP3* by Typhoon scanning (see Methods). Data are expressed as fold increase in the 488 nm fluorescence ± SEM, relative to cells that were not treated with $H_2O_2$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

a compound that inhibits many membrane transporters, prevented *HyPer* oxidation (data not shown). Given the poor specificity of $HgCl_2$, we performed silencing experiments targeting members of the AQP family, membrane channels involved in the transport of water and other solutes (8), including $H_2O_2$ (2, 6). Silencing *AQP8* efficiently prevented $H_2O_2$ entry (Fig. 1B, blue trace), suggesting that this protein plays a major role in our cell model. Transport of $H_2O_2$ was rescued by expression of a Halo-AQP8 chimeric protein, in which the third bases of triplets 12–16 were mutated (red trace). In this way, transgene transcripts were not targeted by silencing oligonucleotides and yet encoded the same amino acids. A similarly engineered C173A-AQP8 mutant was inactive, excluding off-target effects (green trace). Having confirmed that the 488/405 nm ratio remained constant independently from the sensor expression levels (Fig. 1C), we analyzed cells at the population level using single wavelength-automated Typhoon imaging (see Notes). Measuring the increase in 488 nm fluorescence in wells containing $> 10^5$ cells confirmed that AQP8 promotes $H_2O_2$ membrane transport. In contrast, silencing *AQP3* or *AQP4* had only marginal effects (Fig. 1D). The above experiments provided additional evidence that $H_2O_2$ cannot freely permeate through the plasma membrane and identified AQP8 as an efficient transporter.

Next, we investigated whether AQP8 is needed for $H_2O_2$ transport across the mitochondria and ER membranes. Since the silencing of this protein prevented $H_2O_2$ passage across the plasma membrane (Fig. 1B), we treated cells expressing specific sensors in mitochondria or ER (*HyPerMito* or *HyPerERlum*) with digitonin, a detergent that permeabilizes the cholesterol-enriched plasma membrane without significantly affecting the integrity of intracellular membranes (7), and followed the import of added $H_2O_2$. *AQP8* silencing perturbed $H_2O_2$ transport into the ER lumen, but had no significant effects on mitochondria (Fig. 2). The causes and potential physiological significance of the delayed $H_2O_2$ entry into the ER of *AQP8*-silenced cells are presently unclear. Taken

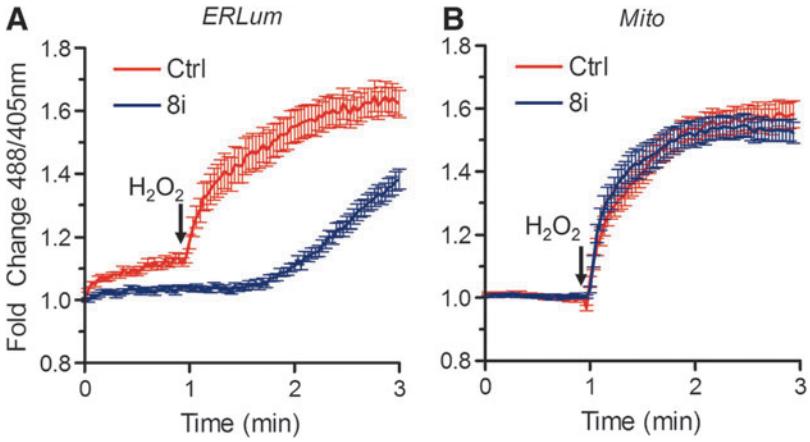


**FIG. 2. $H_2O_2$ transport into the endoplasmic reticulum (ER) is facilitated by AQP8.** HeLa cells expressing *HyPerERLum* **(A)** or *HyperMito* **(B)** were silenced with *AQP8*-specific (blue line) or irrelevant (red line) oligos before semipermeabilization with 40 $\mu$g/ml digitonin (7) to bypass the plasma membrane and allow access to ER and mitochondria. The kinetics of $H_2O_2$ entry into the two organelles were measured by confocal laser scanning. Graphs show mean fluorescence ± SEM. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

together, the above observations suggest that AQP8 is important for $H_2O_2$ to cross the plasma and ER membranes, but dispensable for mitochondrial import.

Epidermal growth factor (EGF)-induced $H_2O_2$ inhibits phosphatases through sulfenylation of their active-site cysteines, potentiating kinase signaling (4). Accordingly, EGF induced a transient spike of cytosolic $H_2O_2$ in our cells (Fig. 3A, red trace). This increase was strongly diminished in *AQP8*-silenced cells (blue trace), suggesting that part of the $H_2O_2$ measured derives from extracellular sources. Hence, when catalase was added to the cell medium before EGF stimulation, the intensity of bands corresponding to tyrosine-phosphorylated proteins was decreased (Fig. 3B, C). Silencing AQP8 expression diminished tyrosine phosphorylation to a level similar to that obtained with catalase. In this way, our results corroborate that, during EGF signaling, AQP8 allows entry of exogenously generated $H_2O_2$, amplifying signal transduction.

## Discussion

Our results indicate that $H_2O_2$ cannot freely diffuse through the plasma and ER membranes and identify AQP8 as an efficient transporter. In our HeLa cell model, entry of exogenous $H_2O_2$ is largely sustained by AQP8. The efficient rescue of $H_2O_2$ entry by AQP8 re-expression excludes off-target effects. Presumably, AQP8 directly transports $H_2O_2$; a less likely possibility is that it controls the expression and/or activity of other transporter(s). Silencing *AQP3*, another family member thought to mediate $H_2O_2$ transport (6), had minor effects, possibly reflecting lower expression levels in our cells. If both AQP3 and AQP8 can allow $H_2O_2$ entry into cells, a differential tissue expression of the two molecules may reflect other metabolic requirements, like glycerol or ammonia transport (8).

Since AQP8 folds in the ER, it is not surprising that downregulation of this channel impacts $H_2O_2$ transport across the membrane of this organelle. The observation that mitochondrial entry was not affected reinforces the view that no functional AQP8 resides in this organelle (9). It will be of interest to determine whether other $H_2O_2$ transporters be present in mitochondrial membranes. The above observations have important functional implications, since both the ER and mitochondria can be sources of abundant $H_2O_2$. During intense metabolic activity or hypoxia, generation of $H_2O_2$ from electron leakage in mitochondria (4) might act as a signaling rheostat. Likewise, it is conceivable that exuberant disulfide bond formation in the ER could modify cytosolic $H_2O_2$ levels with anti- or proapoptotic effects (5). The observation that devoted transporters are needed also for inter-organellar transport delineates yet another possible level at which eukaryotes can exert homeostatic control.

Elegant work from many laboratories demonstrated that $H_2O_2$ increases the strength of tyrosine kinase signaling [(4) and references therein]. *AQP8* silencing strongly reduced the $H_2O_2$ fluxes observed soon upon EGF stimulation. Importantly, also, downstream tyrosine phosphorylation was inhibited. *AQP8* silencing was almost as efficient as extracellular catalase, confirming that most physiologically EGF-related $H_2O_2$ entered through the AQP8 channels. Thus, the regulation of $H_2O_2$ entry could be an additional means to tune signal transduction (Fig. 3D).

## Notes

## Methods

### *Cell culture, plasmids, siRNAs, and transfection*

*HyPer* sensors targeted to the cytosol (*Cyto)* or mitochondria (*Mito*) and the *HyPerC199S* mutant (5) were generous gifts of Dr. V. Belousov (IBCh, Moscow, Russian Federation), while *HyPer ER luminal* (*ERLum*) plasmid (3) was kindly provided by Drs. E. Margittai and M. Geistz (Semmelweis University, Budapest, Hungary). Human AQP8 cDNA was amplified from pCMV6AC-GFP-AQP8 (NM_001169; Origene) and cloned into HaloTag® vector pHTN (Promega). Silencing-resistant Halo-AQP8 was generated by introducing five silent point mutations in the 21 bp *AQP8*-siRNA target sequence, to obtain 5′ TTCGGGAACGATAAA 3′, and used to create the C173A mutant. All constructs and mutations were validated by sequencing. Reagents to silence *AQP3* (5′ GGG UCGUCACUCCUUUAAUU 3′), *AQP4* (5′ GAUCAGCAU CGCCAAGUCU 3′), and *AQP8* (5′ UUUGGCAAUGACA AGGCCA 3′), and an unrelated control (Block-it™) were purchased from Ambion (Life Technologies).

Two or $10 \times 10^4$ HeLa cells were grown overnight in 24- or 6-well plates, transfected with 18 or 90 pmol of siRNA, respectively, using RNAiMAX lipofectamin (Life Technologies) and analyzed after 72 h. Transient transfections were performed by Polyethylenimine as described (7).

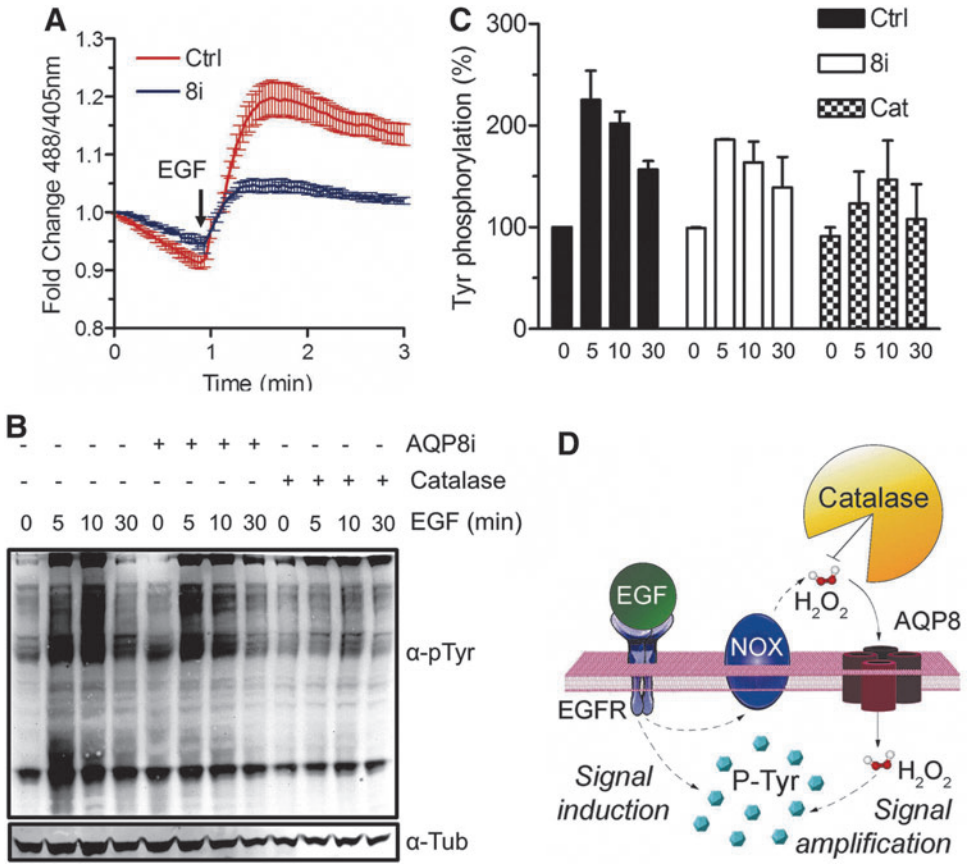


**FIG. 3. Epidermal growth factor (EGF) induces transport of $H_2O_2$ through AQP8. (A)** Time course analysis of $H_2O_2$ uptake by HeLa cells expressing *HyPerCyto* by confocal laser scanning after treatment with 10 $\mu$g/ml EGF. Color code as in Figure 2; **(B)**. Western blot analysis with the indicated antibodies showing changes in phosphorylation of total cell tyrosines on whole HeLa cell extracts that were either silenced for *AQP8* (8i) or treated with catalase before treatment with EGF. **(C)** The intensity of protein phosphotyrosine signals in the blots shown in **B** were quantified by densitometry and normalized to Tubulin. Bars show average band intensities relative to untreated control cells ± SEM. **(D)** A schematic view of $H_2O_2$ generation and transport during EGF signaling. NOX and DuOX enzymes on the cell surface are activated to produce $H_2O_2$. As demonstrated by the inhibitory effects of catalase, extracellularly generated $H_2O_2$ is important for efficient amplification of signaling and enters the cell through AQP8, as indicated by silencing **(B, C)**. The residual activity in silenced cells could reflect incomplete AQP8 downregulation, the presence of additional $H_2O_2$ transporters in the plasma membrane and/or its generation in mitochondria. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

### *Imaging HyPer-oxidation*

Cells were equilibrated in Ringer buffer (140 m*M* NaCl, 2 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 1.5 m*M* $K_2HPO_4$, 10 m*M* glucose, pH 7.3) for 15 min at RT and images taken using a Typhoon FLA 9000 fluorescence scanner (GE Healthcare) equipped with a 488 nm excitation filter 0, 3, 6, or 9 min after treatment with or without 50 $\mu M$ $H_2O_2$ (Sigma-Aldrich). Untransfected HeLa cells were used to calibrate laser sensitivity. Images were analyzed using ImageJ and results presented as fold change increase in 488 nm intensity relative to control cells.

HeLa transfectants were seeded on glass coverslips. Forty-eight hours after transfection, coverslips were equilibrated in Ringer buffer for 10 min at RT before $H_2O_2$ exposure or stimulation with 10 $\mu$g/ml of EGF (Sigma-Aldrich) at 37°C. Confocal images were collected every 2 s for 3 min by excitation with 488 nm argon and 405 nm violet diode lasers on an Ultraview confocal laser scanning microscope using a 40× oil-immersion lens (Perkin Elmer). The 488/405 nm ratios were calculated by ImageJ software in triplicate wells, always averaging in each ≥10 cells, and plotted against time. HaloAQP8-expressing cells were identified by labeling with 5 $\mu M$ HaloTag TMR Ligand (Promega) and analyzed at 532 nm.

To measure intracellular $H_2O_2$ fluxes, transfectants expressing *HyPerMito* or *HyPerERLum* were treated and silenced as above. Coverslips were placed in a live cell chamber at RT, equilibrated in Ringer Buffer for 5 min, and then treated for 5 min with 40 $\mu$g/ml of digitonin (Sigma-Aldrich). The integrity of the ER and mitochondrial membranes was assessed as described previously (7).

### *Antibodies and western blotting*

Mouse anti-PhosphoTyrosine (4G10), mouse anti-tubulin, goat anti-mouse IgG-HRP, or AlexaFluor-647 were from Millipore, Sigma-Aldrich, Zymed, and Invitrogen, respectively. Images were acquired by Chemidoc-it Imaging System (UVP) or Typhoon FLA 9000, processed with ImageJ and

densitometrically quantified by ImageQuant 5.2 (Molecular Dynamic).

To follow EGF responses biochemically, subconfluent HeLa cells were cultured in the FCS-free DMEM for 3 h before incubation with or without 5000 U/ml of catalase and EGF addition. Cells were then washed with ice-cold PBS containing 0.4 m*M* $Na_3VO_4$ to block phosphatases, lysed in sample buffer and analyzed by electrophoresis and western blot.

Further details will be made available upon request (milena.bertolotti@hsr.it)

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## References

1. Belousov VV, Fradkov AF, Lukyanov KA, Staroverov DB, Shakhbazov KS, Terskikh AV, and Lukyanov S. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat Meth* 3: 281–286, 2006.
2. Bienert GP, Moller AL, Kristiansen KA, Schulz A, Moller IM, Schjoerring JK, and Jahn TP. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J Biol Chem* 282: 1183–1192, 2007.
3. Enyedi B, Varnai P, and Geiszt M. Redox state of the endoplasmic reticulum is controlled by Ero1L-alpha and intraluminal calcium. *Antioxid Redox Signal* 13: 721–729, 2010.
4. Finkel T. Signal transduction by reactive oxygen species. *J Cell Biol* 194: 7–15, 2011.
5. Kakihana T, Nagata K, and Sitia R. Peroxides and peroxidases in the endoplasmic reticulum: integrating redox homeostasis and oxidative folding. *Antioxid Redox Signal* 16: 763–771, 2012.
6. Miller EW, Dickinson BC, and Chang CJ. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. *Proc Natl Acad Sci U S A* 107: 15681–15686, 2010.
7. Nerini-Molteni SN, Fassio A, Ciriolo MR, Filomeni G, Pasqualetto E, Fagioli C, and Sitia R. Glutathione limits Ero1-dependent oxidation in the endoplasmic reticulum. *J Biol Chem* 279: 32667–32673, 2004.
8. Verkman AS. Aquaporins. *Curr Biol* 23: R52–R55, 2013.
9. Yang B, Zhao D, and Verkman AS. Evidence against functionally significant aquaporin expression in mitochondria. *J Biol Chem* 281: 16202–16206, 2006.

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**Abbreviations Used**

AQP = aquaporins
EGF = epidermal growth factor
ER = endoplasmic reticulum
SEM = standard error of the mean