

UNIVERSIDADE DE LISBOA
FACULDADE DE FARMÁCIA



Membrane permeability and aquaporin involvement in oxidative stress and cancer

Ana Cláudia Rodrigues Fonseca

Orientadora: Professora Doutora Maria da Graça Soveral Rodrigues

Tese especialmente elaborada para a obtenção do grau de Doutor em Farmácia,
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Júri:

Presidente:

- Doutor António José Leitão das Neves Almeida, Professor Catedrático e Presidente do Conselho Científico da Faculdade de Farmácia da Universidade de Lisboa

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- Doutora Maria da Graça Tavares Rebelo de Soveral Rodrigues, Professora Associada com Agregação da Faculdade de Farmácia da Universidade de Lisboa

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*"Success is not final, failure is not fatal: it is the courage to
continue that counts."*

Winston Churchill

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Abbreviations

AP-1	Activating protein-1
AQP(s)	Aquaporin(s)
cAMP	Cyclic adenosine monophosphate
CFDA	5(6)-Carboxyfluorescein diacetate
CFU	Colony forming units
CM-H₂DCFDA	5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester
H₂DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HER2	Human epidermal growth factor receptor 2
HNE	4-hydroxynonenal
E_a	Activation energy (kcal.mol ⁻¹)
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ERK 1/2	Extracellular signal-regulated kinases 1 and 2
GFP	Green fluorescent protein
GSH	Glutathione
GST	Glutathione-S-transferase
H₂O₂	Hydrogen peroxide
HEK	Human embryonic kidney
HNE	4-hydroxynonenal
<i>k</i>	First-order kinetic rate constant (s ⁻¹)
MAPK	Mitogen-activated protein kinases
MIP	Major intrinsic protein
MUFA	Monounsaturated fatty acid
NOX	NADPH oxidases
NPA	Asparagin-Proline-Alanine
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OD₆₀₀	Optical density (600nm)
PDA	Pancreatic ductal adenocarcinoma
P_f	Water permeability coefficient (cm·s ⁻¹)

P_s	Glycerol permeability coefficient (cm·s ⁻¹)
PKA	Protein kinase A
PUFA	Polyunsaturated fatty acid
RB	Ringer Buffer
ROS	Reactive oxygen species
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SF	Selectivity filter
SFA	Saturated fatty acids
siRNA	Short interfering RNA
VEGF	Vascular endothelial growth factor
YNB	Yeast nitrogen base
WT	Wild-type

List of publications

The major findings were communicated in the form of research papers published in peer reviewed international journals or in preparation, oral communications and poster communications in national and international conferences.

This thesis is based on the following publications:

- Rodrigues, C., Tartaro Bujak, I., Mihaljević, B., Soveral, G. and Čipak Gašparović, A., Yeast aquaporin regulation by 4-hydroxynonenal is implicated in oxidative stress response. IUBMB Life. 69(5):335-362 (2017) doi: 10.1002/iub.1624
- Rodrigues, C.*, Mósca, A. F.*, Martins, A. P., Nobre, T., Prista, C., Antunes, F., Čipak Gašparović, A. and Soveral, G., Rat Aquaporin-5 Is pH-Gated Induced by Phosphorylation and Is Implicated in Oxidative Stress. Int J Mol Sci. 17(12): 2090 (2016) doi: 10.3390/ijms17122090
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- Rodrigues, C., Pimpão, C., Mósca, A. F., Coxixo, A. S., Lopes, D., da Silva, I. V., Pedersen, P. A., Antunes, F., and Soveral, G., Human aquaporin-5 facilitates hydrogen peroxide permeation affecting adaption to oxidative stress and cancer cell migration (submitted)
- Rodrigues, C., Milkovic, L., Tartaro Bujak, I., Tomljanovic, M., Soveral, G. and Čipak Gašparović, A., Lipid profile and aquaporin expression under oxidative stress in breast cancer cells of different malignancy (accepted)

Other publications produced during this period, but of which results have not been used for the elaboration of the thesis:

- Marques CC, Santos-Silva C, Rodrigues C, Matos JE, Moura T, Baptista MC, Horta AEM, Bessa RJB, Alves SP, Soveral G, Pereira RMLN. Bovine oocyte membrane permeability and cryosurvival: Effects of different cryoprotectants and calcium in the vitrification media. Cryobiology (2018) doi: 10.1016/j.cryobiol.2018.03.003
- Mendez-Gimenez, L., Becerril, S., Camões, S. P., da Silva, I. V., Rodrigues, C., Moncada, R., Valenti, V., Catalan, V., Gomez-Ambrosi, J., Miranda, J. P., Soveral, G., Fruhbeck, G. and Rodriguez, A. Role of aquaporin-7 in ghrelin- and GLP-1-induced improvement of pancreatic beta-cell function after sleeve gastrectomy in obese rats. Int J Obes. 44(9): 1394-1402 (2017) doi: 10.1038/ijo.2017.135

The author clarifies that it is her responsibility, except when stated otherwise, to carry out the experiments that allowed the elaboration of the presented results, as well as the interpretation and discussion. The results obtained by other authors were included to facilitate the understanding of the works and the contribution of each author is discriminated.

Resumo

As aquaporinas (AQPs) são membros da família de proteínas membranares MIP (*Major Intrinsic Protein*), expressas em todos os reinos incluindo *Archae*, *Eubacteria*, *Fungi*, *Plantae* e *Animalia*. Desde a sua descoberta, cerca de 450 isoformas foram identificadas possuindo uma elevada homologia entre as sequências das proteínas. De acordo com a complexidade do organismo, o número de AQPs expressas e a sua localização varia. Em microrganismos unicelulares, o número de genes codificantes para AQPs é muito reduzido, como por exemplo a *Escherichia coli* (*E. coli*) que possui apenas dois genes, um codificante para um canal de água e outro para um canal de glicerol. Até ao momento, foram identificadas em humanos 13 isoformas (AQP0-AQP12), sendo diferencialmente expressas em células e tecidos. As AQPs organizam-se em homotetrâmeros e cada monómero é um canal funcional que facilita o transporte bidirecional de água e pequenos solutos de forma rápida, seletiva e regulada, em resposta a gradientes osmóticos ou gradientes de soluto, para manter a homeostasia celular. As AQPs estão divididas em três grupos diferentes de acordo com a sua seletividade e homologia: i) AQPs ortodoxas que são estritamente seletivas para a água; ii) aquagliceroporinas, que para além de permearem água também facilitam o transporte de pequenos solutos não-carregados como o glicerol e a ureia; iii) S-aquaporinas que se encontram maioritariamente expressas em organitos intracelulares, e cuja informação relativamente à sua função ainda permanece pouco conhecida. Recentemente, também foi descrito que algumas isoformas, nomeadamente a AQP3, AQP8 e AQP9, permeiam peróxido de hidrogénio (H_2O_2). As moléculas de H_2O_2 e H_2O apresentam várias semelhanças tais como o tamanho molecular, momento de dipolo e a capacidade de ligação de hidrogénio. Tais semelhanças fazem prever que as AQPs também sejam

capazes de transportar H_2O_2 . O H_2O_2 , apesar de ser uma molécula tóxica, em baixas concentrações pode atuar como molécula sinalizadora em diversas cascatas de sinalização e é maioritariamente produzido pela cadeia respiratória na mitocôndria ou por NADPH oxidases (NOX) localizadas na bicamada lipídica. As NOX reduzem o oxigénio a superóxido que é rapidamente convertido em H_2O_2 e pode ser degradado em H_2O ou transportado pelas AQPs e servir como segundo mensageiro em cascatas de sinalização responsáveis por diversos mecanismos células como proliferação, diferenciação, migração e morte celular.

O papel fisiológico das AQPs tem sido revelado através de estudos em modelos de ratinho *knock-out*. Verifica-se que as AQPs estão envolvidas em processos fisiológicos como secreção de fluído glandular, mecanismo de concentração urinária, excitabilidade neuronal, metabolismo dos lípidos, hidratação epidérmica, balanço de água no cérebro, entre outros. Naturalmente, a disfunção e/ou a expressão anormal de AQPs está associada a inúmeras patologias, incluindo epilepsia, edema cerebral, glaucoma, obesidade e cancro. Para além disso, duas doenças foram diretamente ligadas a aquaporinas, conhecidas como aquaporinopatias: a perda de função da AQP2 causada por uma mutação origina a diabetes insípida e a neuromielite óptica é causada pela presença de anticorpos contra AQP4.

A expressão desregulada de aquaporinas foi encontrada em mais de 20 tipos de cancro e pode ser relacionada com o tipo de tumor, o estadio e a agressividade. Vários estudos demonstram que as AQPs têm um papel importante na tumorigénese contribuindo para a proliferação e migração celular e promove a angiogénese tumoral e formação de metástases. Desta forma, as AQPs têm sido usadas como alvos para o desenvolvimento de fármacos com aplicações terapêuticas no tratamento do cancro, e são também consideradas potenciais biomarcadores de prognóstico. O seu papel em tumores tem sido associado à sua capacidade de transporte de água,

facilitando a formação de protrusões numa das extremidades celulares, e transporte de glicerol aumentando a produção de ATP e a biossíntese de lípidos, funções que contribuem para a migração e a proliferação celular em tumores. As células tumorais são metabolicamente muito ativas e produzem maior quantidade de ROS, incluindo H_2O_2 . A recente descoberta sobre o transporte de H_2O_2 através de algumas AQPs merece especial atenção uma vez que esta molécula serve como segundo mensageiro na ativação de cascatas de sinalização, sugerindo que as AQPs podem participar indiretamente na sinalização redox promovendo diversos processos fisiológicos como sobrevivência, crescimento e proliferação celular. A sobre-expressão de AQPs em tumores, incluindo as três isoformas que se conhecem como transportadoras de H_2O_2 (AQP3, AQP8 e AQP9), sugere que estas proteínas de membrana podem participar na sinalização redox; no entanto o mecanismo molecular ainda permanece por clarificar. Estudos em células de cancro de mama mostraram que o H_2O_2 transportado através da AQP3 regula a migração celular através da oxidação da PTP1B/PTEN que ativa diretamente a via PI3K/Akt. Esta via de sinalização controla a migração de células tumorais e a formação de metástases. A AQP5 é também uma das isoformas que se encontra sobre-expressa em inúmeros tumores e estudos recentes revelaram que AQP5 é um dos principais mediadores de proliferação e migração celular através da via EGFR/ERK/p38 MAPK sugerindo que a AQP5 pode também participar na sinalização redox.

O stress oxidativo é definido como um desequilíbrio entre a produção de ROS e a sua eliminação através de antioxidantes e está diretamente relacionado com as três fases do cancro: iniciação, promoção e progressão. Durante o estadio de iniciação, as ROS podem produzir danos no ADN introduzindo mutações genéticas e alterações estruturais. No estadio de promoção, as ROS podem contribuir para alterações da expressão genética, bloqueio da comunicação célula-célula e modificação de sistemas de

sinalização, resultando num aumento da proliferação celular ou numa diminuição da apoptose. Finalmente, o stress oxidativo pode participar do estadio de progressão introduzindo mais alterações ao ADN. As alterações genéticas que contribuem para o cancro tem maior tendência a afectar três tipos de genes: proto-oncogenes, genes supressores de tumor e genes de reparação de ADN. Até ao momento, é desconhecido o mecanismo molecular que leva à alteração do nível das AQPs expressas nos tumores e não é possível explicar se são uma consequência ou a causa da tumorigénese. No entanto, os resultados obtidos até agora indicam que existe uma possível interação entre as AQPs e oncogenes/oncoproteínas que podem em último caso ativar a transcrição de genes envolvidos em crescimento, transformação e sobrevivência celular.

Tendo em conta a importância das AQPs na iniciação, promoção e progressão do cancro, foi avaliada a importância do perfil lipídico e de produtos de peroxidação lipídica na atividade de uma aquaporina de levedura, num modelo *Saccharomyces cerevisiae* de stress oxidativo. Para além disso, usando um modelo de expressão heteróloga em levedura, foram investigadas novas AQPs de mamífero capazes de transportar H₂O₂. Neste caso, foi utilizada uma estirpe *S. cerevisiae* cujas aquaporinas endógenas foram deletadas, permitindo distinguir a função de cada AQP individualmente expressa. A função das AQPs avaliada em leveduras foi posteriormente validada em linhas celulares de mamífero. Por outro lado, o nível de expressão genética das AQP1, AQP3 e AQP5 foi avaliado em células de cancro após incubação com H₂O₂ para avaliar a sua regulação em condições de stress oxidativo. Por último, os níveis de expressão da AQP1, AQP3 e AQP5 foram avaliados em várias biopsias de tumores de pâncreas como potenciais biomarcadores de prognóstico.

Os resultados obtidos e descritos durante este trabalho revelaram o aumento dos ácidos gordos polinsaturados aumentaram a expressão da

AQY1 e que a sua atividade é inibida pelo produto de peroxidação lipídica HNE. Além disso, a expressão da AQY1 aumentou a sensibilidade ao stress oxidativo facilitando o influxo de H_2O_2 , sugerindo que as AQPs participam na resposta ao stress oxidativo. Para além disso, verificámos que a AQP5, encontrada sobre-expressa em tumores, é transportadora de H_2O_2 e afeta a sinalização redox. Além disso, foi detectado um novo mecanismo de regulação a nível do mRNA em linhas celulares de cancro de mama e colon, que podem ser potencialmente importantes no desenvolvimento de tumores. Por fim, mostrámos que o nível de expressão da AQP1, AQP3 e AQP5 pode ser usado como biomarcador para o prognóstico e diagnóstico do adenocarcinoma ductal pancreático.

Esses resultados abrem uma nova perspectiva para avaliar os mecanismos moleculares associados à função das AQPs nas vias de sinalização em tumores e a sua possível interação com a resposta celular ao stress oxidativo.

Palavras-chave: Aquaporinas; stress oxidativo; regulação; cancro

Abstract

Aquaporins (AQPs) are a family of small transmembrane proteins present in all types of organisms and involved in the selective transport of water and small solutes such as glycerol in response to osmotic or solute gradients. So far, 13 isoforms were identified in humans and can be divided in three different groups according with their selectivity and sequence homology. AQPs were reported to be important in a wide range of physiological functions, such as water and energy homeostasis, exocrine fluid secretion and epidermal hydration. Dysfunction and/or abnormal expression of AQPs have been associated with several chronic diseases including epilepsy, cerebral edema, glaucoma, obesity, and cancer. In cancer, several isoforms were found overexpressed and three of them (AQP3, AQP8 and AQP9) can also transport H_2O_2 . This small molecule can be toxic, but at lower concentration can act as second messenger in signaling pathways responsible for cancer cell migration and proliferation.

Considering the importance of the AQPs in the initiation, promotion and progression of cancer, we first evaluated the effect of lipid peroxidation on the activity of a yeast aquaporin (AQY1), using a *Saccharomyces cerevisiae* oxidative stress model. In addition, we investigated the ability of mammalian AQPs to transport H_2O_2 using a *S. cerevisiae* heterologous expression model. In this case, the yeast strain used was previously deleted of endogenous aquaporins and transformed with mammalian AQPs, allowing to distinguish the function of each individually cloned isoform. It was identified AQPs with promising results and their function was validated in mammalian cell lines. On the other hand, the expression level of AQP1, AQP3 and AQP5 was evaluated in cancer cells after treatment with H_2O_2 to assess their gene regulation under conditions of oxidative stress. Finally, the expression level of AQP1, AQP3 and AQP5 was evaluated in several

pancreatic biopsies in order to determine potential biomarkers for diagnostic and prognostic.

The results obtained and described during this work showed that increased levels of membrane PUFAs triggered AQP1 expression, and its activity is impaired by HNE. Also, our data revealed the ability of AQP5, that is found overexpressed in tumors, as a H_2O_2 channel that can affect redox signaling. In addition, novel regulatory mechanism at mRNA level was detected in breast and colon cancer cells that may be involved in carcinogenesis. Finally, we show that AQP1, AQP3 and AQP5 can be used as biomarkers for pancreatic ductal adenocarcinoma prognostic and diagnostic. These results open a new perspective to assess the underlying molecular mechanisms of AQPs in signaling pathways associated with cancer and the possible interplay with cell oxidative stress response.

Keywords: Aquaporins; oxidative stress; regulation; cancer

Thesis Outline and General Aims

Aquaporins (AQPs) dysfunction and abnormal expression have been associated with several pathologies related to water and energy homeostasis, like kidney disorders, brain edema, obesity and cancer. Nevertheless, detailed mechanisms underlying these disorders remain largely unknown. The work developed in this thesis aimed to identify new AQP isoforms that may transport hydrogen peroxide as well as exploit their possible role in signaling cascades regulated by H_2O_2 . Furthermore, we aimed to evaluate AQPs expression in cancer cell lines and in tumor biopsies to identify new potential biomarkers as well as unveil the involvement of AQP in oxidative stress conditions.

In the first part of the work we used two yeast models, one with increased levels of membrane PUFAs and other with yeast aquaporin-1 (AQY1) overexpression to evaluate their interplay in cell's oxidative status. We also tested the effect of lipid peroxidation product HNE on AQY1 function and correlate with the cell sensitivity to oxidative stress. Then, a yeast cell model silenced on endogenous water channels was used to evaluate mammalian AQP function in their native physiological status expressed in intact yeast cells. Mammalian AQP isoforms were individually cloned in the silenced yeast strain to evaluate AQP function, as water and H_2O_2 channel. Our group has developed a heterologous expression system in yeast silenced on endogenous AQPs, simple and easy to handle and that can be used to characterize AQP function and assess regulatory mechanisms.

Furthermore, AQPs are found overexpressed in many tumors, and it is known that they have a role in tumor biology and thus have a strong potential to be used as cancer biomarkers. Since cancer cells are highly proliferative and metabolic, H_2O_2 production near the tumor is considerably higher than normal cells, and its uptake via AQPs may activate signaling

cascades that control proliferation. Thus, in the second part of the work, breast, colon and pancreatic cancer cells were screened to evaluate the expression profile of AQPs in each cancer cell line. In addition, regulation of AQP expression in oxidative stress conditions was investigated in breast and colon cancer cells. Finally, pancreatic cancer tissues and normal adjacent biopsies were analyzed aiming to correlate *in situ* AQP expression level with tumor aggressiveness, and identifying new potential biomarkers.

Chapter One ||

General Introduction

Water is the most abundant molecule in Nature, mainly responsible for the transport of compounds and solutes between cells and is essential for metabolic reactions and processes in all living cells. Water is vital for maintaining cellular homeostasis and keep a constant internal environment regardless of external changes [1]. Water movement, in response to different osmotic gradients, can occur in parallel between cells (paracellular movement) and through cells (transcellular movement) [2]. Regarding transcellular movement, water can cross biological membranes by three distinct pathways: i) by **diffusion** across the lipid bilayer, in which the energy barrier is too high and therefore the transport is slower and not regulated; ii) by **co-passive transport**, in which water molecules follow the movement of other solutes; iii) via **water channels**, in which transmembrane proteins provide a path through the membrane with a smaller energy barrier allowing a fast, regulated and selective pathway (Fig. I.1) [2, 3].

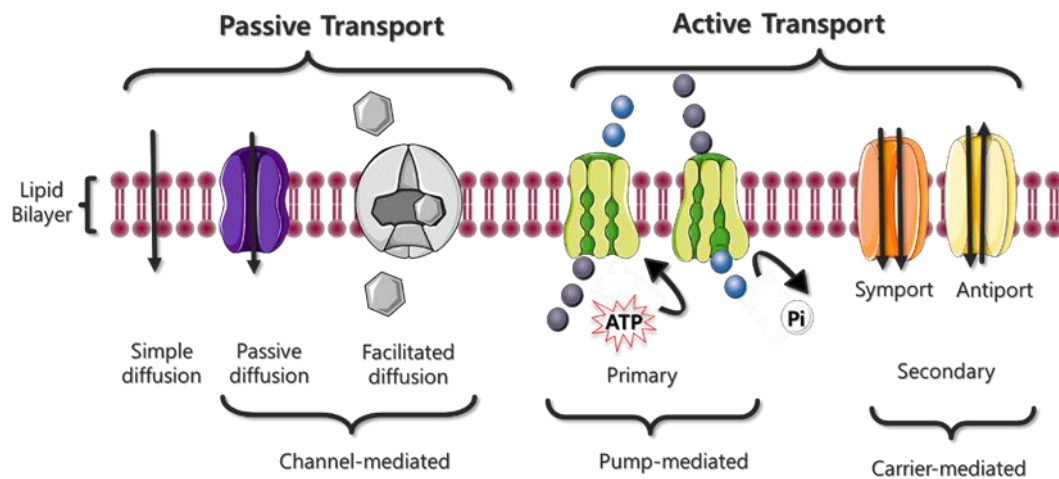


Figure I.1. Schematic representation of the water and solutes transport paths through the membrane by passive and active transport. In passive transport, particles diffuse down a concentration gradient and in active transport, particles diffuse against a concentration gradient and requires energy. Water can diffuse through lipid bilayer by simple diffusion, by co-passive transport following the movement of other solutes and by channel-mediated (aquaporins).

For many years, it was hypothesized the existence of selective pores for water in the plasma membrane, but only many years later experimental evidence based on high osmotic water permeability coefficients and low activation energy proved the existence of selective pores for water in red blood cells (RBC) [4, 5]. Agre and coworkers reported the purification of a membrane protein with 28kDa, named CHIP28, highly expressed in RBC and in renal tubules [6] and with high homology to an old family of transmembrane channels known as Major Intrinsic Proteins (MIP). Afterwards, CHIP28 was cloned in *Xenopus laevis* oocytes and for the first time characterized as a water channel by following the volume change when oocytes were exposed to a hypotonic solution [7]. Later, CHIP28 was renamed aquaporin-1 (AQP1) [8] and Peter Agre was rewarded in 2003 with the Nobel Prize in Chemistry for all the work and progress in physiology by discovery of CHIP28 as a water channel.

1. Aquaporins

Aquaporins are expressed in all living kingdoms [9] and since their discovery around 450 individual AQPs have been identified [10, 11]. The AQP family members have high homology (25%–60%) in amino acid sequence [12] and so far, 13 isoforms (AQP0-12) were identified in humans showing specific organ, tissue and cellular localization [13] thus suggesting a relation between their expression and function in each organ [14].

Regarding their selectivity and primary sequence, AQPs are subdivided in three subgroups: 1) **classical or orthodox aquaporins** (AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8) which are considered selective for water [14]; 2) **aquaglyceroporins** (AQP3, AQP7, AQP9 and AQP10) which, in addition to water, facilitate the transport of small uncharged solutes such as glycerol and urea [15, 16]; and 3) **S-aquaporins** (AQP11 and AQP12) with lower sequence homology, unique subcellular localization and permeability selectivity still not

clear [17] though AQP11 has been recently reported to transport both water and glycerol [18].

1.1. Structural features

In 2001, the high-resolution X-ray structure of AQP1 [19] revealed a tetrameric assembly formed by four identical monomers each corresponding to an individual water channel [11]. The central pore formed by the interaction of each monomer with two of its neighbors, excludes water molecules [20] but has been associated with gas transport and suggested that can act as a gated cation channel [21, 22]. Figure I.2 shows different views of human AQP5 tetrameric structure.

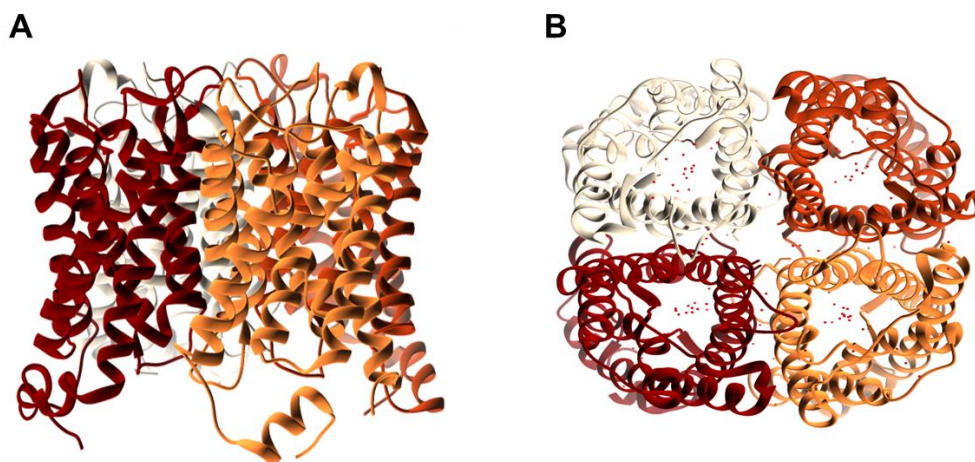


Figure I.2. Tridimensional structure of hAQP5 (*Homo sapiens*). Homotetramer representation of the AQP5 (*Homo sapiens*) determined in 2008 by X-Ray crystallography (2.0Å). **(A)** side view and **(B)** top. Water molecules are depicted in red balls. Picture edited in UCSF Chimera software (PDB code: 3D9S).

Each monomer comprises approximately 300 residues, with six highly hydrophobic transmembrane helices (1 to 6) connected by five loops (A to E) with the N- and C-terminal on the cytoplasmic side. Each individual monomer is constituted by an inner parallel repetition [11], a reflected structural feature due to gene duplication during the evolutionary process [23].

Together, the short helix loop B from the cytosolic side and the loop E from the exoplasmic side form the seventh transmembrane helix containing each one the NPA (Asn-Pro-Ala) sequence, highly conserved in AQPs and considered a signature of the MIP family [24]. NPA motifs are oriented 180° to each other and, in conjunction with internal duplication of the protein sequence in the center of the pore, narrow that region of the pore. It was proposed an hourglass model for the AQPs structure subsequently confirmed by the three dimensional structure of AQP1 [25]. Figure I3 shows the pore region of bovine AQP1 (helices and loops) and in detail the NPA motif and the size (ar/R) exclusion filters.

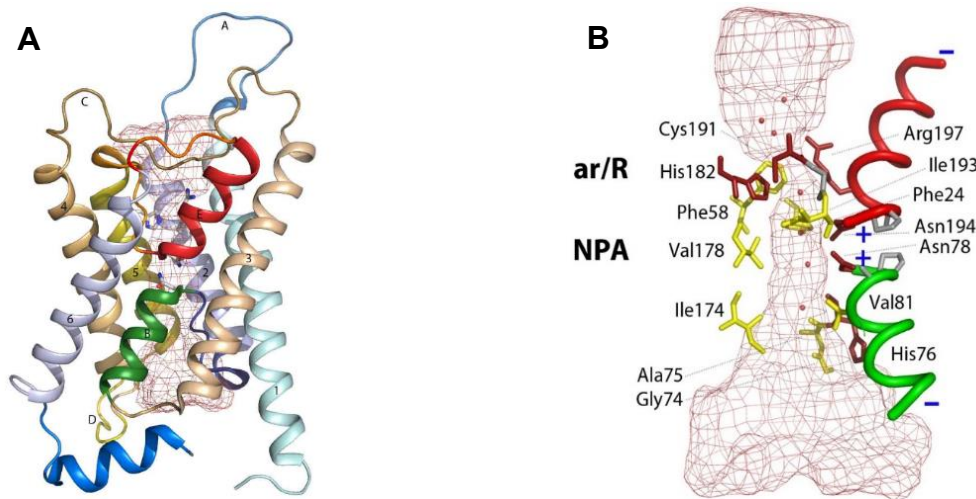


Figure I.3 Tridimensional structure of bAQP1 monomer. (A) Representation of bAQP1 monomer with respective numeration of the helices and loops; (B) detailed representation of the pore and residues (hydrophilic and hydrophobic residues are represented in red and yellow, respectively) located in wall of the channel, relevant to its selectivity. Red mesh indicates the pore region. Adapted from [26].

Each aquaporin has two selectivity filters, the size and charge exclusion filters, responsible for specific permeability [11]. These filters have small differences among the three AQPs subgroups but in general all isoforms are constituted by several carbonyl groups both in cytoplasmic and periplasmatic entry of the pore that facilitate water-wall residues interaction [27]. After passing these interactions, molecules reach the narrowest part of the pore

(**size exclusion filter**) formed by aromatic residues and arginines (ar/R), highly conserved in AQPs and forming a local constriction (Fig. I.3.B).

Structural differences between classical aquaporins and aquaglyceroporins are found in the size exclusion filter and result in a different size of the pore diameter. In classical AQPs, the pore diameter is around 2.8Å and allows the perfect passage of water molecules excluding molecules of larger diameter. In aquaglyceroporins, the size of the pore diameter is about 3.4Å and allows the passage of molecules such as glycerol or other solutes of smaller diameter [11, 27].

The NPA motifs in the central part of the pore constitute **the charge exclusion filter**. As observed in Fig. I.3.B for bAQP1, the hydrophobic Val178 residue intrudes into the pore and repels the water molecules forcing to interact strongly with Asn194 and Asn78 of the NPA loops (Fig. I.3.B). At the NPA region, the water dipoles rotate by 180° due to the local electric field dominated by the macrodipoles of helices B and E to break the hydrogen bonds formed [11]. This mechanism prevents the formation of a proton wire through the pore which ensures the electrochemical gradients across the membrane and is the largest energy barrier for proton conductance [11].

1.2. Water and solute transport

The aquaporin protein family seems to be designed for the selective passage of water or glycerol. Nevertheless, it has been reported that some isoforms can transport unconventional molecules like nonpolar gases (ammonia, carbon dioxide and nitric oxide), reactive oxygen species (H₂O₂) and metalloids (antimonite, arsenite and silicon) [28, 29]. Some of these molecules are part of cell signaling pathways, indicating that AQPs play an important role in other physiological mechanisms as regulators of signal transduction pathways in addition to their well-described involvement in osmotic volume regulation.

Regarding hydrogen peroxide (H_2O_2), it was widely believed that can freely diffuse across the lipid bilayer but the existence of various membrane systems imposing a permeability barrier to H_2O_2 diffusion [30]. This raised the hypothesis that H_2O_2 diffusion might be regulated by changes in membrane composition or by channel proteins, like AQPs [31]. Recently it was reported that AQP3 [32], AQP8 [33] and AQP9 [34] can promote H_2O_2 transport across cellular membranes. H_2O_2 and water show similar features such as molecular size, dipole moment and hydrogen bonding capacity [29]. The physico-chemical properties define the solutes that can be transported by aquaporins and therefore it is not surprising that H_2O_2 was qualified as a possible aquaporin substrate for all isoforms [35]. Although it is not clear that all AQPs function as H_2O_2 channels, at the moment three mammalian isoforms were already identified and may be considered involved in oxidative stress response.

H_2O_2 is a small molecule that belongs to a non-radical group of reactive oxygen species (ROS) and can have dual cellular actions depending on concentration. On one hand it reacts with various cellular targets causing cell damage or even cell death, and on the other hand acts as a signaling molecule in cell growth, cell development and signal transduction pathways both intra and intercellular [31]. ROS, like H_2O_2 , are continuously produced in the mitochondria as a product of aerobic energy metabolism and thus, due to its biological importance, the levels of H_2O_2 should be subjected to tight regulation [31].

1.3. Aquaporin regulation

Nowadays, aquaporins are very well studied at the structural level and the highest aquaporin structure solved to date (AQY1 from *Pichia pastoris*) obtained at 0.88Å provided details on the proton exclusion mechanism [36]. In addition to the selectivity displayed by aquaporins, water transport can be

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regulated post-translationally in response to biological stimuli, either by **gating** or by **trafficking** [27]. Factors like phosphorylation [14], pH [37], divalent cations [38], pressure and membrane tension [39, 40] regulate aquaporins function affecting gating behavior or conformational changes [28, 41]. Trafficking of AQP5 from intracellular organelles to plasma membrane, and vice versa for subcellular AQP5 [28], has been described by site-specific phosphorylation [42], by protein-protein interactions involving C-terminus, by Ca^{2+} -binding sites and by N-terminal conformational switch [27]. Particularly, it was proposed a mechanism for human AQP5 translocation from intracellular storage vesicles to plasma membrane triggered by phosphorylation of Ser156 [43]. This post-translational modification does not induce substantial protein conformational change but triggers AQP5 trafficking to plasma membrane. The short-term regulation of AQP5 alters cell membrane water permeability and it is crucial in physiologic mechanisms.

In plants, aquaporins are additionally regulated by environmental stress such as drought/flooding, salinity, temperature, nutrients deprivation and hypoxia [44]. ROS can act as second messengers in signaling transduction pathways to activate adaptive responses for cellular protection [45] and protect against an invasion by pathogens [46]. For plant AQP5, it was described an oxidative gating of water channels in green alga *Chara corallina* [47] and in roots of corn seedlings [44] by drastic reduction of water transport in both cases. The precise mechanism of the oxidative gating of AQP5 in the presence of ROS is not well understood but two possible mechanisms are proposed. Either aquaporin oxidation by $\cdot\text{OH}$ attacking the channel from inside the pore or by $\cdot\text{OH}$ attack of $\text{C} = \text{C}$ double bonds of the plasma membrane [44]. Anyway, it is needed to consider a relation between oxidative stress and water permeability not only in plant AQP5 but also for other species, including in mammals.

1.4. Aquaporins in diseases

Since the identification of the first AQP isoform, knowledge at the functional and physiological level of the aquaporin protein family has increased and has been extensively studied by analyzing genetically modified phenotypes. These studies revealed that AQPs participate in numerous physiological and pathophysiological mechanisms like renal water absorption, exocrine fluid secretion, epidermal hydration, neuro-homeostasis, tumor angiogenesis and metastasis, and fat metabolism [9, 48]. Although the underlying mechanisms still need clarification, modulation of AQPs expression/function might represent novel target for clinical benefit in treatment of different diseases, such as obesity, epilepsy, glaucoma, brain edema, and cancer [49], encouraging the identification and development of aquaporin modulators with potential pharmacologic applications. Regarding cancer, recent studies showed an abnormal level expression of AQPs and their involvement in physiological processes like cell volume regulation, energy metabolism, cell migration, adhesion, proliferation and differentiation. Concomitantly, AQPs play an important role in tumor growth, proliferation, migration and angiogenesis [50, 51].

ROS are responsible for damage of important biomolecules such as proteins, lipids and nucleic acids [52]. Although there are protective mechanisms against ROS, the misbalance between these species and cellular capacity to their scavenging causes oxidative stress that is associated with pathogenic mechanisms of several chronic diseases like neurodegenerative diseases, diabetes mellitus and cancer [52]. As mentioned above, ROS are products of the aerobic metabolism and can act as signaling molecules but in continued oxidative stress conditions ROS are produced over time and can induce chronic inflammation and somatic mutations [53]. These mechanisms are associated with increased risk of cancer development not only by damage of nucleic acids inducing DNA mutations, but also by chronic

inflammation in which leukocytes are recruited to site of damage with consequently increased uptake of oxygen and ROS production, creating a circle of inflammatory/oxidative environment near the site of damage [53]. Although ROS, like H_2O_2 , can be used as anti-tumorigenic agents, their involvement in three different stages of cancer (initiation, promotion and progression) have been described where their role depends mainly on the concentration, cells and tissues and on the location of ROS production [53].

2. Methods to assess AQP function

AQP function can be assessed either in isolated cells or in vesicles obtained from intracellular or plasma membranes. A heterologous expression cellular system is another approach to evaluate aquaporin activity and experimental assays have been described in *i)* *Xenopus oocytes*, *ii)* yeast silenced on endogenous AQPs; *ii)* AQP-transfected cell-lines and *iv)* mRNA-injected zebra fish embryos (reviewed in [41]). In these cellular systems correct folding and targeting should be verified, usually by analysis of cellular localization of AQP-GFP tagged by fluorescence microscopy.

Different technical approaches can be used to evaluate AQP function based on measuring water or solute permeability of biological membranes after an osmotic shock. Water moves from compartments of lower osmotic pressures to compartments of higher osmotic pressures to equilibrate the concentrations of osmolytes and maintain homeostasis. Thus, functional studies of AQPs can be performed by perturbing the system, generating an osmotic gradient, and measuring the consequent water movement till the establishment of an osmotic equilibrium.

Water flux (J_v , in $\text{cm}^3\cdot\text{s}^{-1}$) through cell membrane is faster than the ion flux and certain solutes such as mannitol. Thus, in the scale of observation of water movement, these solutes like mannitol are considered impermeant or non-diffusible (ND). When cells are exposed to osmotic gradients due to the

addition of ND solutes, the consequent water out-flux produces measurable changes in cell volume (dV/dt , in $\text{cm}^3\cdot\text{s}^{-1}$). The rate and extent to which these changes occur attaining a new osmotic equilibrium depends on the number of AQPs present in the membrane and can be assessed through the evaluation of the water permeability coefficient (P_f in $\text{cm}\cdot\text{s}^{-1}$) [54].

However, there are other solutes such as glycerol or urea that in the scale of observation of water movement are considered permeant or diffusible. In this case, when the cells are subjected to an osmotic gradient by adding a permeant solute (S) it is observed a simultaneous water (J_v in $\text{mol}\cdot\text{s}^{-1}$) and solute flux (J_s , in $\text{mol}\cdot\text{s}^{-1}$) between different compartments thus dissipating the osmotic gradient ($\Delta\Pi$) and the solute gradient (ΔC_s) in order to achieve a final osmotic balance [54].

Stopped-flow spectrophotometry is a method widely used to follow the chemical kinetics of a reaction in solution and relies on light scattering or fluorescence outputs (after loading cells or vesicles with volume-sensitive fluorescent dye). Cell suspensions are subjected to rapid mixing of osmotic solutions and the consequent water flux produced until osmotic equilibrium is attained alters the scatter/fluorescence signal. Water/solute movement is followed and AQP function is assessed due to a linear relation between the optical properties of the system and cell volume. Analysis of the relative volume changes allows estimating water (P_f) or solute (P_s) permeability coefficient [28, 41].

Among other microscopy techniques (reviewed in [54]), characterization of AQP function in cultured cells is extensively performed by fluorescence microscopy that benefits from the properties of volume sensitive fluorescent dyes [55]. Commonly, adherent cells are loaded with a membrane-permeant non-fluorescent precursor that is cleaved intracellularly by non-specific esterases and remains trapped inside the cell (impermeable fluorescent form) [56]. After an osmotic challenge in a perfusion chamber,

alterations in fluorescence intensity due to the fluorescence self-quenching of the fluophore can be monitored in real-time. The osmotic water permeability (P_f) and the solute permeability (P_s) can be estimated from the linear relationship between fluorescence intensity and cell volume [56].

3. References

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Chapter Two ||

Assessment of AQP-mediated H₂O₂ transport

This chapter is adapted from following published and in preparation manuscripts:

- Rodrigues, C., Tartaro Bujak, I., Mihaljević, B., Soveral, G. and Čipak Gašparović, A., Yeast aquaporin regulation by 4-hydroxynonenal is implicated in oxidative stress response. IUBMB Life. 69(5):335-362 (2017) doi: 10.1002/iub.1624
- Rodrigues, C.*, Móscá, A. F.*, Martins, A. P., Nobre, T., Prista, C., Antunes, F., Čipak Gašparović, A. and Soveral, G., Rat Aquaporin-5 Is pH-Gated Induced by Phosphorylation and Is Implicated in Oxidative Stress. Int J Mol Sci. 17(12): 2090 (2016) doi: 10.3390/ijms17122090 *equal contribution to the work
- Rodrigues, C., Pimpão, C., Móscá, A. F., Lopes, D., da Silva, I. V., Pedersen, P. A., Antunes, F., and Soveral, G., Human aquaporin-5 facilitates hydrogen peroxide permeation affecting adaption to oxidative stress and cancer cell migration (submitted)

In biological systems, reactive oxygen species (ROS) are generated endogenously by mitochondrial respiratory chain or in response to extracellular insults. ROS products from biological reduction of molecular oxygen (O₂), like superoxide anion (O₂⁻), hydroxyl radical (OH·) and H₂O₂, contribute to oxidative stress and their pathophysiological actions depend on the intracellular concentration [1]. Oxidative stress arises from imbalance between ROS production and their degradation by the antioxidant system, including enzymatic and nonenzymatic antioxidants. Oxidative stress is responsible for macromolecular damage, including nucleic acids, proteins and lipids [2] and can lead to initiation and progression of several chronic diseases, like atherosclerosis, diabetes, neurodegeneration and tumorigenesis [3, 4]. However, at low concentration ROS can regulate protein expression and/or alter protein function by post-translational modifications that may initiate multiple signaling pathways and regulate biological and physiological processes [5].

1. Hydrogen peroxide as signaling mediator in redox biology

H₂O₂ is mainly generated by the mitochondrial electron transport chain, in particular by complex I, II and III, or by NADPH oxidases (NOX), and is rapidly scavenged by catalase, peroxiredoxins and glutathione peroxidases [6]. In addition, it has been observed increased level of ROS in sequence of external stimuli (cytokines, peptide growth factors, agonists of receptors), mainly by activation of NOX (see review in [7]). ROS level is tightly regulated and can induce protein modification at synthesis level regulating protein expression, or at post-translation level by oxidative modification affecting protein function and stability, as depicted in Fig. II.1.1. The underlying mechanisms can involve: i) ROS direct attack of catalytic cysteines present in several members of signaling cascades including ERK, JNK, p38, MAPK [8] and PI3K/Akt [9]; ii) indirect post-translation modification of redox sensor proteins

by direct oxidative modification or formation of an inter- or intra-molecular disulfide bond, like in some protein tyrosine phosphatases (PTPs) that are an important regulatory family of phosphorylation state [10]; iii) oxidative modification of redox-sensitive interacting proteins leading to dissociation of protein-protein complexes and activation of free proteins, as observed for Nrf2-Keap1 complex [11]; or iv) at transcriptional level, transcription factors can be modulated by conformational alterations (direct cysteine oxidation) or by association to co-activator/repressor, leading to increase or decrease of their affinity towards DNA thus regulating gene expression [12].

Specific cysteines found in redox-sensitive proteins have low pK_as due to surrounding amino acid microenvironment. In this case, thiolate anion of cysteines (Cys-S⁻) is more prone to be oxidized at physiological pH to a sulfenic form (Cys-SOH) and can induce protein modification that prompt transmission of a redox signal [13]. Usually, the sulfenic modifications are more likely to occur near the cellular localization of H₂O₂ accumulation due to its rapid degradation.

Interestingly, serine/threonine protein kinases [8, 14, 15], phospholipases [16] and transcription factors [17, 18] have sensitive thiol groups to H₂O₂. Mild oxidative stress can induce reversible oxidation of amino acid residues in redox sensor proteins leading to structural and functional changes and critically modify cellular signaling mechanism (redox biology). Undoubtedly, ROS generated in response to the activation of various receptors exert their effect as second messenger in several signaling pathways, either by transcriptional regulation or by protein modulation, and regulate physiological processes related to cell survival and cell death.

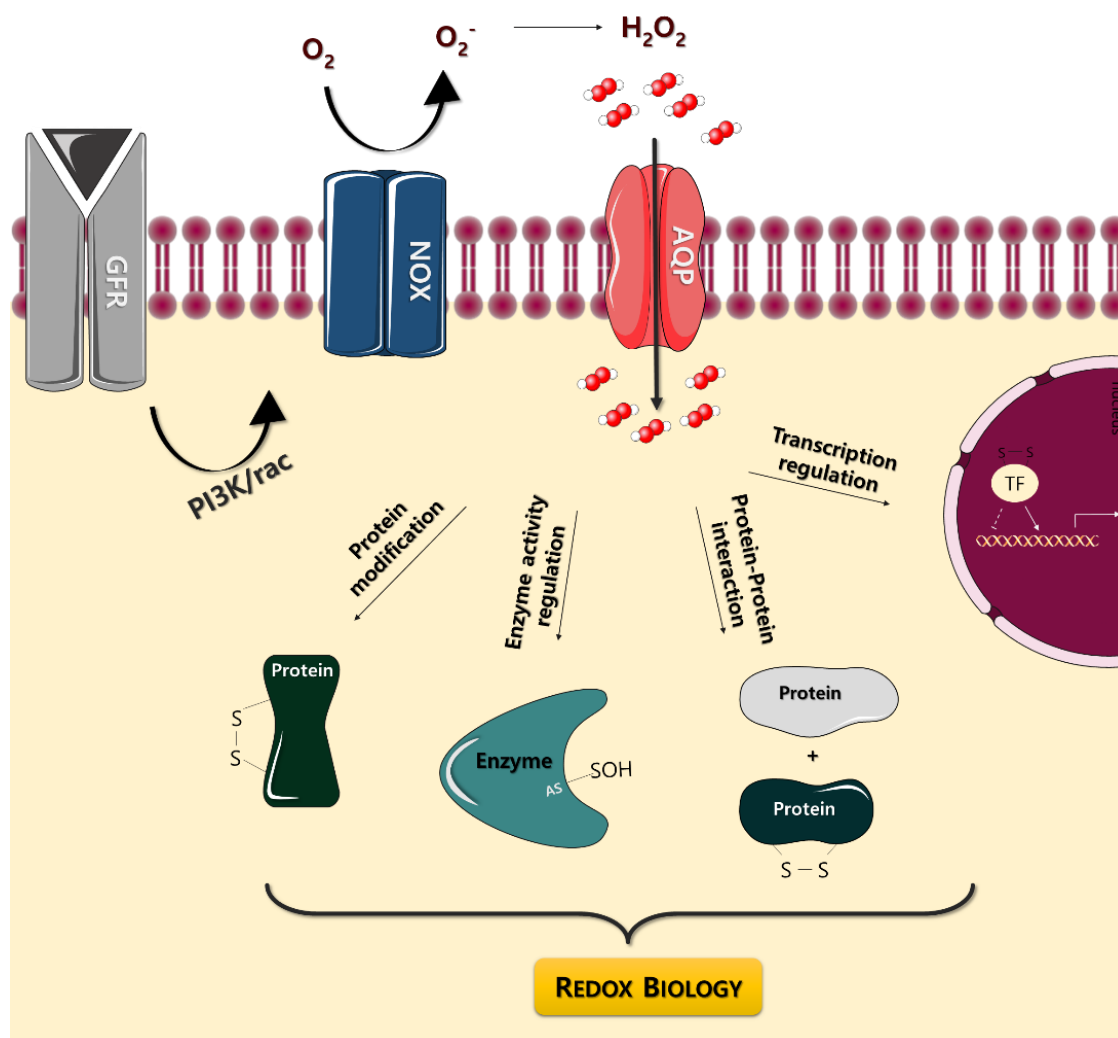


Figure II.1.1. Schematic representation of redox biology. Growth factor receptor is stimulated by ligand binding and mediates the production of superoxide (O_2^-) by NADPH oxidase, through PI3K/Rac pathway. Extracellular superoxide dismutase converts superoxide radical into H_2O_2 that is permeated by some aquaporins. At intracellular level, H_2O_2 can be scavenged by cellular antioxidant and converted into water or can regulate structural and functional changes of redox sensor proteins. Redox-mediated signaling is essential to regulate physiological processes related to cell survival and cell death (redox biology). H_2O_2 can induce thiol modification by direct oxidation, inter- and intramolecular disulfide bridges of redox-sensitive proteins resulting in protein modification or dissociation of protein complexes, including protein phosphatases, protein kinases, phospholipases and ion channels. Also, enzymatic activity can be modified by alteration of oxidative state of catalytic cysteines (protein tyrosine phosphatase) and transcription factors react with H_2O_2 and regulate the affinity towards DNA. **Abbreviations:** AQP, aquaporin; AS, Active site; GFR, growth factor receptor; NOX, NADPH oxidase; SOH, oxidized thiol; TF, Transcription factor.

1.1. Aquaporin-facilitated H₂O₂ transport

The resemblance of H₂O₂ with water molecule in terms of structural and physico-chemical properties, including similar size, dielectric properties, bipolar momentum, and capacity to form hydrogen bonds, suggests that all aquaporins may permeate H₂O₂ and act as peroxiporins [19]. However, so far only three human isoforms showed direct evidence of H₂O₂ transport, namely AQP3 [20], AQP8 [21] and AQP9 [22].

First, Bienert et al. analyzed 24 aquaporins from plants and mammals individually expressed in *S. cerevisiae* strain and observed that hydrogen peroxide transport is facilitated by AQP8 [21]. An indirect assay showed that AQP8 expression impaired yeast growth and survival in solid medium containing H₂O₂ as consequence of failure in ROS detoxification. AQP8-transforming yeast strain did not show alteration of ROS scavengers enzymes, and thus increased sensitivity to H₂O₂ due to impairment in ROS detoxification was discarded. Then, specific AQP8-mediated H₂O₂ flux across plasma membrane was validated using an AQP inhibitor (AgNO₃) with consequent decreased level of the fluorescence signal [21]. The authors used a ROS-sensitive fluorescent dye, 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA). This fluorescent probe is widely used for intracellular H₂O₂ detection but depends on nonspecific oxidation and may give biased information [23].

Therefore, in order to overcome indirect methods, the uptake of H₂O₂ by AQPs has been evaluated using two H₂O₂-selective fluorescent probes, namely HyPer [24] and Peroxy Yellow 1 Methyl-Ester (PY1-ME) [20]. Miller et al. designed and synthesized PY1-ME probe and tested human embryonic kidney cells (HEK293) transfected with AQP1, AQP3 and AQP8 to detected H₂O₂-specific transport [20]. After treatment with H₂O₂, both AQP3- and AQP8-overexpressing cells, but not AQP1, exhibited increase in the fluorescence signal. To validate the results obtained with the new designed PY1-ME probe,

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a secondary method was used to evaluate H₂O₂ influx in HEK293 cells expressing HyPer, a genetically encoded H₂O₂ sensor. Similarly, an enhancement of HyPer fluorescence was observed in AQP3-overexpressing HEK293 cells upon treatment and for the first time it was demonstrated an aquaglyceroporin (AQP3) as H₂O₂ channel [20]. In addition, H₂O₂ uptake was observed in AQP8-overexpressing HEK293 cells expressing the genetically encoded fluorescent H₂O₂ sensor HyPer, validating previous results obtained with AQP8 heterologous expressed in yeast [20].

In order to unveil other AQPs as H₂O₂ channels, chinese hamster ovary (CHO-K1) cells were transfected with AQP9 and were incubated with H₂O₂. The cellular uptake was followed in cells stably expressing HyPer and in cells loaded with fluorescent probe CM-H₂DCFDA [22]. AQP9-facilitated H₂O₂ permeation was detected by increased fluorescence signal after incubation with H₂O₂. Hydrogen peroxide influx was impaired in AQP9 knockdown cells as well as in erythrocytes and bone marrow-derived mast cells from AQP9^{-/-} mice supporting AQP9 function as H₂O₂ channel. Also, cytotoxicity effect of H₂O₂ was mitigated in AQP9 knockdown cells which indicated that AQP9-facilitated H₂O₂ permeability contributes to oxidative stress and can activate apoptotic signaling pathways [22].

Regarding rat and human AQP1 isoforms, controversial results were obtained despite of the high homology between sequences. Comparing growth and survival assay in yeast expressing model, rat AQP1 expression showed increased cell sensitivity to external H₂O₂ [25] but human AQP1 expression did not show significant alteration [21]. The H₂O₂ uptake using a fluorescent probe was evaluated for both isoforms in mammalian cells and again the results were consistent with those obtained for yeast. HEK293 cells transfected with hAQP1 did not show a significant alteration of fluorescence signal [20] whilst enhanced fluorescence signal in rat aortic smooth muscle cells indicated that rAQP1 facilitates H₂O₂ diffusion [26]. Even though the two

AQPs sequences are highly similar and differ in only 18 amino acids, the variation of membrane abundance can explain the difference observed in H₂O₂ transport efficiency. Furthermore, after sequence alignment, five amino acids were found without any similar properties and it will be interesting to thoroughly examine by direct mutagenesis the importance of these amino acids for structural and regulatory mechanisms.

Taking into account the similarity of human aquaporin sequences, we could expect that all 13 human isoforms can transport H₂O₂ (peroxiporins). However this feature remains to be demonstrated.

1.2. Role of H₂O₂-mediated by AQPs in redox signaling

The fact that H₂O₂ can act as second messenger prompted the researchers to investigate the participation of AQPs as H₂O₂ channels in redox biology and in regulation of several physiological and pathophysiological processes. Of several proteins involved in signaling pathways that are regulated by ROS, serine/threonine kinase Akt is one of the most studied because it is a key component in cellular signal transduction which promotes diverse physiological processes, including cell survival, growth and proliferation [27].

In neuroblastoma cells, it was demonstrated that Akt phosphorylation was induced by H₂O₂-mediated activation of PI3K and resulted in Bax inactivation, a key component of the cell death machinery [28]. Post-translational modification of Bax in response of H₂O₂-induced activation of PI3K/Akt suggests that low levels of H₂O₂ can protect from apoptosis and promote cell survival [28]. In gastric cancer cells, matrix metalloproteinases are positively regulated by AQP3 via PI3/Akt signaling pathway [29], possible by AQP3-facilitated H₂O₂ diffusion, although the mechanism remains to clarify. Miller and co-workers assessed the correlation between H₂O₂ uptake by AQP3 and regulation of downstream intracellular signaling [20]. First, the authors

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observed an increase in Akt phosphorylation in response to H₂O₂ treatment, in serum-starved HeLa cells expressing AQP3 but not in control cells, which suggests that AQP3 contributes to downstream signaling [20]. Then, wild-type colon cancer adenocarcinoma cells (HT29), endogenously expressing AQP3, were used together with AQP3-silenced HT29 cells to validate H₂O₂ transport by AQP3 [20]. AQP3-overexpressing HT29 cells treated with epidermal growth factor (EGF) prompted NOX protein activation and consequent increase in Akt phosphorylation. Cells treated with extracellular catalase, diphenyleneiodonium (NOX inhibitor) and AQP3 silencing resulted in inhibition of EGF-induced Akt phosphorylation. Thus, it was suggested that H₂O₂ produced by activation of NOX upon EGF stimulation is transported by AQP3 and regulates intracellular redox signaling [20]. In breast cancer, *in vitro* and *in vivo* assays were performed to ascertain the mechanistic involvement of AQP3 in cell migration during metastasis via CXCL12-CXCR4 signaling [30]. The authors observed that CXCL12/CXCR4 axis induces H₂O₂ production by NOX activation and it is the transported by AQP3. Localization of AQP3-NOX2 complex at the cell leading edge reinforces the idea that AQP3-mediated H₂O₂ transport is required for directional migration. In AQP3^{-/-} breast cancer cells, it was observed a reduction in the level of oxidized PTEN/PTP1B protein as well as in Akt phosphorylation after stimulation with H₂O₂ or CXCL12. These data suggest that AQP3 is required for breast cancer cell migration by a mechanism involving CXCL12/CXCR4-dependent H₂O₂ production by NOX activation and subsequently AQP3-facilitated H₂O₂-dependent PTEN/PTP1B oxidation, followed by activation of the Akt pathway and regulation of directional cell migration [30].

In another context, AQP3 plays an important role in inflammatory response. Colonic epithelium forms the physical barrier against external insults and controls inflammation. AQP3 was found expressed in epithelial cells and is dysregulated in intestinal inflammation by TNF- α [31], a pro-inflammatory

cytokine that can be modulated by redox status [32]. AQP3-expressing colon epithelial cells (Caco-2) showed an increased HyPer fluorescence signal after treatment with H₂O₂ [33], consistent with those previously obtained. Epithelial cells from AQP3^{-/-} mice support the evidence obtained with monolayer colon cells [33]. AQP3 is required for H₂O₂ uptake generated by NOX activation, upon response to injury and host defense against pathogen infection, and can act as a signaling molecule for epithelial migration and wound repair [33]. A recent study explored the molecular mechanism of AQP3 role in psoriasis, and reported that AQP3-dependent H₂O₂ transport in keratinocytes is crucial for the pathogenesis of psoriasis [34]. AQP3 deficiency in mice conferred resistance to psoriasis when stimulated with a known psoriasis-inducer (IL-23). In IL-23-mediated induction of psoriasis mice model there was a constant NF-κB activation in response to TNF-α-induced production of extracellular H₂O₂ followed by its intracellular transport by AQP3 [34]. In this case, membrane-associated AQP3-NOX2 complex observed in keratinocytes appears to be crucial for H₂O₂ uptake produced extracellularly by NOX2 activation in response to TNF-α stimulus. This interplay constitutes an important role in the pathogenesis of psoriasis since allows a dynamic control of intracellular H₂O₂ concentration to achieve the required concentration to oxidized the critical cysteine residue present in protein phosphatase 2A (PP2A) that regulates NF-κB activation [34], a known mediator in the pathogenesis of psoriasis [35]. These results are in agreement with described previously, showing that NF-κB transcription factor activation is dependent of TNF-α stimulation, which regulation depends on H₂O₂ levels [36]. In addition to the implication of AQP3-facilitated H₂O₂ in NF-κB signaling in keratinocytes, AQP3 is required for T-cell chemotaxis to the skin, an important factor to the onset of psoriasis. AQP3 deficiency resulted in impaired T-cell migration into the dermal and epidermal layers and reduced downstream immune response during the development of this disease [34]. In addition, impairment in migration of CD4⁺ T cells from

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AQP3^{-/-} mice toward to chemokine CXCL12, along with deficient actin polymerization and inadequate develop of distinct uropods was restored after exogenous H₂O₂ supplementation and supports the evidence that AQP3-mediated H₂O₂ transport participates in T cell chemotaxis [37]. Impairment in chemotaxis efficiency was also observed in neutrophils from AQP9^{-/-} mice, but the underlying mechanism remains unclear [22]. Vascular endothelial growth factor (VEGF) is related to angiogenic signaling, including in leukemia cells, possible through NOX-generated H₂O₂ [38]. A recent study observed that VEGF-dependent H₂O₂ generation and intracellular uptake via AQP8 in B1647 leukemia cells is crucial for activation of cell proliferation pathways [39]. AQP8-mediated H₂O₂ permeability triggers the phosphorylation of both PI3K and p38 MAPK, two kinases involved in angiogenesis, and appears to have an important role in redox signaling associated to leukaemia cell proliferation [39]. Similarly, in HeLa cell model silenced for AQP8, cytosolic H₂O₂ level upon EGF induction was strongly diminished along with inhibition of tyrosine phosphorylation [40]. Also, addition of extracellular catalase to WT HeLa cells reduced tyrosine phosphorylation protein similar to that obtained for AQP8-silenced cells, which proposes that AQP8-mediated H₂O₂ permeability exerts its effect by regulating downstream tyrosine kinase signaling [40]. In addition, it is proposed that AQP8 permeability is regulated by cellular stress possible by redox regulatory modification of cysteine 53 [41]. The expression of AQP8 mutant (C53S), resistant to inhibition induced by stress, diminished phosphorylation of p38 and restricted stress-induced apoptosis. The data point to a key physiological role of conserved cysteine residue in AQP8 gating and regulates cell signaling and survival during stress [41]. In human hepatocarcinoma-derived cell line HepG2, mitochondrial AQP8 silencing diminished mitochondrial H₂O₂ efflux and leads to membrane depolarization along with loss of cell viability [42] but the molecular mechanism remains unclear. Loss of cell vitality was also observed in activated marine teleost

spermatozoa with a direct involvement of mitochondrial AQP8-mediated H₂O₂ efflux (scavenging) and preservation of spermatozoa motility by ROS detoxification [43], later on observed in human sperm cells [44] but further studies are needed to unveil the mechanism.

1.3. Polyunsaturated fatty acids and cancer

Fatty acids are carboxylic acids with long unbranched aliphatic tail chains. Natural fatty acids commonly have a chain of 4 to 28 carbons, which according to its saturated state and structural and functional groups can be classified as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA). PUFA contain two or more carbon-to-carbon double bonds and there is increasing evidence that can play a role in cancer risk and progression [45]. When compared to normal cell, the level of ROS is increased in many type of cancer cells [46] which can alter the membrane bilayers by lipid peroxidation of PUFA generating several reactive aldehydes including 4-hydroxynonenal (HNE) [47]. HNE is the lipid peroxidation product most studied and can easily react with important biomolecules including proteins, DNA, and phospholipids containing amino group [48]. Lipid peroxidation products, especially HNE, can act as signaling regulator molecules and modulate redox cell signaling pathways inducing cancer cell proliferation, differentiation and apoptosis (reviewed in [49]). In addition, the lipid content and the breakdown of lipids with the formation of reactive aldehydes can lead to changes in the permeability and fluidity of the cellular membrane and can induce alterations in cell migration and invasiveness [50].

2. Yeast as a simple eukaryotic model

The yeast *S. cerevisiae* is one of the best characterized eukaryotic model and is widely used to study proteins in general due to the broad library of strains available and due to their easy and inexpensive genetic manipulation

when compared to mammalian cell cultures. Yeast cells possess high functional homology to higher eukaryotes including mammals and can endure severe external environments that allows setting several experimental conditions that could not be applied to mammalian cells.

Yeast *S. cerevisiae* cells express two endogenous AQPs, namely AQY1 and AQY2, and constitute a powerful model for studies of osmoregulation [51]. In addition, *S. cerevisiae* do not synthesize PUFA but only monounsaturated fatty acids. For this reason, *S. cerevisiae* is a perfect system to be genetically manipulated to express delta12 desaturase, responsible for PUFA synthesis, to study the effect of PUFAs at the membrane level as well as the effect of lipid peroxidation on AQP function and correlate with the cell sensitivity to oxidative stress.

Altogether, it became clear that AQPs participate actively in physiological and pathophysiological processes by controlling transmembrane diffusion of H₂O₂ and subsequently regulation of downstream signaling and cascade activation. Also, peroxidation of PUFA generates several reactive aldehydes including HNE that activates signaling pathways and consequently modulate cellular processes leading to proliferation differentiation or apoptosis. Thus, we hypothesized if HNE could modulate AQP function and we evaluated their interplay in cell's oxidative status. In addition, during this work we proposed to characterize the function of AQP5, found upregulated in many tumors, as a potential H₂O₂ channel. Also, we also investigate the role of AQP5 in resistance to oxidative stress and the importance AQP5-mediated H₂O₂ transport in cancer cell migration.

3. Material and methods

3.1. Mammalian cell culture and culture medium

Biopsy xenograft of Pancreatic Carcinoma line-3 (BxPC3) was obtained from ATCC with catalog no. CRL-1687 and cultured at 37°C in 5% CO₂. Cells

were grown in RPMI1640 medium with 10% FBS and 1% penicillin/streptomycin. Medium was changed every 2-3 days and experiments were performed with 70% to 80% cell confluence.

3.2. Transfection with siRNA for AQP silencing

Short interfering RNA (siRNA) targeting human AQP3 (ID: s1521), human AQP5 (ID: s1527) were purchased from Ambion. Silencer® Negative Control siRNA #1 (Ambion) was employed as the negative control to ensure silencing specificity in all the experiments. Briefly, the medium was removed and then Opti-MEM I reduced serum medium without antibiotics (Opti-MEM) (Life technologies) was added to the cells. siRNA (30 pmol) were diluted in Opti-MEM and mixed with Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) pre-diluted in Opti-MEM according to the manufacturer's instructions. After 5 min incubation at room temperature, the mix was added to the cells and incubated at 37°C in 5% CO₂. For double silencing, both AQP3-RNAiMAX and AQP5-RNAiMAX complexes were added to the cells. After 48 h of incubation, the knockdown efficiency was evaluated by quantitative real-time RT-PCR, as below.

3.3. RNA isolation and RT-qPCR

Total RNA was extracted from cultured cells using TRIzol Reagent (Invitrogen), according to the manufacturer's protocol. The RNA was treated with RNase-free DNase I (Sigma) to avoid contamination with genomic DNA. Extracted RNA was quantified with Nanodrop™ 2000c spectrophotometer. For template cDNA synthesis, 1 µg of total RNA was reverse transcribed in a 20-µL final volume using random hexamers primers (Roche Applied Science, Penzberg, Germany) and 200 units of M-MLV reverse transcriptase (Invitrogen) as previously described [52].

Real-time PCR reactions were carried out using a CFX96 Real-Time System C1000 (BioRad, Hercules, CA, USA), the TaqMan Universal PCR Master

Mix (Applied Biosystems Thermo Fisher Scientific, Waltham, MA, USA) and the following specific TaqMan pre-designed gene expression primers: AQP1 (Hs01028916_m1), AQP3 (Hs01105469_g1), AQP5 (Hs00387048_m1) and ACTB (Hs99999903_m1) (Applied Biosystems). The cDNA was amplified at the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

The relative quantification of gene expression was determined using the $2^{-\Delta Ct}$ method (adapted from [53]). Using this method, we obtained the fold variation in AQP gene expression normalized to an endogenous control (β -actin).

3.4. Yeast strains and growth conditions

Saccharomyces cerevisiae strains used in Chapter 2 were BYctrl, BYdesa, ANTctrl, ANTaqy1, control (pUG35 empty vector), rat-AQP5 and human-AQP5 (both cloned in pUG35 vector) and the details of the genetic background and plasmids are presented in Table II.2.1. BYctrl and BYdesa strains were kindly given by Dr. Ana Čipak Gašparović (Institut Ruđer Bošković, Zagreb, Croatia). ANTctrl and ANTaqy1 were kindly given by Dr. Patrick Van Dijck (VIB, Leuven, Belgium) and transformed yeast strain with human-AQP5 (cloned in pUG35) was kindly given by Dr. Pederson (University of Copenhagen, Copenhagen, Denmark)

BYctrl and BYdesa strains were grown in minimal synthetic-defined (SD) media (1% w/v glucose, 0.17% Difco Yeast Nitrogen Base) supplemented with 0.004% w/v adenine, 0.002% w/v arginine, 0.002% w/v histidine, 0.01% w/v leucine, 0.003% w/v lysine, 0.002% w/v methionine, 0.01% w/v threonine, and 0.002% w/v tryptophan [54]. ANTctrl and ANTaqy1 strains were grown at 28°C in yeast nitrogen base (YNB) medium (2% w/v glucose, 0.67% Difco Yeast Nitrogen Base) supplemented with 0.004% w/v leucine, 0.002% w/v histidine, 0.003% w/v tryptophan and 0.002% w/v uracil [55]. Transformed yeast strain

with pUG35 (empty vector), rat-AQP5 and human-AQP5 were grown in same medium as ANTctrl, ANTa_{qy1} without uracil. Yeast strains were maintained in the same medium with 2% (w/v) agar. For all experiments, cells were grown to mid exponential phase (OD₆₀₀ 1.0).

Table II.2.1. Strains and plasmids used in Chapter 2.

Yeast strains (Name in this work)	Genotype/Relevant characteristics	Source/Reference
BYctrl	BY4742 (MAT α his3 Δ 1 leu2 Δ 1 lys2 Δ 0 ura3 Δ 0) transformed with pYES2	[54]
BYdesa	BY4742 (MAT α his3 Δ 1 leu2 Δ 1 lys2 Δ 0 ura3 Δ 0) transformed with pYES2-desa1	[54]
ANT29 (ANTctrl)	10560-6B/pYX012 KanMX MAT leu2::hisG trp1::hisG his3::hisG ura3-52	Dr. Patrick Van Dijck
ANT 27 (ANTa_{qy1})	10560-6B/pYX012 KanMX AQY1-1 MAT α leu2::hisG trp1::hisG his3::hisG ura3-52	Dr. Patrick Van Dijck
pUG35 empty vector (Control)	10560-6B MAT α leu2::hisG tpr1::hisG his3::hisG ura3-52 aqy1D::KanMX aqy2D::KanMX transformed with pUG35	Our lab
Rat AQP5 isoform (rAQP5)	10560-6B MAT α leu2::hisG tpr1::hisG his3::hisG ura3-52 aqy1D::KanMX aqy2D::KanMX transformed with pUG35-rAQP5	Our lab
Human AQP5 isoform (hAQP5)	10560-6B MAT α leu2::hisG tpr1::hisG his3::hisG ura3-52 aqy1D::KanMX aqy2D::KanMX transformed with pUG35-hAQP5	Prof. Per Amstrup Pedersen

Plasmids (Name in this work)	Relevant characteristics	Source/Reference
pYES2 (empty vector)	GAL1/10 promoter, Amp ^r , URA3 selection marker and CYC1 transcriptional terminator	Invitrogen
pYES2-desa1	Fatty acid-Δ12 desaturase (H. brasiliensis) gene cloned into pYES2	[56]
pUG35 (empty vector)	MET25, Amp ^r , URA3 selection marker, CYC1 transcriptional terminator and yEGFP N-terminal fusion protein	Our lab
pUG35-rAQ5	Rat AQP5 (<i>Rattus norvegicus</i>) gene cloned into pUG35	Our lab
pUG35-hAQ5	Human AQP5 (Homo sapiens) gene cloned into pUG35	Prof. Per Amstrup Pedersen

3.5. Cloning and heterologous expression of AQP5 in *S. cerevisiae*

This section was performed according to previously described methods [57]. Plasmid (pcDNA3) with *Rattus norvegicus* AQP5 cDNA (pcDNA3-AQP5), kindly provided by Prof. Miriam Eschevarría (Virgen del Rocio University Hospital, Seville, Spain), was used for AQP5 cDNA amplification. The centromeric plasmid pUG35 was used for cloning AQP5, conferring C-terminal GFP tagging, MET25 promoter, and CYC1-T terminator [58]. For plasmids propagation, *Escherichia coli* DH5α was used as host [59]. *E. coli* transformants were maintained and grown in Luria-Bertani broth (LB) supplemented with ampicillin (100 µg·mL⁻¹), at 37°C [60]. Plasmid DNA was extracted from *E. coli* using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). AQY-null *S. cerevisiae* (10560-6B MATa leu2::hisG tpr1::hisG his3::hisG ura3-52 aqy1D::KanMX aqy2D::KanMX) was used as host strain for heterologous

expression of rat-AQP5. The *aqy*-null strain was grown and maintained in YPD medium (2% w/v peptone, 1% w/v yeast extract, 2% w/v glucose). Then, rat AQP5-specific primers modified to incorporate restriction sites for *SpeI* (underlined) and *Clal* (underlined) (5'-GGACTAGTCCT ATG AAA AAG GAG GTG TGC TCC CTT GC-3' and 5'-CCATCGATGGA GTG TGC CGT CAG CTC GAT G-3', respectively) were designed and used for PCR amplification of rat AQP5 cDNA (carried out in an Eppendorff thermocycler using Taq Change DNA polymerase from NZYTech). The PCR product was digested with *SpeI* and *Clal* restriction enzymes, purified (using a Wizard® SV Gel and the PCR Clean-Up System kit Promega) and cloned (using T4 DNA Ligase Roche) into the corresponding restriction sites of pUG35 digested with the same restriction enzymes, behind the MET25 promoter and in frame with the GFP sequence and CYC1-T terminator, according to standard protocols [60], to construct the expression plasmid pUG35-rAQP5. This plasmid was propagated in *E. coli* DH5α. After extraction and purification, fidelity of constructs and correct orientation of rAQP5-cDNA were verified by PCR amplification and DNA sequencing. Transformation of the *S. cerevisiae* *aqy*-null strain with pUG35-rAQP5 was performed using the lithium acetate method described in [61]. The same strain was also transformed using an empty pUG35 vector to be used as a control (further indicated as control strain). Transformants were selected on YNB medium without uracil as auxotrophic marker.

For human AQP5 and site-directed mutagenesis, two sets of expression plasmids were generated. One set expresses non-tagged AQP5 while the other set expresses AQP5 C-terminally tagged with yeGFP. All plasmids were constructed by homologous recombination in yeast strain YSH1770 by co-transformation of AQP5 derived PCR fragments and *Bam*HI, *Sall*, *Hind*III digested pUG35 as described before [62]. Primers used are shown in Table II.2.2. The nucleotide sequence of all constructs was verified by DNA sequencing at Eurofins Genomics, Germany.

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Table II.2.2. Nucleotide sequences of the PCR primers used for generating the mutants analyzed in the present study. Nucleotide sequences in turkish are for homologous recombination with pUG35, the red sequence is a yeast Kozak sequence while AQP5 sequences are shown in black. The codon changed in each primer is underlined. Non-tagged AQP5 mutations were made by transforming YSH1770 with BamHI, Sall, HindIII digested pUG35 and two PCR products; one generated by AQP5fw + a mutant rv primer the other by AQP5rv + the corresponding mutant fw primer. GFP tagged version were made in the same except for using the AQP5-GFPv primer instead of AQP5rv. Non-tagged and GFP tagged wild type AQP5 were generated by transforming YSH1770 with BamHI, Sall, HindIII digested pUG35 and PCR products generated by AQP5fw + AQP5rv and AQP5-GFPfw + AQP5-GFPv, respectively.

Abbreviations: Fw, forward; Rv, reverse.

Construct	Primer sequences
AQP5	Fw: 5' ATACATAGATACAATTCTATTACCCCATCCATAC TAAGATAATT ATGAAGAA GGAGGTGTGCTC 3'
	Rv: 5' ACAACACCAGTGAATAATTCTTCACCTTTAGACATTCAGCGGGTGGTCAGCT C 3'
AQP5-GFP	Rv: 5' ACAACACCAGTGAATAATTCTTCACCTTTAGACATGCGGGTGGTCAGCTCCA 3'
AQP5-GFP H173A	Fw: 5' ACCCTGGGCGCACTTGTCTGGAATC 3'
	Rv: 5' GATTCCGACAAGTGCAGCCAGGGT 3'
AQP5-GFP H173W	Fw: 5' ACCCTGGGCTGGCTTGTCTGGAATC 3'
	Rv: 5' GATTCCGACAAGCCAGCCAGGGT 3'
AQP5-GFP S156A	Fw: 5' CGCCGCACCGCACCTGTGGGCT 3'
	Rv: 5' AGCCACAGGTGCGGTGCGGCG 3'
AQP5-GFP S156E	Fw: 5' CGCCGCACCGAACCTGTGGGCT 3'
	Rv: 5' AGCCACAGGTTCGGTGCAGGCG 3'

AQP5-GFP S183A	Fw: 5' CACTGGCTGCGCAATGAACCCAGC 3'
	Rv: 5' GCTGGGTTCATTGCGCAGCCAGTG 3' (continue)
AQP5-GFP S183A	Fw: 5' CACTGGCTGCGAAATGAACCCAGC 3'
	Rv: 5' GCTGGGTTCATTTGCGCAGCCAGTG 3'

3.6. AQP5 subcellular localization by fluorescence microscopy

For subcellular localization of GFP-tagged AQP5 in *S. cerevisiae*, yeast cells in the mid-exponential phase were observed using a Zeiss Axiovert 200 fluorescence microscope (Zeiss Axiovert), at 495 nm excitation and 535 nm emission wavelengths. Fluorescence microscopy images were captured with a digital camera (CoolSNAP EZ, Photometrics) and using the Metafluor software (Molecular Devices). AQP5 membrane expression was measured by evaluating GFP-protein fluorescence intensity according to [63, 64]. A linear profile that crosses the cell membrane was generated and analyzed using the software ImageJ (<https://imagej.net>). The intensity profile along the line path from at least 30 cells in each experimental condition ($n = 3$) was recorded, and for each cell three profile lines were taken. The background intensity along the same distance was measured and subtracted from the peak fluorescence intensity over each line, and the obtained difference divided by the maximal fluorescence to calculate the relative membrane expression.

3.7. Aquaporin functional assessment

For permeability assay, yeast strains grown as described, with orbital shaking, at 28°C up to OD₆₀₀ 1.0 (Gallenkamp Cooled Orbital Incubator), were harvested by centrifugation (5000g; 5 min; 4°C) (Allegra® 6 Series Centrifuges, Beckman CoulterVR) washed 3 times and resuspended in ice cold sorbitol (1.4 M) K⁺-citrate (50 mM pH 5.1 or 7.4) buffer up to a concentration of 0.3 g·mL⁻¹

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¹ wet weight and cells were incubated on ice for at least 90 min. Prior to permeability assays, cells were preloaded with the non-fluorescent precursor 5(6)-carboxyfluorescein diacetate (CFDA, 1 mM, 20 min at 30°C) (Sigma), which is intracellularly hydrolyzed yielding the impermeable fluorescent form, as previously described [65]. Cells were then diluted (1:10) in 1.4 M sorbitol buffer and immediately used for experiments. Equilibrium cell volumes (V_0) were obtained after loading the cells with CFDA under an epifluorescent microscope (Zeiss Axiovert) equipped with a digital camera. Cells were assumed to have a spherical shape with a diameter calculated as the average of the maximum and minimum dimensions of each cell.

Permeability assays were performed by stopped-flow fluorescence spectroscopy as previously described [65], using a HI-TECH Scientific PQ/SF-53 stopped-flow apparatus, which has a 2-ms dead time, controlled temperature, interfaced with a microcomputer. Experiments were performed at temperatures ranging from 9 to 34°C. Four runs were usually stored and analyzed in each experimental condition. In each run, 0.1 mL of cell suspension was mixed with an equal volume of hyperosmotic sorbitol buffer (2.1 M sorbitol, 50 mM K⁺-citrate, pH 5.1 or 7.4) producing an inwardly directed gradient of the impermeant sorbitol solute that induces water outflow and cell shrinkage. Fluorescence was excited using a 470 nm interference filter and detected using a 530 nm cut-off filter. The time course of cell volume change was followed by fluorescence quenching of the entrapped fluorophore. The recorded fluorescence signals were fitted to a single exponential from which the rate constant (k) was calculated. The osmotic water permeability coefficient, P_f , was estimated from the linear relationship between P_f and k [65], $P_f = k(V_0/A)(1/V_w(\text{osm}_{\text{out}}))$, where V_w is the molar volume of water, V_0/A is the initial volume to area ratio of the cell population, and (osm_{out}) is the final medium osmolarity after the osmotic shock. The osmolarity of each solution was determined from freezing point depression by a semi-micro-

osmometer (Knauer GmbH). The activation energy (E_a) of water transport was evaluated from the slope of the Arrhenius plot ($\ln P_f$ as a function of $1/T$) multiplied by the gas constant R .

3.8. External pH dependence and *in vivo* PKA phosphorylation

Yeast cells were grown and prepared as above described and incubated in isotonic sorbitol buffer (1.4 M sorbitol, 50 mM K-citrate) at two different pH (5.1 and 7.4) for at least 90 min. Deprived of a carbon source and incubated in ice for a long period, yeast cells are considered in starvation. The production of intracellular cAMP and phosphorylation was triggered immediately before water permeability measurements by the addition of 0.1 M glucose (adjusted to pH 5.1 or 7.4) to starved cells [66, 67].

3.9. Hydrogen peroxide consumption

The H₂O₂ consumption was measured in intact yeast cells. Cells were harvested by centrifugation (5000g; 10 min; RT) (Hettich Rotofix32), resuspended in fresh growth media and incubated at 30°C with orbital shaking. Hydrogen peroxide (50 μ M) was added to intact cells and the consumption of H₂O₂ was measured by following O₂ release with an oxygen electrode (Hansatech Instruments Ltd.) after the addition of catalase [68]. Direct consumption of H₂O₂ (18 μ M) was measured in the same conditions using a H₂O₂ electrode (World Precision Instruments). In both cases, H₂O₂ consumption was reported as a first order rate constant (s^{-1}), obtained from the slope of a semi-logarithmic plot of H₂O₂ concentration versus time.

3.10. Intracellular ROS analysis

To evaluate H₂O₂ influx, oxidation kinetics of non-fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂-DCFDA, Invitrogen, ThermoFisher, Massachusetts, USA) to fluorescent 2',7'-dichlorofluorescein was

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measured until 60 min from H₂O₂ administration, according to previously described methods [69].

Yeast transformants were grown up to OD₆₀₀ 1.0, harvested by centrifugation (4000g; 10 min; 4°C), washed 3 times with phosphate buffer 0.1 M pH 5.0 and resuspended in the same buffer to OD₆₀₀ 1.4. Then, cells were incubated with 5 µM H₂-DCFDA for 45 min at 30°C and washed one time with phosphate buffer 0.1 M. Cells were transferred to black multi-well plates and incubated with several concentrations of H₂O₂ (0.5 – 50 mM). Fluorescence intensity was measured after addition of H₂O₂ over time until 60 min in a microplate reader at an excitation/emission of 485/520 nm (FLUOstar Omega, BMG Labtech). As control, H₂O₂ non-treated strains was incubated with 5 µM H₂-DCFDA and fluorescence intensity was followed. The intracellular ROS accumulation was calculated from the slope of a plot of fluorescence intensity versus time and normalized to non-treated cells.

BxPC3 cells were seeded in 6-well microplates at a density of 0.15x10⁶ cells/well and were allowed to adhere for 24 h prior to AQP silencing. After 48 h incubation with silencing reagent, H₂O₂ transport was measured in individual adherent cells on a coverslip. Briefly, cells were loaded with 10 µM H₂-DCFDA for 30 min at 37°C in 5% CO₂. After, cells were washed twice with Ringer Buffer (RB) pH 7.4 (140 mM NaCl, 2 mM CaCl₂, 1 mM MgSO₄, 1.5 mM K₂HPO₄, 10 mM Glucose) and the coverslips with the cells were mounted in a perfusion chamber (Warner Instruments, Hamden, CT) on the stage of a Zeiss Axiovert 200 inverted microscope, using a x40 epifluorescence oil immersion objective. Fluorescence was excited at wavelength 495/10 nm; emission fluorescence was collected with a 515/10 nm band pass filter. Data were recorded and analyzed using the Metafluor Software (Molecular Devices) connected to a CCD camera (Cool SnapTM EZ Photometrics). Cells were equilibrated in RB pH 7.4 for 2 min and then 100 µM H₂O₂, freshly prepared in RB, was added directly to the cells. H₂-DCFDA fluorescence was scanned every 10 s. For the inhibition studies,

cells were incubated with 10 μ M Auphen for 15 min or 0.1 mM HgCl₂ for 5 min at 37°C in 5% CO₂. H₂O₂ consumption is reported as a first order rate constant obtained from the slope of a semi-logarithmic plot of H₂O₂ concentration versus time.

3.11. Yeast sensitivity assay

Acute stress — Yeast cells were grown overnight to mid exponential phase (OD₆₀₀ 1.0). Ten-fold serial dilutions (up to 10⁻⁴) were prepared for each strain and 100 μ L of highest dilution was plated in triplicate. BYctrl, BYdesa, ANTctrl and ANTaqy1 cells were treated with 0.25, 0.5, 1, and 2 mM HNE or with 1 mM H₂O₂ for 1 h on an orbital shaker at 28°C. AQY-null strains transformed with empty vector (pUG35) or with rat AQP5 isoform were treated with 0.5 mM H₂O₂ for 5, 15, 30, 45 and 60 min on an orbital shaker at 28°C. After each incubation time, 100 μ L aliquot was taken from each condition, diluted to 10⁻⁴ and 100 μ L was plated in triplicate. As a control for maximum viability, aliquots of 100 μ L cells of the same serial dilution without treatment were also plated in triplicate. Agar plates were then incubated for 3 days at 28°C, till visible growth was observed and colonies were then counted. Results are expressed as percentage of the time 0 (non-treated cells) colony number. For HNE, IC₅₀ was calculated using nonlinear regression curve fitting of log(inhibitor) versus normalized response and variable slope with a least squares (ordinary) fit of GraphPad Prism 5 software.

Chronic stress — Growth assays were performed on solid YNB medium, supplemented with 2% (w/v) glucose. Solid YNB medium with 1.5 mM H₂O₂ was freshly prepared at the time of inoculation for oxidative stress experiments. Yeast strains were grown in liquid YNB medium, with orbital shaking, at 28°C up to OD₆₀₀ $\approx$ 1.0 corresponding to 1 $\times$ 10⁷ cells/mL (Gallenkamp Cooled Orbital Incubator). Cells were harvested by centrifugation (4000g; 10 min; 24°C) (Centrifuge 5810 R Eppendorf), washed once in sterile

distilled water, and re-suspended to OD₆₀₀ $\approx$ 10. Multi-well plates were prepared with serial 10-fold dilutions of the original concentrated culture up to 10⁻⁵, 3 μ L suspensions were spotted with replica platter for 96-well plates device on plates containing YNB solid medium with and without H₂O₂ and incubated at 28°C. Differences in growth phenotypes of yeast strains were recorded after 1 and 2 weeks of incubation.

3.12. Migration assay

BxPC3 cells were seeded in 6-well microplates at a density of 0.15x10⁶ cells/well and were allowed to adhere for 24 h prior to AQP silencing. After 48 h incubation with silencing reagent, a wound was made with an even trace in the middle of the monolayer using a sterile 10- μ L pipette tip. After washing three times with phosphate buffered saline (PBS) to remove cell debris, cells were incubated with low serum (2% FBS) RPMI medium or with 100 μ M H₂O₂ prepared in same medium. The cells were then incubated at 37°C in a 5% CO₂ incubator, and images of the wound were captured at intervals of 2 hours. The distance of the wound was measured under a light microscope and analyzed using the software ImageJ (<https://imagej.net>). Wound closure was normalized to original wound area at time 0. All samples were tested in triplicate, and the data are expressed as the mean $\pm$ SD.

3.13. Preparation of cell lysates for colorimetric assays

For antioxidant measurements, yeast strains cultivated to OD₆₀₀ 1.0, were harvested by centrifugation (3000g; 5 min; 4°C) (Eppendorf® Centrifuge 5702), and washed with dH₂O and dry pellets were stored at -80°C until analysis. Dry pellet was resuspended in PBS and disrupted mechanically by vigorous agitation with acid washed glass beads for 7 one-minute intervals with cooling intervals between each agitation cycle. After disruption, cell lysates were cleared by centrifugation (14000g; 15 min; RT) (Eppendorf® Minispin®) and the supernatants were used for assays. Prior to performing the assays,

protein concentration of cell lysates was determined according to Bradford, using bovine serum albumin as a standard [70].

3.14. Catalase activity analysis

The catalase activity was measured by modified method of Goth [71]. This method is based on the measurement of H₂O₂ degradation in cell lysate, which occurs mostly by catalase activity as it has one of the highest turnover numbers among all the enzymes. For catalase activity assay, 40 µL of supernatant was mixed with 65 mM H₂O₂ for the start of the reaction. Different dilutions of hydrogen peroxide (0–75 mM) were used for standards. The reaction was stopped after 5 min by addition of 100 µL of 200 mM ammonium molybdate and color development was measured spectrophotometrically in a plate reader at 405 nm (Shimadzu UV-1601). One unit of catalase activity is defined as the amount of enzyme needed for degradation of 1 µmol of H₂O₂/min at 25°C. Catalase activity was expressed as units of catalase per milligram of proteins in cell lysate (U·mg⁻¹).

3.15. Determination of GSH levels

The intracellular GSH content was measured by modification of the protocol described by Tietze [72]. Briefly, samples were diluted to 0.03 mg/mL protein and 150 µL of each was used for the assay. Reduced glutathione in serial dilutions (0–20 mg/mL) was used as a standard. Reaction was started by addition of freshly prepared reaction mix: 1.8 mM 5,5-dithio-bis-2-nitrobenzoic acid, 0.4 U GSH reductase, and 0.6 mM NADPH in phosphate buffer (100 mM NaH₂PO₄, 5 mM EDTA pH 7.4). The formation of 2-nitro-5-thiobenzoic acid was monitored spectrophotometrically in a plate reader at 405 nm (Shimadzu UV-1601). GSH concentration in cell lysates was expressed as µM of GSH per milligram of total protein (nmol·mg⁻¹).

3.16. Fatty acid analysis

The samples were evaporated and lipid extracts were treated with 0.5 M KOH/MeOH [73, 74] for 10 min at RT and extracted with hexane. The corresponding fatty acid methyl esters were analyzed by gas chromatography (GC). GC analysis of total fatty acids were performed by Varian 450-GC equipped with a flame ionization detector. A Stabilwax column (crossbond carbowax polyethylene glycol, 60 m 3 0.25 mm) was used as a stationary phase at a programmed temperature with helium as the carrier gas. The heating was carried out at 80°C followed by an increase of 40°C/min up to 250°C for 15 min. Methyl esters were identified by comparison with the retention times of authentic samples.

3.17. Statistical analysis

Stopped-flow experiments were performed in decaplicates, while all other experiments and assays were carried out in triplicate. Mean values were compared using ANOVA followed by unpaired t-test. Statistically significant outcomes are represented as $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***); ns is not significant. Statistical analysis were performed using the Graph Prism 5 software.

4. Yeast aquaporin regulation by 4-hydroxynonenal is implicated in oxidative stress response

4.1. Abstract

Reactive oxygen species, especially H₂O₂, contribute to functional molecular impairment and cellular damage, but also are necessary in normal cellular metabolism, and in low doses play stimulatory role in cell proliferation and stress resistance. In parallel, reactive aldehydes such as HNE, are lipid peroxidation breakdown products which also contribute to regulation of numerous cellular processes.

Recently, channeling of H₂O₂ by some mammalian aquaporin isoforms has been reported and suggested to contribute to aquaporin involvement in cancer malignancies, although the mechanism by which these membrane water channels are implicated in oxidative stress is not clear. In this study, two yeast models with increased levels of membrane PUFA and aquaporin AQY1 overexpression respectively, were used to evaluate their interplay in cell's oxidative status. In particular, the aim of the study was to investigate if HNE accumulation could affect aquaporin function with an outcome in oxidative stress response.

Our data show that induction of aquaporin expression by PUFAs results in increased water permeability in yeast membranes and that AQY1 activity is impaired by HNE. Moreover, AQY1 expression increases cellular sensitivity to oxidative stress by facilitating H₂O₂ influx. On the other hand, AQY1 expression has no influence on the cellular antioxidant GSH levels and catalase activity. These results strongly suggest that aquaporins are important players in oxidative stress response and could be contribute to regulation of cellular processes by regulation of H₂O₂ influx.

Keywords: Reactive oxygen species (ROS); Oxidative stress;

4-Hydroxynonenal (HNE); Polyunsaturated fatty acid (PUFA); Aquaporins (AQPs)

4.2. Introduction

For a long time polyunsaturated fatty acids were considered merely to regulate membrane fluidity. Discovery of the arachidonic acid as precursor for prostaglandin synthesis introduced PUFAs as molecules that modulate inflammation and also raised the question about their possible role in other cellular processes [75]. Since then, PUFAs function is being clarified; but still, due to the complex composition of mammalian cell membranes, the development of a simplified eukaryotic model enabling to assess the influence of PUFAs on cell membrane behavior and its outcome on cell signaling was imperative.

Yeasts are eukaryotic cells that do not synthesize PUFAs but only monounsaturated fatty acids and that are easily genetically manipulated, thereby being an attractive system for the study of the PUFAs effect at the membrane level. In fact, introduction in the yeast genome of the inducible delta12 desaturase gene proved to enable the modulation of its membrane fatty acid content [56]. In such yeast system, cells produced linoleic acid and incorporated it in membranes and, under mild oxidative stress conditions, showed increased sensitivity to oxidative stress [56]. During the period of increased sensitivity to oxidative stress, changes in gene expression occur. Upregulation of the pentose phosphate and fatty acid beta oxidation pathway genes as well as aquaporin genes have been reported, concomitantly with downregulation of components of electron transport chains, thereby decreasing the potential source of ROS generation [76]. In addition, antioxidant activities are altered. Altogether, these events change the cell response to oxidative stress and the initial sensitivity is shifted to stress resistance after 3 days of PUFAs presence in the yeast membrane [54].

Interestingly, these events are similar to those observed in mammalian cells where mild oxidative stress stimulates the cellular antioxidative system causing adaptation and even cell proliferation [77]. It is known that adaptation to mild oxidative stress is a result of activation of Nrf2 signaling pathway [78, 79] and that moderate oxidative stress also activates signaling pathways such as MAPK and NF κ B pathways [80, 81], modulating inflammation. In addition to the interaction of oxidants with redox sensitive proteins, oxidative stress also affects other cellular molecules such as lipids. In turn, peroxidation of PUFAs in particular, results in the production of great variety of reactive aldehydes that also play an active role in cellular signaling [82, 83]. Among these aldehydes, HNE was the first shown to modulate cell growth [84] but only recently the mechanisms by which HNE is involved in signaling pathways are getting revealed [85]. HNE activates most of the pathways triggered by oxidative stress, by binding to redox sensitive molecules [86]. HNE-protein adducts were detected in numerous pathologies like cancer [82, 87]. A recent study reported that HNE was found bound to a membrane protein channel aquaporin-4 (AQP4) in epilepsy and suggested it could be a new player in oxidative neuronal damage [88]. Aquaporins are membrane channels that facilitate the rapid movement of water and small uncharged solutes across membranes and were found upregulated in a variety of systemic malignancies [89, 90]. Recently, an increasing number of reports showed that AQPs are abundantly expressed in different tumors [90]. While the mechanisms explaining AQPs upregulation in cancer are still under debate, AQPs implication in water and energy homeostasis facilitating cell migration and proliferation as well as their interaction with signal transduction pathways involved in cell growth and proliferation, have been reported [91]. Additionally, channeling of H₂O₂ by some AQP isoforms [21, 92] has been considered as a possible mechanism explaining their involvement in cell redox status and tumorigenesis [93]. The fact that yeast cells transformed with delta12

desaturase gene show PUFA peroxidation, increased HNE production and concomitantly exhibit an increase in aquaporin gene (AQY1) expression [54, 76], prompted us to investigate the significance of aquaporin upregulation in this oxidative stress cell model. Thus, in this study we evaluated AQY1 functional activity in desa-transformed yeast cells by measuring water permeability and activation energy of water transport, a valuable parameter to identify AQP activity [65, 94, 95]. Moreover, since HNE is prone to form aquaporin-adducts as reported for AQP4, we investigated the effect of HNE on AQP activity. AQP involvement in H₂O₂ permeation, ROS production and correlation with sensitivity to oxidative stress indicate that it may have a role in oxidative cell response.

4.3. Results and Discussion

4.3.1. PUFA increases expression of functional aquaporins

In order to confirm previously observed overexpression of AQY1 [76], we tested AQY1 activity by evaluating yeast membrane water permeability. Water transport activity in BYdesa strain (PUFA-producing yeast) was evaluated by monitoring the shrinkage rate of cells after a hyperosmotic challenge (Fig. II.4.1.A). It can be noticed that, after a hyperosmotic shock, the BYdesa cells adapted to a new equilibrium volume faster than the BYctrl. The osmotic water permeability coefficient (P_f) estimated for the BYdesa ($(12.3 \pm 0.08) \times 10^{-4} \text{ cm}\cdot\text{s}^{-1}$) is 1.9 times higher than P_f estimated for BYctrl ($6.57 \pm 0.73 \times 10^{-4} \text{ cm}\cdot\text{s}^{-1}$) (Fig. II.4.1.B). At the same time, the activation energy (E_a) of water transport was also determined for both strains. The E_a is a valuable parameter enabling to distinguish water permeation through lipid bilayers from proteic water channels pathways; the high E_a values observed for lipid bilayers decreases significantly when aquaporins are expressed and are functional [94, 95]. All the obtained E_a values are in accordance with the previously published data on yeast aquaporins [95]. As shown in Figure II.4.1.C, the lower E_a obtained for

BYdesa (10.9 ± 0.1 kcal·mol⁻¹ compared to 13.5 ± 0.4 kcal·mol⁻¹ for BYctrl, $p < 0.01$) corroborate the higher P_f value and indicate that BYdesa strain is expressing functional AQY at the plasma membrane although the number of active channels may be low.

Our results indicate the PUFA presence in yeast cell membrane increases functional expression of aquaporin. Interestingly, a reduced AQP9 level in n3-PUFA depleted rats with no decrease in mRNA was previously reported and was attributed to hyperinsulinemia or lack of PPAR γ activation of aquaporin transcription rather than to direct effect of PUFA depletion [96].

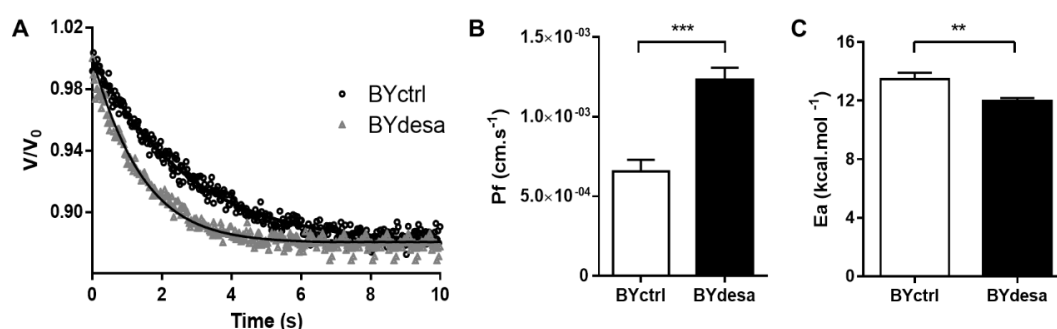


Figure II.4.1. Water permeability of BY strains. (A) Time course of cell volume change (V/V_0) after a hyperosmotic shock. BYdesa strain shows a faster volume adaptation to the new osmotic equilibrium; (B) Water permeability coefficient (P_f) at 23°C. Values are means $\pm$ SD of 10 measurements; (C) Activation energy (E_a) of osmotic water transport. For each strain, 4–8 replicates were analyzed for each experimental condition. Values are means $\pm$ SD. Significance levels: **, $p < 0.01$; ***, $p < 0.001$.

4.3.2. HNE inhibits osmotic water permeability

Previously, we have shown that BYdesa generated HNE as a result of linoleic acid peroxidation [56]. Thus, we aimed to investigate the effect of HNE on AQY1 activity. For all the strains inhibitory concentrations of HNE for 50% (IC_{50}) of the cells were determined and were 1.4 mM, 0.9 mM, 1.1 mM and 1.0 mM for ANTctrl, ANTaqy1, BYctrl and BYdesa, respectively. The observed IC_{50} for all the strains were higher than the previously published data [97], but the strain used is different than the one in this paper. Also, sensitivity to HNE is

strain specific, as observed for the ANTctrl and BYctrl strains. Therefore, 500 μ M HNE, being below IC₅₀, was selected for all further analysis. After incubation of cells with HNE prior to permeability assays a significant decrease of P_f on BYdesa (18.4%, $p < 0.001$) was induced by 0.5 mM HNE, while BYctrl was not affected (Fig. II.4.2.A).

To further confirm that the observed inhibition was specific for aquaporin activity, the strain ANTa_{qy1} (overexpressing AQY1 in wild type background) was tested for water permeability and HNE effect. The basal permeability of ANTa_{qy1} at 23°C, ca. 2-fold the permeability of the control, was decreased (11.4%, $p < 0.05$), and curiously, a similar inhibition was also observed for the ANTctrl strain (13.7%, $p < 0.05$), which may be explained by the endogenous AQY1 expression of this wild-type control strain.

Knowing that the activation energy of water transport (E_a) can be used to detect AQP function and discriminate between lipid diffusion and aquaporin water permeation [65], we computed E_a values for both strains with and without HNE. Water flux through a hydrophilic channel pore needs lower activation energy than fluxes across a hydrophobic lipid bilayer, and thus high permeability and low E_a indicate permeation via AQPs (reviewed in [51, 65]). Results show that while incubation with HNE did not change E_a for the ANTctrl, whose AQY expression levels are relatively low, a significant increase was detected for the ANTa_{qy1} (8.54 ± 0.43 kcal·mol⁻¹ to 12.83 ± 0.75 kcal·mol⁻¹, $p < 0.001$) (Fig. II.4.2.B), clearly demonstrating an HNE effect on AQY1 permeability.

In addition, since previous results from our group showed an increased content of PUFA in the BYdesa strain concomitantly with aquaporin upregulation, we investigated if AQY1 overexpression would in turn affect membrane lipid saturation content of the ANTa_{qy1} strain. Membrane analysis of saturated (C16:0 and C18:0) and monounsaturated (C16:1 and C18:1) fatty acids, compared to control, showed a slight, but not significant, decrease on

both C18 (C18:0 and C18:1) and increase in both C16 (C16:0 and C16:1) fatty acids content (Fig. II.4.2.C). This result confirms that AQY1 expression does not influence the overall level of fatty acid saturation in membrane, but instead the degradation products of PUFA peroxidation (such as HNE) may affect AQY1 activity. The fact that HNE can form covalent adducts with cellular proteins such as AQP4 in neurons and glial cells thus affecting water and ion balance [88], indicates that lipid peroxidation aldehydes can indeed contribute to modulation of aquaporin activity. In addition, transcription factors like AP1 and SP1 regulating aquaporin transcription [96] are in part regulated by HNE [98].

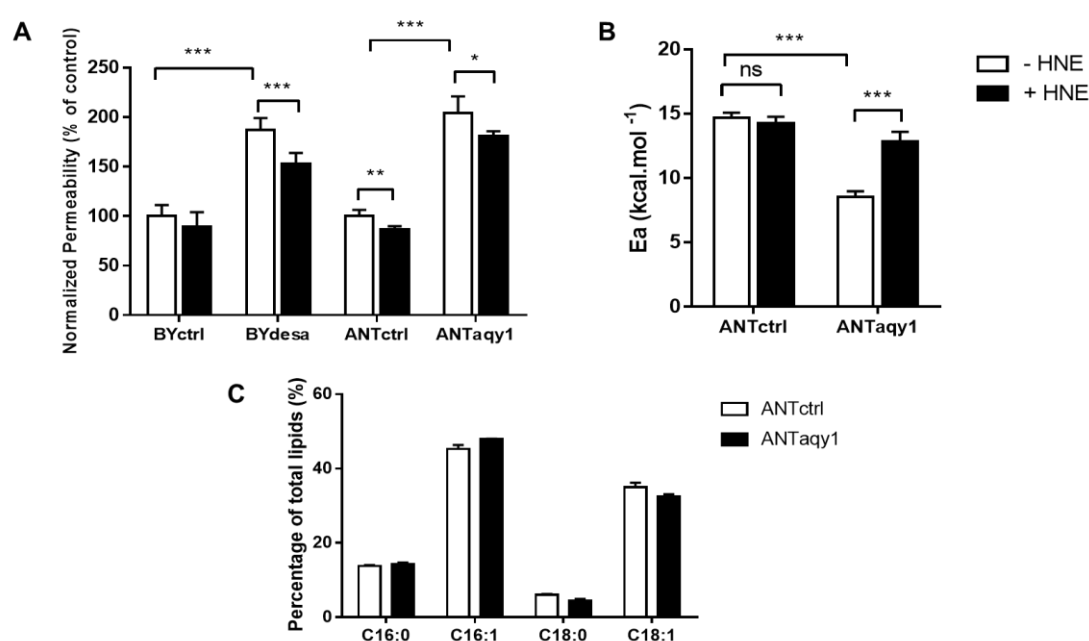


Figure II.4.2. Effect of HNE on yeast membrane water permeability and membrane fatty acid content. (A) Water permeability (percentage of control) of BYdesa and ANTAqy1 strains before and after treatment with 0.5 mM HNE. Values are means $\pm$ SD of 10 measurements; (B) Activation energy (E_a) of water transport of ANT strains before and after treatment with 0.5 mM HNE. For each strain, 4–8 replicates were analyzed for each experimental condition. Values are means $\pm$ SD. Significance levels: ns, non significant, * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$. (C) Membrane fatty acid content of control and ANTAqy1 strains. [Data of fatty acid content was obtained by Ivana Tartaro Bujak and Branka Mihaljević].

4.3.3. Aquaporin expression increases sensitivity to H₂O₂ and HNE

Among the many changes induced by PUFAs in yeast cellular function, an increasing sensitivity to H₂O₂ 24 h after functional induction of the desaturase gene has been reported [54]. Interestingly, this study reported that when PUFAs were present in the membrane for 3 days, the initial sensitivity changed to resistance [54]. This result led us to investigate the influence of AQY1 expression on oxidative stress sensitivity by testing BYdesa and ANTaqy1 strains for survival to HNE and H₂O₂ compared with the respective control strains. In order to facilitate the comparison and conclusions between effects of PUFAs and aquaporin expression, previously published results obtained on BY-based strains were included in figures when needed. Interestingly, ANTaqy1 was more sensitive to HNE, while BYdesa showed the same trend but lacking statistical significance. In addition, both ANTaqy1 and BYdesa strains were more sensitive to H₂O₂ compared to the respective controls (Fig. II.4.3.A and B), a behavior already reported for BY strains [56]. These results indicate that AQY1 contributes to sensitivity to oxidative stress and that it may also be responsible for cell sensitivity to peroxidation products such as HNE.

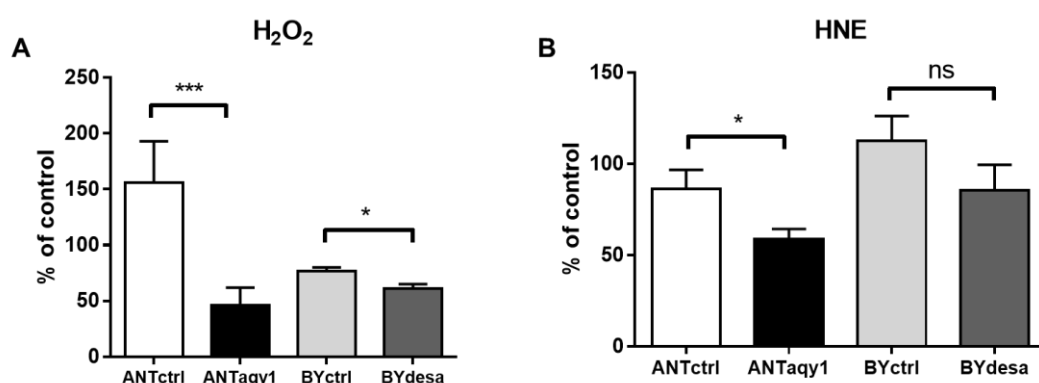


Figure II.4.3. Acute stress response of BY and ANT strains to HNE and H₂O₂. Cell survival test in liquid culture after treatment with (A) 1 mM H₂O₂ or (B) 0.5 mM HNE, 60 min at 28°C with orbital shaking. Percentage survival is expressed relative to the respective untreated strains. Values are means $\pm$ SD of triplicates. Significance levels: ns, non significant, * $p < 0.05$, *** $p < 0.001$.

4.3.4. Aquaporin expression does not influence the antioxidant defense system

The cellular antioxidative defense system of ANT strains was assessed by measuring GSH levels and catalase activity. There were no statistical differences in GSH level between ANTaqy1 and its control strain, as well as between BYdesa and its control strain (Fig. II.4.4.A). Interestingly, catalase activity was not changed when compared to respective controls in both, ANTaqy1 and BYdesa strains (Fig. II.4.4.B) which is in accordance with previously published data for BY strains [54]. Therefore, aquaporin expression and short-term presence of PUFAs in yeast membrane did not change antioxidant defense system. As GSH and catalase were not affected by either PUFAs or aquaporin, it can be assumed that genetic manipulation does not essentially facilitate increased intracellular oxidative stress. Nevertheless, PUFAs and aquaporins contribute to intracellular oxidative stress and result in increased sensitivity. In support of our findings is the study of AQP4 modulation, which did not affect GSH [99]. Still, yeast aquaglyceroporin Fps1 expression increased under arsenic stress but only if rice glutaredoxins were expressed. These yeast cells extrude arsenic ions via Fps1 together with GSH extrusion (not via same channels during arsenic stress), in an attempt to neutralize arsenic ions [100]. The mechanism that links these two proteins probably includes maintenance of cellular redox status and activation of the corresponding signaling pathways. However, so far there is no literature about the changes of catalase expression or activity in response to aquaporins.

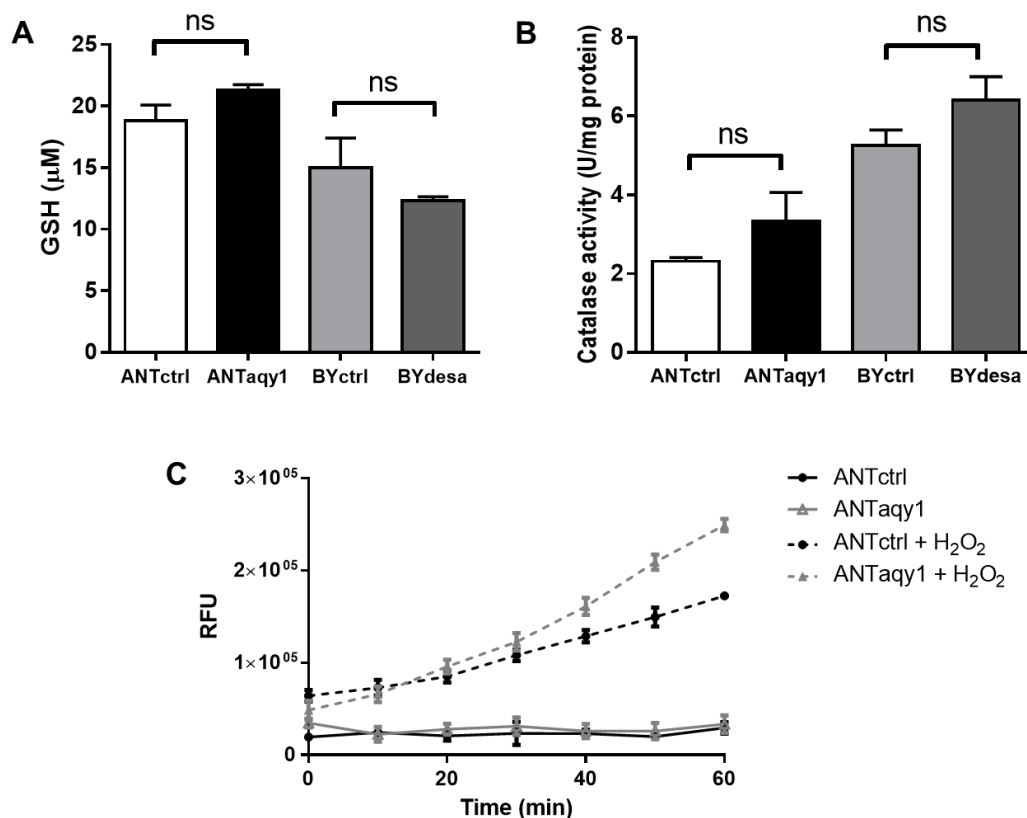


Figure II.4.4. Antioxidant defense system evaluation and ROS production after acute stress. (A) Total intracellular GSH levels and (B) Catalase activity of control and BY and AQY1 strains; (C) Control and AQY1 strains were treated with 10 mM H₂O₂ and the increase in fluorescence due to intracellular ROS production was measured every 10 minutes for 60 min. Values are means ± SD of triplicates. Significance level: ns, non significant. [Data of intracellular ROS production was obtained by Ana Čipak Gašparović].

4.3.5. Aquaporin facilitates influx of H₂O₂

The fact that yeast strains expressing AQY1 have unchanged GSH levels and catalase activity and at the same time show increased sensitivity to H₂O₂, suggests that this protein channel may also be able to facilitate H₂O₂ diffusion through membranes, as reported for other plant and mammalian aquaporin isoforms [21]. Thus we tested the influx of H₂O₂ in ANT strains by measuring the increase in DCFH fluorescence triggered by the addition of 10 mM H₂O₂ to the media. As shown in Fig. II.4.4.C, ANTaqy1 and ANTctrl displayed the same basal levels of ROS.

After H₂O₂ addition, both strains showed an increase in fluorescence;

however, the rate of fluorescence increase was higher for the strain that overexpresses AQY1 (ANTaqy1) compared to the wild type strain with an endogenous level of aquaporin expression (ANTctrl), indicating that AQY1 transports H₂O₂. Therefore, the increased sensitivity to H₂O₂ observed for AQY1 expressing strains seems to be correlated to the enhanced H₂O₂ transmembrane diffusion rate conferred by AQY1.

In conclusion, the link between aquaporins, membrane lipid content and lipid peroxidation is not nearly to be understood, although the interaction of AQP4 and HNE has been reported, implying possible role in regulation of these channels [88]. Our study showed that HNE inhibited aquaporin activity in PUFA-producing strain and in AQY1 overexpressing strain. Further, both strains with increased AQY1 expression showed decreased survival to H₂O₂ challenge and increased ROS accumulation triggered by exogenous H₂O₂, indicating a role of aquaporin in cellular sensitivity to oxidative stress. Further investigation is needed to untangle the underlying mechanisms of aquaporin involvement in cell oxidative stress response.

5. Rat aquaporin-5 is pH-gated induced by phosphorylation and is implicated in oxidative stress

5.1. Abstract

Aquaporin-5 (AQP5) is a membrane water channel widely distributed in human tissues that was found up-regulated in different tumors and considered implicated in carcinogenesis in different organs and systems. Despite its wide distribution pattern and physiological importance, AQP5 short-term regulation was not reported and mechanisms underlying its involvement in cancer are not well defined. In this work, we expressed rat AQP5 in yeast and investigated mechanisms of gating, as well as AQP5's ability to facilitate H₂O₂ plasma membrane diffusion. We found that AQP5 can be gated by extracellular pH in a phosphorylation-dependent manner, with higher activity at physiological pH 7.4. Moreover, similar to other mammalian AQPs, AQP5 is able to increase extracellular H₂O₂ influx and to affect oxidative cell response with dual effects: whereas in acute oxidative stress conditions AQP5 induces an initial higher sensitivity, in chronic stress AQP5 expressing cells show improved cell survival and resistance. Our findings support the involvement of AQP5 in oxidative stress and suggest AQP5 modulation by phosphorylation as a novel tool for therapeutics.

Keywords: aquaporin; yeast; permeability; phosphorylation; pH gating; reactive oxygen species; hydrogen peroxide; oxidative stress

5.2. Introduction

Aquaporins are a family of highly conserved transmembrane channels that transport water and, in some cases, small solutes such as glycerol, driven by osmotic or solute gradients [94, 101]. The 13 known mammalian isoforms (AQP0–12) are differentially expressed in several tissues/organs and show different permeability, structural features, and cellular localization. Importantly, these proteins are involved in many biological functions including transepithelial fluid transport, brain edema, neuroexcitation, cell migration, adhesion, proliferation, differentiation, and metabolism [102, 103]. Their numerous roles in physiology make these proteins essential for health, suggesting that modulation of AQP's function or expression could have therapeutic potential in edema, cancer, obesity, brain injury, glaucoma, and several other conditions [104].

Besides being widely distributed among the human body, AQP5 was found expressed in salivary and lacrimal glands and showed to play a major role in saliva secretion [105]. AQP5 transport defect was also associated with Sjögren's syndrome, a chronic autoimmune disease that destroys the salivary and lacrimal glands [105]. More recently, AQP5 gained attention due its potential implication in carcinogenesis in different organs and systems [90]. AQP5 was found overexpressed in cancer cells and tumor tissues, strongly suggesting that it may be implicated in tumor formation by contributing to cell differentiation and migration through mechanisms involving AQP5 interplay with intracellular signaling transduction pathways. In tumors, the cAMP-dependent phosphorylation of AQP5 by PKA activates the RAS/Mitogen-activated protein kinases (MAPK) pathway involved in cell proliferation and survival [106-108]. In addition to AQP5 interaction with oncogenes, its function as a water channel was proposed to be involved in cell migration, since AQP5 expression may facilitate changes in cell volume and shape that are crucial for migration [109].

Recently, permeation of H₂O₂ by some AQPs revealed that these channels could influence regulatory complex signaling pathways involved in pathological states. It is now well established that ROS, particularly H₂O₂, participate in cell signaling transduction pathways affecting cellular growth and proliferation mechanisms involved in cancer development [110, 111].

Although it is generally assumed that H₂O₂ is highly diffusible across the membrane lipid bilayer, it was shown that actually the membrane lipid bilayer slows down permeation of H₂O₂, leading to the formation of gradients [68]. This barrier, however, may be overcome by the presence of some AQPs recently shown to be capable of transporting extracellular H₂O₂ into the cells [112]. In mammalian cells, AQP3, AQP8, and AQP9 were shown to mediate H₂O₂ membrane transport [20, 22, 39], which may be used for intracellular signaling in cancer cells [30]. Consequently, due to its involvement in cancer progression, we anticipated that AQP5 might also be able to facilitate H₂O₂ permeation and have a role in cell oxidative stress response explaining, at least in part, its overexpression in cancer tissues.

Interestingly, a recent study showed that AQP5 membrane abundance is regulated by phosphorylation [63], and in fact, the contrasting phosphorylation status between cancer and normal tissues suggests that AQP5 role in tumorigenesis is related with its phosphorylation [90]. Furthermore, gating mechanisms that induce a change in the 3D-channel structure and consequently affect its transport activity [94] have been described for several eukaryotic AQPs [95, 113-115], among which pH and phosphorylation represent a short-term regulatory mechanism commonly used by several AQP family members [115, 116]. However, besides its post-translational modification by phosphorylation, gating mechanisms for AQP5 channel activity regulation have not been reported so far.

An important tool to investigate AQP's function and regulation is the yeast heterologous expression system [94]. Yeast cells lacking endogenous

AQPs have been used to detect water transport capacity of mammalian [57, 117] and plant [118, 119] aquaporins and recently we used this system to characterize the pH gating of AQP3 [57].

In the current work, we cloned rat AQP5 in the yeast *S. cerevisiae* and investigated its channel activity regulation by external pH and phosphorylation. We observed that AQP5 does not change its activity by external acidification, but phosphorylation makes the AQP5 channel prone to pH sensing. Moreover, AQP5 is able to modulate H₂O₂ transport through the plasma membrane and this feature interferes with oxidative cell response with dual effects: acute oxidative stress induces an initial higher sensitivity while long-term exposure and chronic stress conditions increase cell survival and resistance to the oxidative stress insult.

Thus, the current findings support a direct role of AQP5 in cancer development by mediating H₂O₂ membrane permeation, affecting redox signaling, and influencing signaling transduction pathways involved in tumorigenesis.

5.3. Results

5.3.1. Subcellular localization and water permeability of rat AQP5 expressed in yeast

Yeast cells made devoid of endogenous aquaporins (aqy-null) were transformed with either the empty plasmid pUG35 (control cells) or the plasmid containing the rat AQP5 gene (mentioned as AQP5 cells, for clarity). The expression of AQP5 in the *S. cerevisiae* model was assessed by fluorescence microscopy, using GFP tagging. In transformed cells, AQP5–GFP is localized at the cellular membrane, as depicted in Fig. II.5.1.A.

The permeability of yeast expressing AQP5 was evaluated by stopped-flow fluorescence after loading cells with the volume sensitive dye carboxyfluorescein. When cells are exposed to hyperosmotic shock with

2 Assessment of AQP-mediated H₂O₂ transport

impermeant solutes, water outflow induces cell shrinkage. Water permeability is then evaluated by monitoring the time course of fluorescence output that reflects the transient volume change.

As depicted in Fig. II.5.1.B, cells expressing AQP5 show a much faster volume change after a hyperosmotic shock. The water permeability coefficient P_f was 12-fold higher for AQP5 cells ($(4.94 \pm 0.40) \times 10^{-3} \text{ cm}\cdot\text{s}^{-1}$ and $(0.41 \pm 0.05) \times 10^{-3} \text{ cm}\cdot\text{s}^{-1}$ for AQP5 and control, respectively) (Fig. II.5.1.C). The activation energy for water transport E_a was concomitantly lower for AQP5 cells ($6.52 \pm 0.82 \text{ kcal}\cdot\text{mol}^{-1}$) compared to the control ($15.16 \pm 0.85 \text{ kcal}\cdot\text{mol}^{-1}$) (Fig. II.5.1.D), corroborating the increase in membrane water permeability conferred by AQP5 expression. Although AQP5 behavior as a water channel is well known in the literature, these data validate the use of the yeast system to detect AQP5 function and further explore mechanisms of regulation.

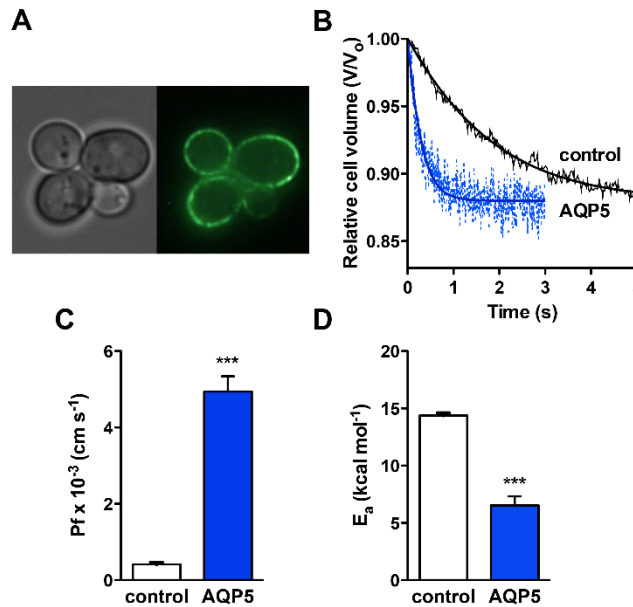


Figure II.5.1. Expression and function of rat AQP5 (Aquaporin-5) in yeast. (A) Epifluorescence images of GFP-tagged AQP5 localization (green) in yeast cells (100x objective); (B) Representative time course of the relative cell volume (V/V_0) changes after a hyperosmotic shock inducing cell shrinkage (pH 7.4); (C) Water permeability coefficients of control ($P_f = (0.41 \pm 0.05) \times 10^{-3} \text{ cm}\cdot\text{s}^{-1}$) and cells expressing AQP5 ($P_f = (4.94 \pm 0.40) \times 10^{-3} \text{ cm}\cdot\text{s}^{-1}$), measured at 23°C and pH 7.4. Data are mean $\pm$ SD of 10 measurements; (D) Activation energies (E_a) for water permeation of control and AQP5 cells (15.16 ± 0.85 and 6.52 ± 0.82

kcal·mol⁻¹, respectively). Data are mean ± SD. Significance level: *** $p < 0.001$. [Data was obtained by Andreia Mósca].

5.3.2. Effect of pH and glucose-stimulated phosphorylation on rat AQP5 permeability

AQPs can be subjected to regulation via different mechanisms, among which pH regulation has been disclosed for plant [118] and for a few mammalian AQPs, such as AQP0, AQP3, and AQP6 [120-122]. It is also known that eukaryotic AQPs can be gated by phosphorylation [115].

A gating mechanism regulating human AQP5 activity has been proposed by molecular dynamics simulations [123]. This study revealed that the AQP5 channel could change between an open and closed state by a tap-like mechanism at the cytoplasmic end, induced by a translation of the His67 inside the pore, blocking the entrance of the channel. Moreover, when in the open state, the selectivity filter (SF) can regulate the flow rate of water molecules by exhibiting two different conformations (wide or narrow). These two conformations are decided by the side chain orientation of His173 and the proximity to Ser183—when His173 is close to Ser183, the SF is in the narrow conformation and the water passage is restricted. The trigger for this gating mechanism has not been described; in addition, this *in silico* approach has not been so far experimentally validated. A similar gating mechanism for human AQP4 was recently described [124], where two putative gate regions formed by two residues on the cytoplasmic side (His95 and Cys178) and the other two on the SF region (Arg216 and His201) modulate opening and closure of the AQP4 pore along four possible conformational states. The relative stability of the two resulting states, open and closed, may depend on small changes in the microenvironment, such as variations of pH. Indeed, a pH-dependent gating mechanism was recently obtained from *in silico* and *in vitro* studies [125], ascribing to His95 located in AQP4 cytoplasmic end the role of regulating channel permeability. Phosphorylation of AQP4 has also

been demonstrated with opposed effects depending on the residue that is phosphorylated. AQP4 is inhibited when Ser180 is phosphorylated in loop D and is activated when Ser111 in loop B is phosphorylated [99]. These observations prompted us to investigate if AQP5 would be gated by pH or by phosphorylation.

Our group has recently characterized the pH dependence of AQP3 permeability using the yeast heterologous expression system [57]. Thus, we first decided to investigate the effect of external pH on AQP5 activity of rat AQP5-transformed yeasts. Although our yeast cells express rat AQP5, sequence alignment of human and rat AQP5 isoforms showed a sequence identity of 91% [126] (Fig. S1. Supplementary Materials). From the analysis of the amino acid sequences, we can infer that human and rat AQP5 may share the same gating mechanism.

Since the physiological pH of yeast's natural environment is acidic (3.5–6.5) [127], and conditions at which cells show optimal growth and expression and trafficking mechanisms are expected to be fully active, we chose the external pH 5.1 to test yeast membrane water permeability. In addition, considering that a mammalian aquaporin is being expressed, permeability was also measured at the mammalian physiological pH 7.4. Permeability experiments with AQP5 and control cells incubated at pH 5.1 and 7.4 showed that, by changing external pH channel, activity was not altered (Fig. II.5.2.A). At pH 5.1, $P_f = (0.35 \pm 0.01) \times 10^{-3} \text{ cm} \cdot \text{s}^{-1}$ and $E_a = 14.38 \pm 0.22 \text{ kcal} \cdot \text{mol}^{-1}$ for control cells, and $P_f = (4.77 \pm 0.32) \times 10^{-3} \text{ cm} \cdot \text{s}^{-1}$ and $E_a = 7.69 \pm 0.86 \text{ kcal} \cdot \text{mol}^{-1}$ for AQP5 cells, were not different from the respective values at pH 7.4 (detailed above, Fig. II.5.1.). These results indicate that the acidic external pH does not affect AQP5 water permeability.

It is known that AQP5 expression and trafficking can be regulated by phosphorylation, but whether phosphorylation also regulates channel activity and contributes to gating still remains uncertain. Several studies reported

AQP5 redistribution in plasma membrane of animal cells initiated by phosphorylation [63, 128]. Post-transcriptional regulation of AQP5 function in response to stimuli such as neurotransmitters, hormones, and cyclic adenosine monophosphate (cAMP), has been reported (for a review see [90]. cAMP regulates aquaporin-5 expression at both transcriptional and post-transcriptional levels through a protein kinase A (PKA) pathway [129], increasing AQP5 abundance on the apical membrane of lung epithelial cells after long-term exposure [130]. Besides translocation to the plasma membrane, phosphorylation of AQP5 was shown to promote cell proliferation [106] and, interestingly, AQP5 Ser156 was found preferentially phosphorylated in tumor cells [131], supporting AQP5 phosphorylation involvement in cell proliferation.

In yeast *S. cerevisiae*, the basal intracellular cAMP concentration is low [132]. However, addition of glucose or related fermentable sugars after a period of glucose-starvation triggers the Ras/PKA pathway, creating a sudden and transient increase in intracellular cAMP levels that induce a protein phosphorylation cascade [66, 67]. Activation of this pathway by glucose mimics the well-known hormonal-induced phosphorylation pathways that occur in animal cells [133]. Therefore, to examine the effect of phosphorylation on AQP5 water permeability, glucose starved yeast cells were incubated with 100 mM glucose for 5 min before P_f measurements. As shown in Fig. II.5.2.B, glucose addition did not affect P_f of control or AQP5 cells at pH 5.1 (Fig. II.5.2.B). However, at pH 7.4, glucose pulse resulted in a significantly two-fold increased P_f in AQP5 cells ($P_f = (10.60 \pm 0.53) \times 10^{-3} \text{ cm}\cdot\text{s}^{-1}$) and a 30-fold increase compared with basal levels of control cells (Fig. II.5.2.B).

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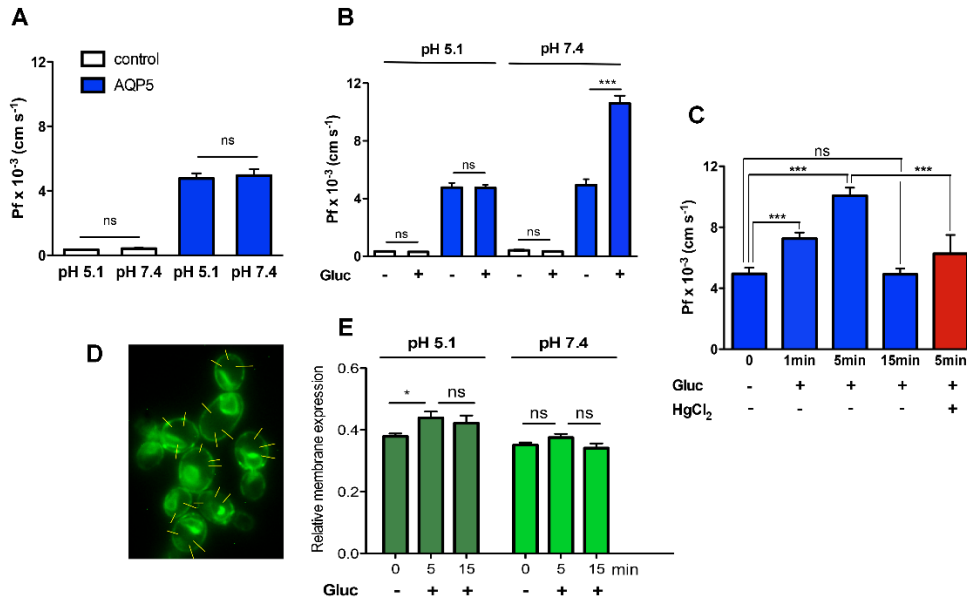


Figure II.5.2. Regulation of AQP5 water permeability. (A) Water permeability P_f at pH 5.1 and pH 7.4 of control cells ($P_f = (0.35 \pm 0.01) \times 10^{-3}$ and $(0.41 \pm 0.05) \times 10^{-3} \text{ cm} \cdot \text{s}^{-1}$, respectively) and yeast cells expressing AQP5 ($P_f = (4.77 \pm 0.32) \times 10^{-3}$ and $(4.94 \pm 0.40) \times 10^{-3} \text{ cm} \cdot \text{s}^{-1}$, respectively); (B) Water permeability P_f at pH 5.1 and pH 7.4 upon an external glucose pulse; (C) Time course of glucose-induced phosphorylation (1, 5 and 15 min, pH 7.4) and inhibition of AQP5 by HgCl₂ 0.5 mM. Data are mean $\pm$ SD of 10 measurements; (D) Representative epifluorescence images of GFP-tagged AQP5 localization in yeast cells (100x objective); linear intensity profiles are indicated (yellow lines); and (E) Relative membrane expression of AQP5 calculated from fluorescence intensity profiles (30 cells in each experimental condition, 3 profiles for each cell, from at 3 independent experiments). Significance levels: ns, non significant, * $p < 0.5$, *** $p < 0.001$.

In addition, we investigated the time course of AQP5 activation after glucose addition at pH 7.4 (Fig. II.5.2.C). Following a glucose pulse, a transient strong increase of cAMP with peak values around 1–2 min that progressively decay to about their basal levels, was previously reported in yeast cells [134, 135]. Our data show a significant increase of AQP5 water permeability after 1 min that was further increased at 5 min; after 15 min, glucose exposure no longer produces effect on AQP5 permeability, possibly due to the decay of cAMP synthesis. Yeast cells were subsequently incubated for 5 min simultaneously with glucose and HgCl₂, a well-known aquaporin inhibitor (Fig. II.5.2.C). In this case, the glucose-induced increase in P_f of AQP5 cells was

partially abolished ($P_f = (6.26 \pm 1.23) \times 10^{-3} \text{ cm} \cdot \text{s}^{-1}$). The inhibitor alone had no effect on P_f of control cells.

Afterwards, to determine whether cAMP-mediated increase in water permeability was due to AQP5 increased trafficking and abundance or to opening of the channel, we measured GFP-tagged AQP5 relative membrane expression (Fig. II.5.2.D). Before glucose addition, the membrane abundance measured at pH 5.1 and pH 7.4 was not significantly different (0.37 ± 0.07 and 0.35 ± 0.07 , respectively) (Fig. II.5.2.E). Although membrane abundance was slightly increased after 5 min glucose pulse (implying that also trafficking is triggered by phosphorylation, as previously reported by Kitchen et al. [63]), this effect was only detected at extracellular pH 5.1 (0.49 ± 0.08 and 0.47 ± 0.12 at 5 and 15 min, respectively). Interestingly, at pH 7.4, no significant difference could be detected after 5 min (0.37 ± 0.07), and at both pH the membrane abundance was kept stable at least for 15 min (Fig. II.5.2.E).

Also to consider is the fact that, if a carbon source is available, external shifts in proton concentration in the pH range from 3.0 to 7.5 do not significantly affect yeast internal pH values due to ATPase activity [127]. However, a two-fold increase in P_f with concomitant reduction of E_a after glucose addition only happened at pH 7.4 (Fig. II.5.2.B). No increase was seen at pH 5.1. Interestingly, pH 5.1 is in the range of yeast physiological pH at which the machineries for protein expression, transcription, and trafficking are expected to be in place. If the increase in P_f observed would be simply due to an increase in AQP5 membrane abundance, then it should also be detected at pH 5.1. Hence, since we observe differences in permeability mediated by phosphorylation when the external pH is changed, changes in the channel structure activity rather than in AQP5 membrane abundance might be responsible for the measured difference in permeability.

Figure II.5.3.A displays the structure of human AQP5 with several consensus phosphorylation sites at cytoplasmic loop D (Ser152 and Ser156)

and at C-terminal (Ser231, Ser233, and Thr242) [136]. In Fig. II.5.3.B the top view of the monomer is depicted, with His173 and Ser183 located in the selectivity filter (SF). The distance between these two residues (8.4 Å) was proposed to correspond to the pore wide conformation [123]. All these residues are conserved in rat AQP5 sequence (Fig. S1. Supplementary Materials) with the exception of Ser233. Phosphorylation of Ser156 was reported to play an important role in AQP5 translocation to the plasma membrane in HEK293 cells [63]. In contrast, another study observed that a mutation on Ser156 had no effect on membrane trafficking but instead affected cell proliferation [131].

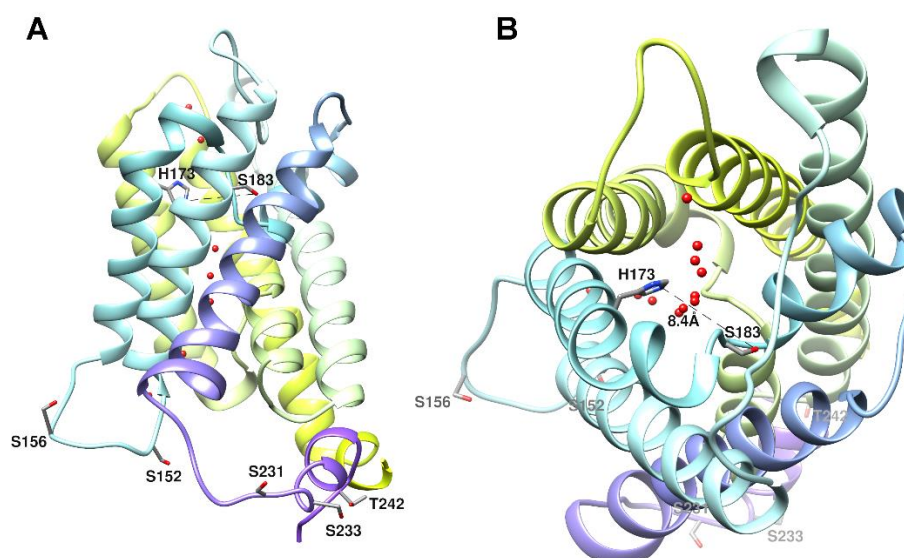


Figure II.5.3. Structure of human AQP5 monomer. (A) Side view of the monomer with several phosphorylation consensus sites in the cytoplasmic region (Ser152, Ser156, Ser231, Ser233, and Thr242) shown in licorice representation. Thr259, also a phosphorylation site, is not represented due to the inexistent electron density beyond Pro245 for hAQP5 structure [136]; (B) Top view of the monomer with His173 and Ser183 in the selectivity filter (SF) shown in licorice representation. The distance between these two residues (8.4 Å) corresponds to the proposed distance for the SF wide conformation [123]. Water molecules are shown as red spheres along the channel pore. [Figure generated by Andreia Mósca with Chimera (<http://www.cgl.ucsf.edu/chimera>) and based on AQP5 X-ray structure (PDB databank code 3D9S)]

Thr259 is not represented in Fig. II.5.3.A due to the inexistent electron density beyond Pro245 in hAQP5 structure. However, this residue is also an

interesting phosphorylation site due to the homology to Ser256 in hAQP2. It has been demonstrated that phosphorylation of Ser256, besides triggering AQP2 insertion at apical plasma membrane, is also essential to modulate AQP2 function, increasing water permeability of the individual channel [137, 138]. In a recent study, extracellular acidic pH was shown to attenuate AQP2 hormone-induced phosphorylation and membrane apical trafficking, probably by inhibition of vasopressin V2 receptor-G protein-cAMP-PKA actions [139].

From a structural point of view, Ser152 and Ser156 are strong candidates prone to induce conformation changes at loop D with impact on the protein channel monomeric conformation. However, phosphomimetic mutations of Ser156 were able to increase membrane expression but did not cause any significant structural change [63]. It is thus reasonable to anticipate that more than one phosphorylation site is necessary to produce a measurable conformation change. Phosphorylated AQP5 (Ser and Thr residues facing the cytoplasmic region, Fig. II.5.3.A) may induce a change in channel conformation, and in this new conformation de-protonation of His13 residue (facing the outer membrane) may occur at pH 7.4, with widening of the channel pore (Fig. II.5.3.B). While at pH 7.4 the channel is wide open, at pH 5.1 the protonated residues and putative hydrogen-bond interactions hold the channel in the narrow open conformation, with lower permeability. Interestingly, the human AQP5 crystals were obtained at pH 7.0–7.6, the pH range where, in this study, phosphorylated AQP5 shows increased permeability.

5.3.3. Hydrogen peroxide consumption

AQP5 has been implicated in carcinogenesis and its tissue expression might be associated with cancer aggressiveness [90]. The mechanisms underlying AQP5 involvement in cancer are still unclear, but in addition to participation in intracellular signaling transduction pathways and interaction with oncoproteins, AQP5 channel activity facilitating rapid changes in cell volume and subsequent changes in cell shape, was proposed to be crucial for

cell migration [90, 109]. Furthermore, AQP5 permeation of H₂O₂ and subsequent implication in cell oxidative stress would help explaining its participation in tumorigenesis.

To investigate if AQP5 expression increases the rate of H₂O₂ diffusion through membranes, we measured the consumption of external H₂O₂ in control and AQP5 expressing cells by electrochemical assays, using O₂- and H₂O₂-specific electrodes. After the external addition of H₂O₂, the rate constant of H₂O₂ consumption by cells was 1.4-fold increased ($p < 0.05$) in AQP5 cells ($k_{\text{Control}} = (1.68 \pm 0.12) \times 10^{-3} \text{ s}^{-1}$) compared to control ($k_{\text{AQP5}} = (2.39 \pm 0.15) \times 10^{-3} \text{ s}^{-1}$) (Fig. II.5.4.A), indicating that AQP5 cell membranes possess a facilitated H₂O₂ diffusion pathway. To validate this result and further investigate if AQP5 would be mediating H₂O₂ permeation, we then followed H₂O₂ cell consumption using a specific H₂O₂ electrode and checked whether the aquaporin inhibitor HgCl₂ quenches the uptake. The obtained results ($k_{\text{Control}} = (1.44 \pm 0.49) \times 10^{-3} \text{ s}^{-1}$ and $k_{\text{AQP5}} = (4.13 \pm 0.26) \times 10^{-3} \text{ s}^{-1}$) corroborate the previous increased diffusion rate of H₂O₂ consumption by AQP5 cells (Fig. II.5.4.B). In addition, HgCl₂ showed a significant inhibitory effect, reducing approximately five-fold the rate of consumption ($p < 0.001$) and not affecting the control. Therefore, these data strongly suggest that AQP5 can mediate H₂O₂ diffusion through membranes.

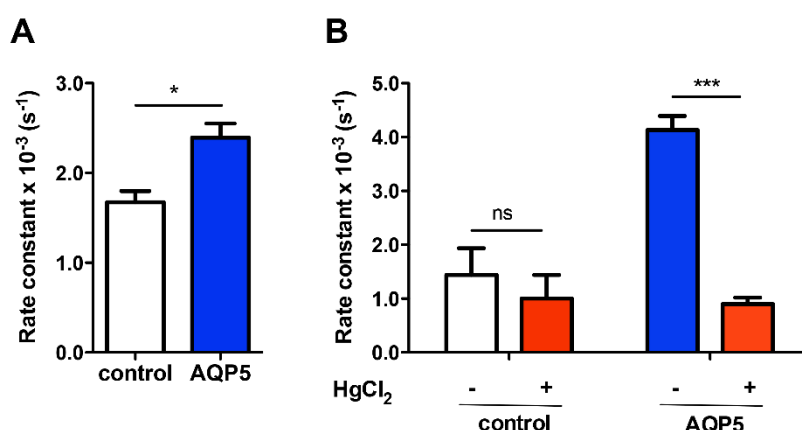


Figure II.5.4. AQP5-dependent H₂O₂ consumption of yeast cells. (A) First-order kinetic rate constant (s⁻¹) of H₂O₂ consumption measured with the Clark electrode (O₂ measurement); (B)

First-order kinetic rate constant (s^{-1}) of the H₂O₂ consumption measured with the H₂O₂ electrode, before (white and blue bars) and after incubation with 0.5 mM HgCl₂, 5 min at RT (red bars). Values are mean $\pm$ SD of triplicates. Significance levels: ns, non significant, * $p < 0.05$, *** $p < 0.001$.

5.3.4. AQP5 implication on cell oxidative status

In order to assure that the disappearance of extracellular H₂O₂ was due to cellular uptake rather than extracellular degradation, we measured the intracellular levels of ROS after acute stress induction with 20 mM H₂O₂. As expected, a higher intracellular level of ROS was detected for AQP5 cells (Fig. II.5.5.A). Although control cells also respond to oxidative stress induction, which may be explained by basal H₂O₂ membrane lipid diffusion [140], ROS content was significantly increased in AQP5 cells after approximately 40 min of stress induction. Thus, the extracellular disappearance of H₂O₂ measured by electrodes is in agreement with intracellular ROS production, supporting AQP5-dependent H₂O₂ consumption.

To further confirm that the increase in external H₂O₂ consumption and concomitant intracellular ROS levels were mainly due to AQP5 expression and activity, we assessed the cells' antioxidant defense system by measuring catalase activity and GSH level in basal conditions (before addition of H₂O₂). An increase in these scavengers could explain the previously obtained results without the influence of AQP5.

Catalase activity, as part of the antioxidative defense system, was 30% reduced (0.235 ± 0.012 and 0.163 ± 0.008 U/mg protein, for control and AQP5, respectively) (Fig. II.5.4.B) and GSH level was also slightly diminished in AQP5 cells (1.94 ± 0.18 μ M for control and 1.53 ± 0.20 μ M for AQP5) (Fig. II.5.5.C), indicating that these scavengers are not contributing for the observed higher H₂O₂ consumption and ROS production in AQP5 cells.

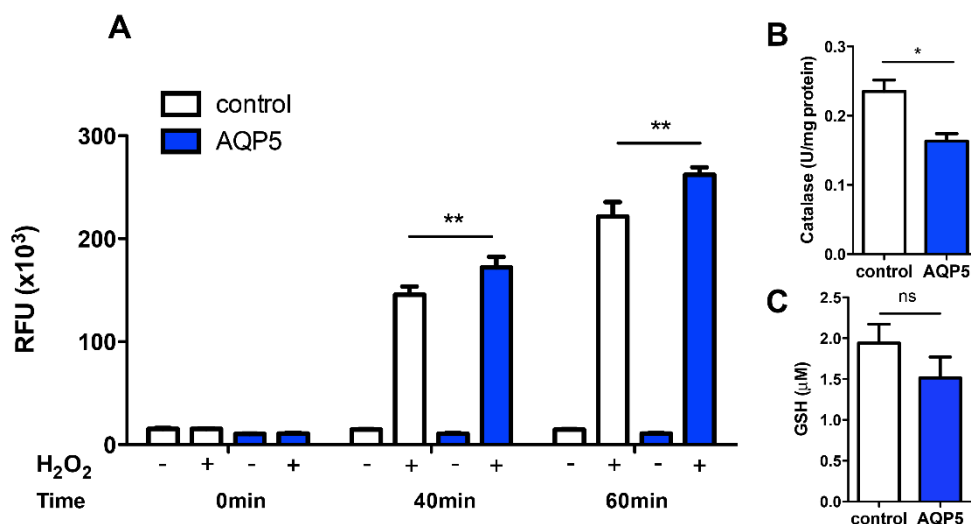


Figure II.5.5. Cellular levels of ROS (oxidant), GSH and catalase (antioxidants). (A) Time course of Intracellular ROS production after acute stress induction with 20 mM H₂O₂; (B) Catalase activity and (C) total intracellular GSH content of yeast strains. Values are means ± SD of triplicates. Significance levels: ns, non significant, * $p < 0.05$, ** $p < 0.01$. [Data obtained by Ana Čipak Gašparović].

5.3.5. Yeast sensitivity to hydrogen peroxide

ROS are no longer known only as purely harmful but instead they are described as important regulators of signaling pathways [141]. The increased intracellular levels of ROS in AQP5 cells after acute stress induction with H₂O₂ prompted us to investigate its outcome on yeast cell survival at shorter and longer periods after inducing the oxidative stress.

In a first approach, cells suspended in liquid medium were treated with 0.5 mM H₂O₂ and, after incubation for different time intervals, the ability of plated cells to survive and form colonies was evaluated. Interestingly, as depicted in Fig. II.5.6.A, a different and opposed behavior was detected in a 60 min time range. While initially AQP5 cells were more sensitive to H₂O₂ than control, longer incubation periods, for more than 30 min, reveal that AQP5 cells survive better and are significantly more resistant to the oxidative stress insult. Thus, the initial H₂O₂-triggered harmful effects and lower cell survival for AQP5 cells ($p < 0.05$ and $p < 0.01$) are reversed after a while, showing that after 45 min incubation cells are able to survive significantly better than control

($p < 0.001$). It is worth mentioning that the initial sensitivity of AQP5 cells may be due to the cells' lower antioxidant defense system, as suggested by catalase activity and GSH level, thus rendering AQP5 cells more prone to ROS effects. However, after 45 min of incubation with H₂O₂, it is clear that AQP5 cells are more resistant than control.

Measurements of membrane abundance showed that AQP5 expression was not affected after treatment with 0.5 mM H₂O₂ (Fig. II.5.6.B). Indeed, these results are expected from the cloning method as the AQP5 gene was cloned under inducible MET25 promoter. MET25 promoter provides an inducible expression system in the absence of methionine, but renders the expression of AQP5 insensitive to stimuli or inhibition, which would normally occur, such as possible regulation by oxidative challenge.

These same conclusions can be drawn when cells were grown in solid media containing H₂O₂ (Fig. II.5.6.C). In this assay, the long-term stress response given by the ability to grow under oxidative stress was evaluated after two weeks. A clear resistance of AQP5 cells can be observed.

Altogether, these results indicate that AQP5 expression contributes to activation of either the cell antioxidant defense response or the signaling transduction pathways triggered by H₂O₂. The increased resistance displayed by AQP5 cells might be the result of a faster metabolic adaptation to the oxidative cell status conferred by AQP5 expression.

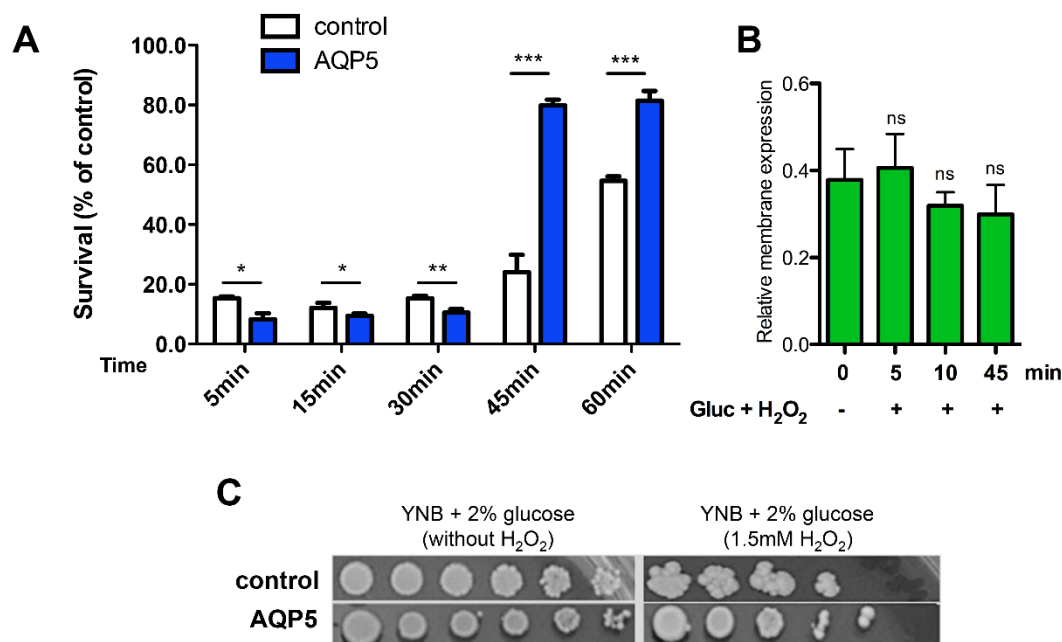


Figure II.5.6. Stress response of yeast strains to H₂O₂. (A) Acute stress response. Time course of cell survival of yeast cells in liquid culture after treatment with 0.5 mM H₂O₂. Percentage survival is expressed relative to untreated controls. Values are means $\pm$ SD of triplicates. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; (B) Relative membrane expression of AQP5 calculated from fluorescence intensity profiles (30 cells in each experimental condition, 3 profiles for each cell, from 3 independent experiments). ns, non significant; (C) Chronic stress response. Growth assay of yeast cells under oxidative stress. Yeast suspensions were spotted in 10-fold dilution on solid YNB plates without or with 1.5 mM H₂O₂. Growth was recorded after two weeks at 28°C. Photographs shown are representative of three independent experiments with consistent results. [Data of acute stress response was obtained by Ana Čipak Gašparović].

5.4. Discussion

The present study provides experimental evidences for the direct regulation of AQP5 water permeability by pH dependent on phosphorylation and for AQP5 involvement in cell oxidative stress response.

Human AQP5 was the first aquaporin crystalized in full tetrameric assembly [136] and its trafficking and membrane expression are regulated by phosphorylation [63] through PKA and RAS signaling pathways that promote cell proliferation [106]. The fact that AQP5 was found preferentially phosphorylated in tumor cells strongly suggests that its regulation might be involved in tumorigenesis. In addition to trafficking, AQP5 channel activity may

be crucial for cancer cell migration and proliferation [90, 109]. Therefore, it is reasonable to hypothesize that AQP5 ability to permeate H₂O₂ in addition to water, could also contribute to its up-regulation in cancer tissues.

In this context, this study aimed at investigating two mechanisms supporting AQP5 involvement in tumorigenesis: (i) its ability to be short-term regulated or gated, either by acidic conditions or by phosphorylation; and (ii) its ability to permeate H₂O₂ in addition to water and consequent outcome in oxidative cell response.

Our data show that AQP5 can be gated by pH in a phosphorylation dependent manner. Glucose addition with consequent PKA pathway activation may simultaneously stimulate AQP5 trafficking and abundance at the plasma membrane and contribute to opening of the channel at physiological pH 7.4; the measured increase in water permeability in AQP5 cells may reflect the sum of the two processes, trafficking and gating.

In our assays, a small increase in AQP5 membrane expression 5 min after the glucose pulse was detected, but only for extracellular pH 5.1. At pH 7.4 the increase in membrane abundance was not observed and cannot explain the higher P_f measured. It is worth mentioning that, although at different extracellular pH, yeasts are able to maintain the intracellular pH close to neutrality if a carbon source is available. Thus, the extracellular pH is not expected to greatly influence inner pH and is not likely to induce changes in membrane trafficking. In fact, the average membrane expression was similar at both pH. Since phosphorylation occurs intracellularly, one may speculate that it is the direct AQP5-phosphorylation that alters protein conformation and, in this new conformation, channel widening results from de-protonation of residues at pH 7.4. Therefore, in addition to promoting membrane trafficking, phosphorylation may endorse an AQP5 channel with the ability of pH sensing.

At mammalian physiologic conditions (pH 7.4) phosphorylated AQP5 enables larger fluxes of water through membranes, which may contribute to rapid changes in cell volume and shape and thus facilitate cell migration. Extracellular pH shifts acidic conditions favors channel narrowing, allowing fine-tuning of cell volume. Notably, although natural for yeast cells, the low pH tested is far below the pH found in mammalian tissues, even in solid tumors where it can reach pH 6.5 [142]. There are, however, a few mammalian acidic physiological conditions where pH gets close to this value. For instance, sweat shows acidic pH levels, between 4.6 and 5.4 [143], and in the skin the protective sweat acid mantle acidity ranges from 4 to 5.5 [143]; the pH of human stomach is highly acidic, usually from 1 to 2; osteoclasts' acidic microenvironment below pH 5.5 is critical for the bone resorption [144]; an acidic pH luminal fluid microenvironment is important for sperm maturation [145]. Interestingly, AQP5 was found expressed in sweat glands [146], gastric mucosa [147], bone cells [148], and in the epididymis [145]. In all these tissues, effective mechanisms of water flux regulation induced by pH fluctuations might be advantageous to prevent excessive water reabsorption and control cell volume and shape.

Furthermore, this study demonstrates that AQP5 is able to permeate H₂O₂. AQP5-transformed cells show a high rate of H₂O₂ consumption, indicating that AQP5 facilitates H₂O₂ membrane permeation. Data of H₂O₂ consumption, ROS intracellular levels, and resistance to H₂O₂ suggest that AQP5 is involved in cell oxidative stress response and point to a novel mechanism explaining AQP5 contribution to tumorigenesis.

Noteworthy, the initial higher sensitivity to H₂O₂ displayed by AQP5-expressing cells turned into higher resistance in chronic oxidative stress conditions. Yeast cells possess a limited pool of antioxidant enzymes that protect against ROS but are not sufficient to protect cells from sudden and high oxidative challenges. The onset of oxidative stress usually induces an early

response, where the intracellular antioxidant system provides instant protection against the initial toxic accumulation of ROS. Stress signals such as H₂O₂ can activate transcription factors which upregulate the expression of many genes including the ones encoding enzymatic (e.g., catalases) and non-enzymatic antioxidants (e.g., GSH) [12]. The subsequent syntheses of antioxidant defenses promote ROS scavenging, repair oxidized biomolecules, and restore cellular redox balance [149, 150]. These response mechanisms help ensure the survival of non-lethally damaged cells [141]. Additionally, exposure of cells to severe oxidative stress can elicit lethal response pathways such as autophagy, apoptosis, and necrosis [149]. We may speculate that AQP5-facilitated H₂O₂ permeation induces a faster activation of transcription factors and antioxidant system stimulation, contributing to the pro-survival cellular responses to oxidative stress.

The results obtained in this study with yeast cells can possibly be translated to mammalian cells. For instance, *S. cerevisiae* contains three conserved signaling modules that control transcriptional regulation triggered by oxidative stress, and two of them have homology to others species, including mammalian [151]. Furthermore, the activating protein-1 (AP-1) family of transcription factors that regulate cellular processes such as proliferation, differentiation, apoptosis, and stress response, exists in both mammalian and yeast cells and can be activated by H₂O₂ [152]. In yeast, the AP-1 homolog Yap1 functions as an oxidative stress sensor and regulates the expression of antioxidant genes in response to stress, including thioredoxin, thioredoxin reductase, glutathione reductase, and γ -glutamylcysteine synthase [151], and, interestingly, the AQP5 5'-flanking region has a consensus binding site for AP-1 [153]. Thus, a mechanism where H₂O₂ permeated by AQP5 contributes to activation of AP-1 transcription factor that in turn stimulates antioxidant genes expression and possibly AQP5 gene expression as well, cannot be disregarded. A major issue that remains to be resolved is the precise connection between

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AQP5 phosphorylation via the cAMP-PKA pathway and the oxidative stress cell resistance conferred by AQP5. Further experiments using mammalian cells and the human AQP5 gene will help to untangle the mechanisms underlying AQP5 involvement in oxidative stress response.

The present study provides experimental evidences for the direct regulation of AQP5 water permeability by phosphorylation at mammalian physiological pH. In addition to triggering AQP5 trafficking and increasing its membrane abundance, AQP5 phosphorylation promotes an increase in water permeability of cell membranes, which may be needed for rapid volume adaptation and for changes in cell shape crucial for cell migration and proliferation as seen in cancer cells. AQP5 is also shown to participate in cell oxidative cell response after a stress insult, probably in a dual mechanism where AQP5–H₂O₂ permeation activates signal transduction cascades that stimulate antioxidants gene expression. While an initial acute stress induces cell sensitivity, under chronic stress conditions similar to tumor cell niches, AQP5 may facilitate metabolic adaptation, cell survival, and resistance. In this context, AQP5 modulation by phosphorylation may represent a novel strategy with potential application in cancer treatment.

5.5. Supplementary Materials

	
	10 20 30 40 50
rAQP5	MKKEVCSLAF FKAVFAEFLA TLIFVFFGLG SALKWPSALP TILQISIAFG
hAQP5	MKKEVCSVAF LKAVFAEFLA TLIFVFFGLG SALKWPSALP TILQIALAFG
	*****: ** :***** ***** ***** *****: **
	
	60 70 80 90 100
rAQP5	LAIGTLAQAL GPVSGGHINP AITLALLIGN QISLLRAVFY VAAQLVGAI A
hAQP5	LAIGTLAQAL GPVSGGHINP AITLALLVGN QISLLRAFFY VAAQLVGAI A
	***** ***** *****: ** *****: ** *****
	
	110 120 130 140 150
rAQP5	GAGILYWLAP LNARGNLAVN ALNNNTTPGK AMVVELILTF QLALCIFSST
hAQP5	GAGILYGVAP LNARGNLAVN ALNNNTTQGQ AMVVELILTF QLALCIFA ST
	***** : ** ***** ***** * : ***** *****: **
	
	160 170 180 190 200
rAQP5	DSRRTSPVGS PALSIGLSVT LGHLVGIYFT GCSMNPARSF GPAVVMNRF S
hAQP5	DSRRTSPVGS PALSIGLSVT LGHLVGIYFT GCSMNPARSF GPAVVMNRF S
	***** ***** ***** ***** *****
	
	210 220 230 240 250
rAQP5	PSHWVFWVGP IVGAMLAAIL YFYLLFPSSL SLHDRVAVVK GITYEPEEDWE
hAQP5	PAHWVFWVGP IVGAVLAAIL YFYLLFPNSL SLSERVAIIK GITYEPDEDWE
	*:***** ****:***** *****: ** ** :***: * *****: ****
	
	260
rAQP5	DHREERKKI ELIAH
hAQP5	EQREERKKM ELITR
	:*****: ***:

Figure S1. Sequence alignment of the full-length amino acid sequences of *Rattus norvegicus* AQP5 and human AQP5. The alignment was generated with CLUSTAL W (using BioEdit software). The amount of conservation of each amino acid is represented by symbols ('*', exact match in all sequences; ':', conserved substitution; '.', semi-conserved substitution). The two conserved NPA signature motifs are underlined. Ser152, S156, Ser231, Ser233, Thr242, Thr259 and Thr263 (colored green) are consensus phosphorylation sites of hAQP5; His173 and Ser183 (colored yellow) are the residues involved in the switch between narrow and wide conformations in the gating mechanism of human AQP5 proposed by Janosi et al. (doi 10.1371/journal.pone.0059897).

6. Human aquaporin-5 facilitates hydrogen peroxide permeation affecting adaption to oxidative stress and cancer cell migration

6.1. Abstract

ROS, including H₂O₂, contribute to oxidative stress and can lead to cancer initiation and progression. However, at low concentrations H₂O₂ can regulate signaling pathways modulating cell growth, differentiation and migration. A few mammalian aquaporins (AQPs) facilitate H₂O₂ diffusion across membranes and participate in tumorigenesis. AQP3 and AQP5 are strongly expressed in cancer tissues and AQP3-mediated H₂O₂ transport was related to breast cancer cell migration, but studies with human AQP5 were lacking.

Here we report that human AQP5 facilitates H₂O₂ membrane diffusion in addition to water, and modulates cell growth of AQP5-transformed yeast cells in response to oxidative stress. Mutagenesis studies revealed that residue His173 located in the selective filter is crucial for AQP5 permeability and possibly interacts with phosphorylated S183 for permeability regulation and pore blockage. Moreover, in human pancreatic cancer cells the measured AQP5-mediated H₂O₂ influx rate indicates a highly efficient peroxiporin activity. Cell migration was similarly suppressed by AQP3 or AQP5 gene silencing and was recovered by external oxidative stimulus.

Altogether these results unveil a major role of AQP5 in dynamic fine-tuning of intracellular H₂O₂ to activate signaling networks related to cell survival and cancer progression, highlighting AQP5 as a promising drug target for cancer therapies.

Keywords: aquaporin; permeability; hydrogen peroxide; oxidative stress; cancer.

6.2. Introduction

Aquaporins constitute a highly conserved group of transmembrane proteins expressed in almost every organism and tissues that are crucial for cell homeostasis and volume regulation. AQPs are assembled in a tetrameric structure in membranes, each monomer being a functional channel that facilitates rapid bidirectional flux of water, and in some cases, small uncharged solutes like glycerol, in response to osmotic or solute gradients [101]. In humans, thirteen isoforms (AQP0-AQP12) are differentially expressed in cells and tissues, and are grouped according to their selectivity and sequence homology: classical or orthodox aquaporins are considered mainly selective to water (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, AQP8), aquaglyceroporins that also transport glycerol and urea (AQP3, AQP7, AQP9, AQP10), and S-aquaporins, with lower homology and found in subcellular membranes (AQP11, AQP12). Among these AQPs some isoforms were also shown to permeate ammonia [101, 154]. More recently, a fourth sub-group comprising isoforms that facilitate the diffusion of H₂O₂ through cell membranes has been identified and named peroxiporins [20-22, 92, 155].

The participation of AQPs in several biological functions related to water homeostasis and energy metabolism has been extensively reported [103, 156], and more recently a growing number of studies reported AQPs involvement in cell proliferation, angiogenesis and migration [109, 157] and participation in cell resistance to oxidative stress [92]. It is well known that high intracellular levels of ROS unbalancing the cellular redox state play a key role in tumorigenesis either by damage of proteins, lipids and nucleic acids, or by abnormal induction of signaling pathways [158]. However, ROS also act as signaling molecules regulating physiological processes [159]. H₂O₂ is a small molecule produced by aerobic metabolism or in response to extracellular insults, being the main ROS participating in oxidative sensing and signaling [12]. Depending on its intracellular concentration, H₂O₂ can have a dual effect

[7]. In this context, the facilitated diffusion of H₂O₂ across cell membranes may play a crucial role in the fine-tuning of cell oxidative status and its biological consequences.

Several studies reported the overexpression of AQPs in human tumors of different origin, frequently correlated with tumor stage and aggressiveness [109], unveiling a putative association with their role as peroxiporins [30, 90, 155, 160]. In particular AQP3 and AQP5 were found aberrantly expressed in a variety of human tumors and have been considered potential therapeutic targets and biomarkers with prognostic value (for a recent review see [161]). The expression level of AQP3 has been evaluated and correlated with cancer cell migration through a mechanism involving its hydrogen peroxide transport function [20, 30].

In our previous study we showed that rat AQP5 also mediates H₂O₂ membrane diffusion and is involved in oxidative cell response [92]. In addition, the transport activity of phosphorylated rAQP5 could be regulated by acidification, a condition that is favored in cancer tissues. Thus we hypothesized that human AQP5 may also channel H₂O₂ in addition to water, and that the high level of expression and peroxiporin activity may have a key role in cellular adaption to oxidative stress, with impact on cancer cell migration.

Thus, in this study we expressed human AQP5 in a yeast cell model and firstly investigated its functional regulation by phosphorylation and pH, disclosing the important residues for functional regulation as well as the ability of AQP5 to facilitate H₂O₂ across cell membranes. Secondly, we evaluated the contribution of AQP5 to cell survival under oxidative conditions and to cell adaptation to an oxidative environment. Finally, we measured the H₂O₂ influx rate mediated by AQP5 in human pancreatic cancer cells and revealed its impact on cell migration under oxidative stress.

We found that AQP5 expressed in yeast localizes at the plasma

membrane increasing 10-fold the membrane water permeability compared to the control. Investigation of residues important for functional regulation revealed H173 localized in the selectivity filter as crucial for channel permeability, independent of pH. Phosphorylation of S183 impaired AQP5 function probably due to its proximity to H173, interacting with the negative charge of the phosphate group. Importantly, we show for the first time that human AQP5 transports H₂O₂ and modulates cell resistance to oxidative stress.

Moreover, in human pancreatic cancer cells the measured AQP5-mediated H₂O₂ influx rate indicates a highly efficient peroxiporin activity. Cell migration was similarly suppressed by AQP3 or AQP5 gene silencing, and was promoted by AQP-facilitated H₂O₂ transport. Altogether these results unveil a major role of AQP5 in cancer progression, highlighting its potential as drug target for cancer therapies.

6.3. Results

6.3.1. Human AQP5 is localized and functional at the yeast plasma membrane

To evaluate human AQP5 function, yeast cells depleted of endogenous aquaporins (aqy-null) were transformed with either the empty plasmid pUG35 (control cells) or the plasmid encoding hAQP5. Prior to functional analysis, expression and localization of AQP5 at the yeast plasma membrane were verified by fluorescence microscopy using GFP-tagging (Fig. II.6.1.A). AQP5 function was evaluated by stopped-flow fluorescence. Cells were loaded with the volume sensitive dye carboxyfluorescein and exposed to hyperosmotic solution with an impermeant solute inducing cell shrinkage. Water permeability was evaluated by monitoring the time course of fluorescence output that reflects the transient volume change. As shown in Figure II.6.1.B, when exposed to an hyperosmotic solution cells expressing AQP5 readjust the final volume and reach the new osmotic equilibrium faster than control cells,

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evidencing water channeling. The water permeability coefficient (P_f) is 10-fold higher for AQP5-transformed yeast cells ($(3.70 \pm 0.31) \times 10^{-3} \text{ cm s}^{-1}$ and $(0.37 \pm 0.03) \times 10^{-3} \text{ cm s}^{-1}$ for AQP5 and control, respectively). Incubation with 0.5 mM HgCl₂ markedly reduced water permeability of AQP5-transformed yeast cells ($\approx 64\%$) without affecting control cells (Fig. II.6.1.C), indicating that AQP5 is a mercury-sensitive water channel. Finally, the activation energy for water transport (E_a), which distinguishes passive water diffusion through lipid bilayer from AQP-mediated diffusion, was lower for AQP5 cells ($5.63 \pm 0.36 \text{ kcal mol}^{-1}$) compared to the control ($12.66 \pm 0.69 \text{ kcal mol}^{-1}$) (Fig. II.6.1.D). These results show that AQP5 is assembled into a functional water channel in yeast.

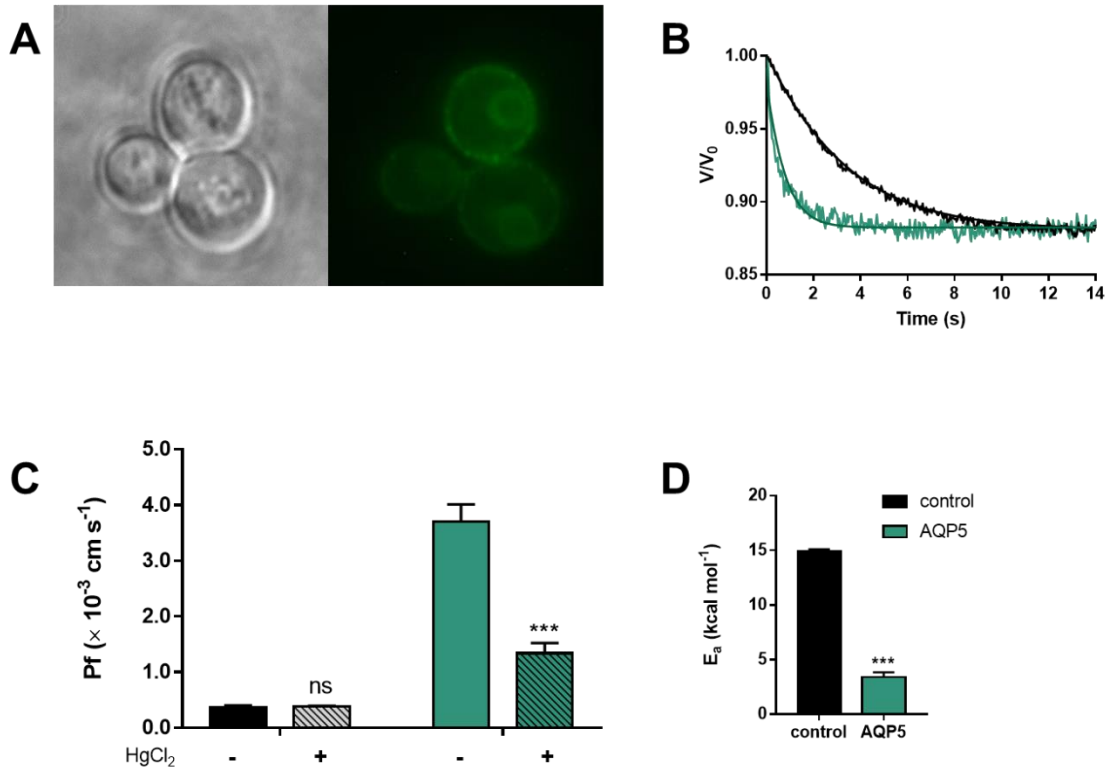


Figure II.6.1. Expression and function of human AQP5 in transformed *S. cerevisiae* cells.

(A) Phase contrast (left) and epifluorescence (right) microscopy images of yeast aqy-null cells transformed with GFP-tagged human AQP5 (100× objective); **(B)** Representative time course of the relative cell volume (V/V_0) changes after a hyperosmotic shock inducing cell shrinkage at pH 5.1; **(C)** Water permeability coefficients of control and cells expressing human AQP5 before and after HgCl₂ incubation for 5 min at RT, measured at 23°C and pH 5.1. Data are shown as mean $\pm$ SD of 10 measurements; **(D)** Activation energy (E_a) for water permeation of

control and AQP5 cells. Data are shown as mean $\pm$ SD of three independent experiments. Significance levels: ns, non-significant; *** $p < 0.001$. [Data was obtained by Andreia Mósca].

6.3.2. S183 and H173 are important residues for AQP5 gating

Recent evidence supports that human AQPs can be gated via different mechanisms including pH and phosphorylation [116, 162]. Regarding AQP5, regulation was proposed to involve phosphorylation at Ser156 in cytoplasmic loop D to rapidly and reversibly regulate AQP5 plasma membrane abundance [Kitchen, 2015 #582]. Phosphorylation of AQP5 in its PKA consensus site (S156) induced colon cancer cell proliferation via Ras/ERK/Rb pathway [Kang, 2008 #621]. In addition, *in silico* studies suggested a second gating mechanism [123] where the AQP5 monomer undergoes conformational changes varying between an open/close state and wide/narrow state. The authors proposed that AQP5 channel could change from open to closed by a tap-like mechanism at the cytoplasmic end, induced by translation of the His67 side chain inside the pore, blocking the water passage, and that the selectivity filter (SF) regulates the rate of water flux when the channel is open. In this case, AQP5 channel could exhibit two different conformations (wide and narrow) determined by the proximity of H173 side chain to S183: when these residues get close (<5.5 Å), the SF turns to the narrow conformation and water passage is restricted. The channel constriction induced by H173 side chain orientation determines the two states wide/narrow when the cytoplasmic end gate switches from close to the open state. In addition, our recent study with rAQP5 indicated that channel widening results from deprotonation when the protein is in the phosphorylated state [92]. Thus, using the same yeast system, here we investigated mechanisms of human AQP5 gating by phosphorylation and pH.

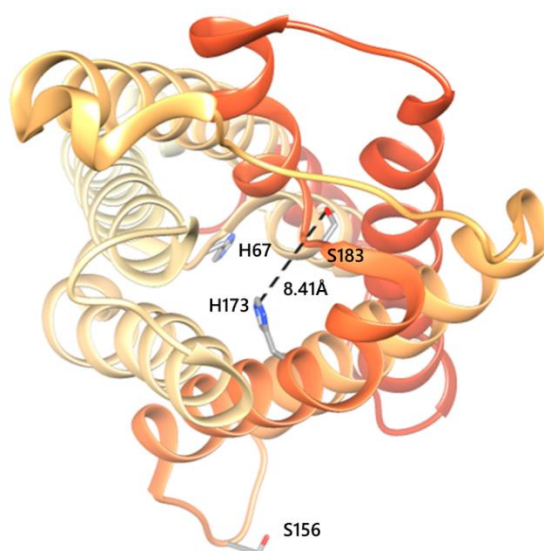


Figure II.6.2. Structure of human AQP5 monomer. Top view of AQP5 monomer with phosphorylation consensus sites Ser156 localized in intracellular loop D and Ser183 localized in the selectivity filter along with His173. As proposed, when His67 side chain rotates outside the pore it allows the water passage through the pore (open state) [123]. In such case, if the proximity of His173 to Ser183 (D1) is $7\text{\AA} < D1 < 10\text{\AA}$, the AQP5 monomer shows a wide conformation. Structures were generated with Chimera (<http://www.cgl.ucsf.edu/chimera>) and are based on AQP5 X-ray structure (PDB databank code 3D9S).

We generated point mutations in AQP5 aromatic/arginine region and in intracellular loop D (Fig. II.6.2.). Mutations to change wide and narrow state were obtained by substitution of histidine (H) 173 with alanine (A) and with tryptophan (W), respectively. Mutations preventing phosphorylation of S156 and S183 were obtained by substitution of serine (S) with alanine (A). Mutations to mimicking the charge state of AQP5 phosphorylated at the same serine residues were performed by substitution of serine (S) with glutamic acid (E). Water permeability of yeast cells expressing wild-type AQP5 (WT) or AQP5 mutants was determined at 23°C at both pH 5.1 and pH 7.4 (Fig. II.6.3.A). Expression and localization of all AQP5 mutants were confirmed at pH 5.1 and pH 7.4 by fluorescence microscopy using GFP-tagging (Figure S1 supplementary materials). All yeast clones displayed similar GFP-fluorescence intensity at the plasma membrane (Fig. S1 and II.6.3.B) indicating that the

observed differences in permeability cannot be assigned to impairment of AQP5 trafficking due to mutations.

Permeability experiments with AQP5 WT, mutants and control cells performed at two pHs (pH 5.1 and 7.4), showed that alteration of external pH did not affect water permeability and that this is not dependent on protein phosphorylation. A recent study reported that phosphomimetic mutation of S156E increased membrane expression of AQP5 in HEK293 cells [63]. In our work, preventing or mimicking S156 phosphorylation did not alter AQP5 membrane expression (Fig. II.6.3.B) nor modified AQP5 activity at any pH tested, which may in part be explained by a different signaling pathway for AQP5 trafficking used by yeasts. However, water permeability was fully blocked when histidine was mutated (H173A and H173W). Histidine is a highly conserved residue in the selective filter of water-specific aquaporins and is considered crucial for selectivity and transport [163]. The observed impairment of water permeability by H173 mutation (Fig. II.6.3.A) validates the previously reported *in silico* data.

To experimentally investigate the phosphorylation gating mechanism previously proposed *in silico* [123], we measured water permeability of AQP5-S183A and AQP5-S183E mutant yeast cells. Impairment of S183 phosphorylation by mutation to alanine (S183A) did not affect water permeability, suggesting that the AQP5 channel remains in a wide conformation probably because the distance between H173 side chain and A183 is above 5.5 Å. However, the phosphomimetic mutation of S183 to glutamate (S183E) impaired permeability, indicating that the proximity of S183 to H173 mainly supported by the negative charge of the phosphate group is responsible for pore constriction. This new AQP5 gating mechanism, involving phosphorylation of S183 at the selectivity filter (SF) has never been reported.

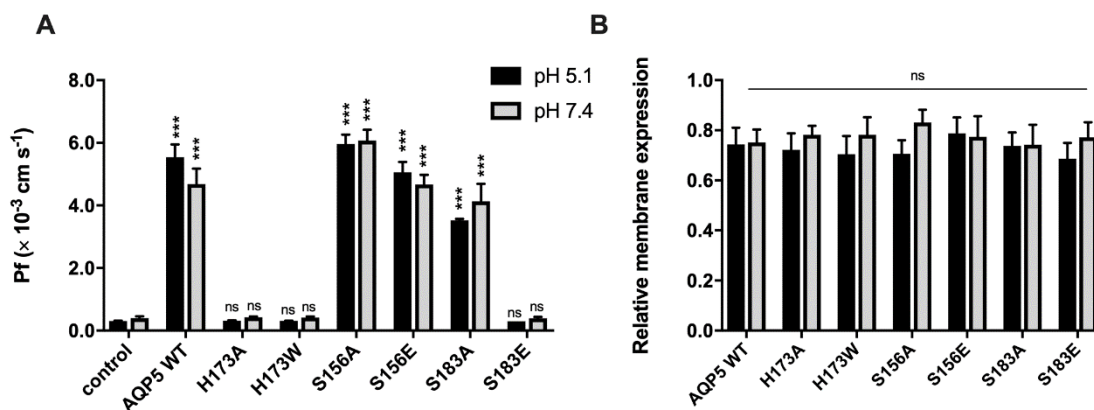


Figure II.6.3. Residues involved in water permeability through AQP5. (A) Water permeability coefficients of control and cells expressing AQP5 WT and mutants, performed at 23°C and pH 5.1 and 7.4. Data are shown as mean $\pm$ SEM of 10 measurements; (B) Relative membrane expression of AQP5 WT and mutants at pH 5.1 and pH 7.4 calculated from fluorescence intensity profiles (10 cells in each experimental condition, 4 profiles for each cell, from at least 3 independent experiments). Significance levels: ns, non significant, *** $p < 0.001$, compared to control.

6.3.3. Human AQP5 transports hydrogen peroxide

Several studies reported H₂O₂ transport by a few human AQP isoforms, including AQP3 [20], AQP8 [21, 40] and AQP9 [22]). Regarding AQP5, our group revealed that the rat isoform could also mediate H₂O₂ transport [92]. Sequence alignment of human and rat AQP5 isoforms reveals 91% sequence identity and lead us to investigate if human AQP5 can facilitate H₂O₂ permeation through membranes.

Thus, we measured consumption of external hydrogen peroxide by yeast cells expressing human AQP5 using an electrochemical assay (O₂ electrode). Briefly, hydrogen peroxide was added to cell suspensions and H₂O₂ uptake was evaluated by monitoring its conversion into O₂ with a Clark electrode after addition of catalase in excess to samples of the cell suspension. Since we found that human AQP5 is not pH regulated, we performed the assays at pH optimal for yeast cells (pH 5.1). As depicted in Fig. II.6.4.A, the rate constant of H₂O₂ consumption was 3-fold higher for yeast cells expressing

AQP5 comparing to control cells. In addition, incubation with 0.5 mM HgCl₂, shown above to inhibit AQP5 water permeability, reduced H₂O₂ consumption to the basal level corresponding to diffusion through the lipid bilayer, not affecting control cells. These data confirm that AQP5 can facilitate H₂O₂ membrane permeability. Since H₂O₂ uptake followed by the Clark electrode relies on its extracellular disappearance, and to assure that the high rate of H₂O₂ consumption observed for AQP5-yeast cells was due to cellular uptake, we loaded the cells with the ROS sensitive fluorescence probe H₂-DFCDA and followed the intracellular increase in fluorescence after incubation with a range of H₂O₂ concentrations. As depicted in Fig. II.6.4.B, the rate of ROS accumulation was dependent of H₂O₂ concentration largely facilitated by AQP5 expression.

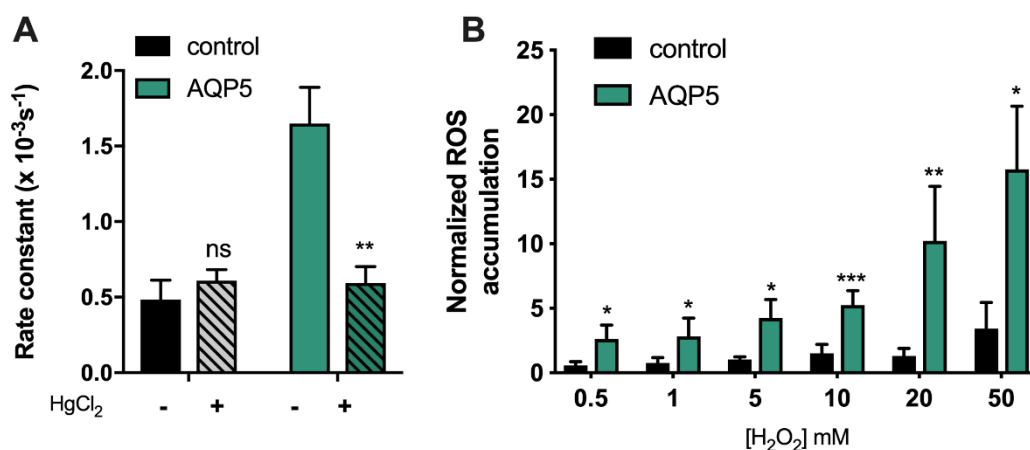


Figure II.6.4. AQP5-mediated H₂O₂ uptake. (A) First-order kinetic rate constant (s⁻¹) of H₂O₂ consumption of yeast cells measured with the Clark electrode (O₂ measurement), before and after incubation with 0.5 mM HgCl₂ for 5 min at RT; (B) AQP5-facilitated H₂O₂ consumption of yeast cells is concentration-dependent. Cells were incubated with a range of H₂O₂ concentration from 0.5 mM to 50 mM and ROS accumulation was recorded over 60 min, following H₂-DCFDA fluorescence signal. Rate constant of ROS accumulation without H₂O₂ for each yeast strain was used to normalized rate constant of ROS accumulation. Values are shown as mean ± SD of at least 3 independent experiments. Significance levels: ns, non significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.3.4. AQP5 regulates cellular resistance to oxidative stress

The involvement of ROS in initiation, promotion and progression of cancer and their effect on cell behavior depends on concentration, time of exposure, cellular antioxidant defense, among other factors [164]. In fact, ROS can contribute to regulation of cell fate, including anti-cancer actions (e.g. by promoting senescence, apoptosis, necrosis or other types of cell death, and inhibiting angiogenesis) or pro-cancer actions (promoting proliferation, invasiveness, angiogenesis, metastasis, and suppressing apoptosis) [164]. Recent evidence showed a novel role for AQP3-mediated H₂O₂ transport in the mechanism of breast cancer cell migration [30]. Therefore, we aimed to investigate the physiological role of AQP5-mediated H₂O₂ transport in cell resistance and growth.

First, we evaluate the effect of long exposure to oxidative stress (Fig. II.6.5.A). Yeast cells were grown on solid medium containing 1 mM H₂O₂ for two days and it could be observed that cells expressing AQP5 are much more sensitive to oxidative stress than control cells, which were only slightly affected.

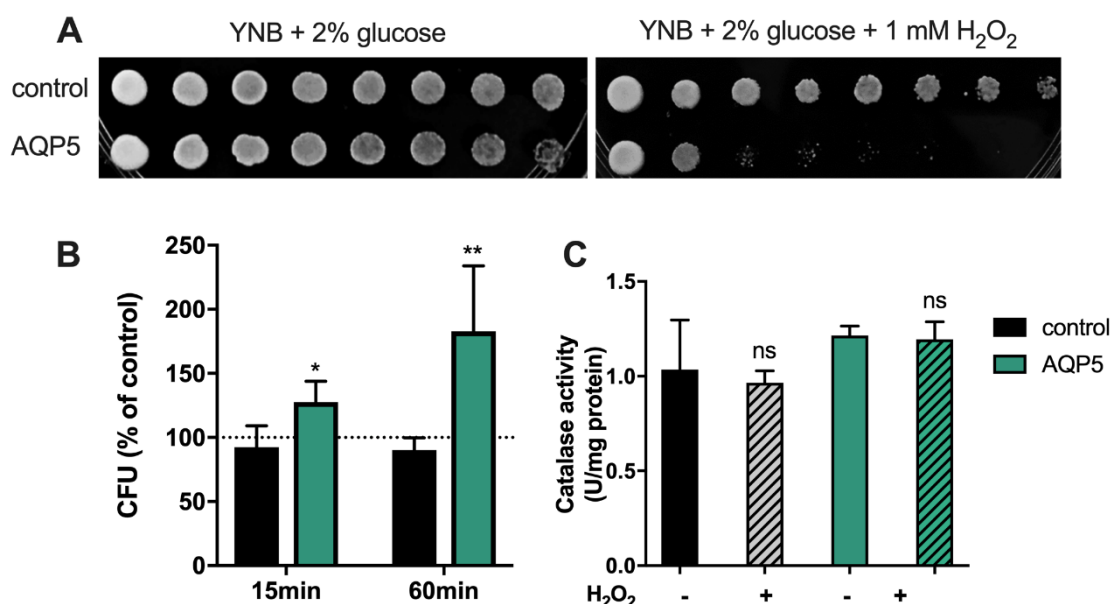


Figure II.6.5. Yeast cell sensitivity assay and catalase activity under oxidative stress. (A) Growth assay of yeast cells on medium containing H₂O₂. Yeast suspensions were spotted at 10-fold dilutions on solid YNB plates without or with 1 mM H₂O₂. Growth was recorded after two days at 28°C. Photographs shown are representative of three independent experiments with consistent results. **(B)** Survival test of yeast cells in liquid culture after treatment with 1 mM H₂O₂ for 15 and 60 min at 30°C. Survival is expressed by the percentage of colony forming units (CFU) relative to untreated controls. Significance levels: * $p < 0.05$ and $p < 0.01$ vs. control. **(C)** Catalase activity before and after treatment with 1 mM H₂O₂ for 60 min at 30°C. Values are mean $\pm$ SEM of triplicates. Significance level: ns, non-significant vs. non-treated cells.

Subsequently, cells were exposed to short-term oxidative stress, *i.e.* in the presence of 1 mM H₂O₂ for 15 and 60 min (Fig. II.6.5.B). Results indicate that, when exposed to H₂O₂, cells expressing AQP5 can not only survive to oxidative stress but also grow better (1.5- and 2-fold after 15 and 60 min respectively, compared to non-treated cells). This "overcoming" effect was not observed in control cells that are depleted of endogenous aquaporins and H₂O₂ slowly diffuses through membrane lipids. As a control, catalase activity was also evaluated in non-treated cells and after incubation with 1 mM H₂O₂ for 60 min; absence of statistical differences indicate that this antioxidant

scavenger is not responsible for the observed cellular resistance of AQP5-yeast cells.

6.3.5. AQP5 shows high peroxiporin activity in pancreatic cancer cells

The BxPC3 pancreatic cancer cell line was tested to confirm the yeast results and further investigate the biological relevance of AQP-mediated H₂O₂ transport. Evaluation of relative gene expression by quantitative real-time RT-PCR demonstrated that both AQP3 and AQP5 are expressed in BxPC3, although with different levels of expression (Fig. II.6.6.A).

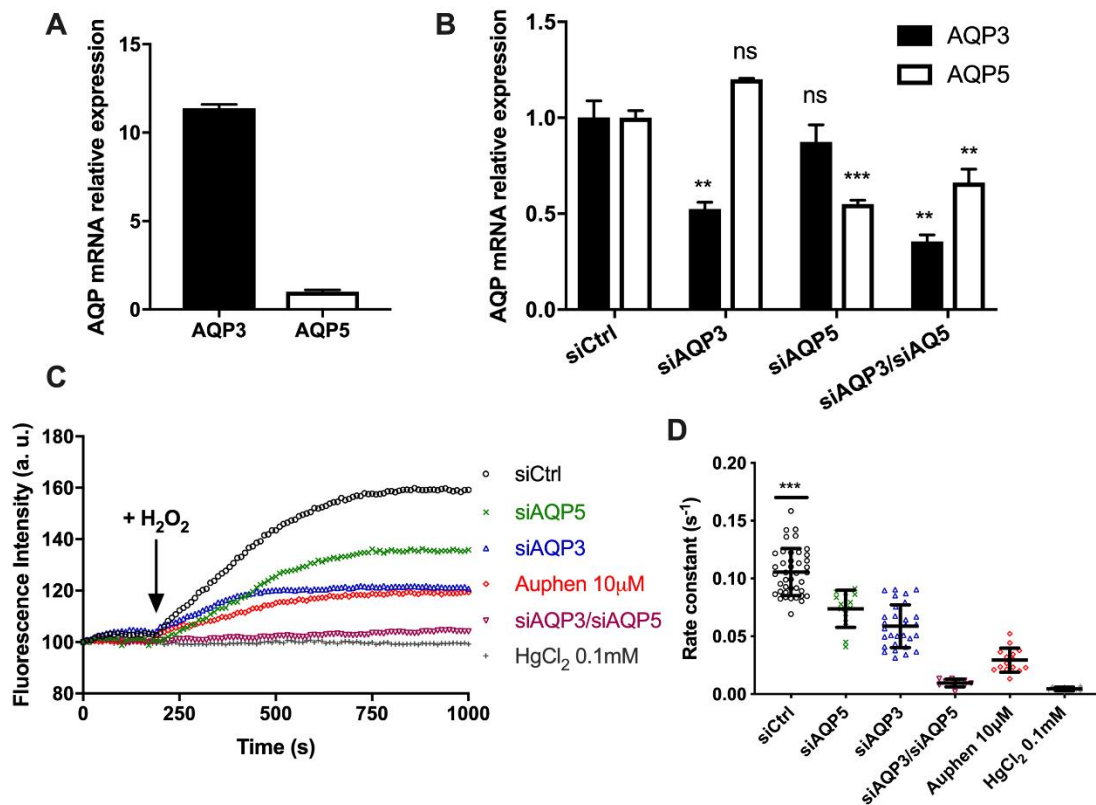


Figure II.6.6. AQP5-mediated H₂O₂ uptake in pancreatic cancer cells. (A) AQP3 and AQP5 mRNA expression levels in BxPC3 cells using β -actin as a reference; (B) AQP3 and AQP5 mRNA expression levels in BxPC3 cells transiently transfected with siAQP3 and siAQP5. Data represent mean $\pm$ SEM from three independent experiments. Significance levels: ns, non significant, ** $p < 0.01$, *** $p < 0.001$ vs. siCtrl; (C) Representative traces of H₂-DCFDA fluorescence increase after addition of 100 μ M H₂O₂; (D) First-order kinetic rate constant (s⁻¹) of H₂O₂ influx through endogenous AQP3 and AQP5 expressed in BxPC3 cells after addition of 100 μ M H₂O₂. Data shown are individual values and mean $\pm$ SEM of three independent experiments. Significance

levels: *** $p < 0.001$ vs. siAQP5, siAQP3, siAQP5, siAQP3/siAQP5, Auphen 10 μ M and HgCl₂ 0.1 mM.

Since both AQP5 and AQP3 transport H₂O₂ and to distinguish the contribution of each isoform to H₂O₂ permeability, we used BxPC3 cells silenced for AQP3, for AQP5, or double silenced for AQP3-AQP5 to evaluate H₂O₂ permeability. The knockdown efficiency was evaluated by RT-qPCR after 48h and compared with the non-targeting control construct, showing a reduction of AQP3 and AQP5 expression around 50% (Fig. II.6.6.B).

H₂O₂ membrane transport was evaluated by epifluorescence microscopy. The rate of fluorescence increase of individual cells incubated with 10 μ M H₂-DCFDA was monitored before and after addition of 100 μ M H₂O₂ (Fig. II.6.6.C). As depicted, the rate of H₂O₂ uptake is maximal for control cells that express both AQP3 and AQP5. Cells silenced for AQP5 (siAQP5) still expressing AQP3, and cells silenced for AQP3 (siAQP3) still expressing AQP5, respond to H₂O₂ addition with a similar rate of fluorescence increase, approximately half the control (Fig. II.6.6.D). In AQP3-AQP5 silenced cells, H₂O₂ uptake is almost abolished and a similar effect is seen when cells are treated with the non-specific aquaporin inhibitor HgCl₂. Moreover, when control cells are incubated with the AQP3 inhibitor Auphen [165], inhibiting AQP3 but not AQP5, the rate of H₂O₂ uptake is considerably decreased but still significant due to AQP5 peroxiporin activity.

Interestingly, the level of AQP3 expression in BxPC3 cells is 11-fold higher than AQP5 and the silencing was equally efficient for both AQPs. Yet, the measured rate of H₂O₂ permeation was similar for AQP3 and AQP5 suggesting that AQP5 may have higher capacity for H₂O₂ flux resulting in a highly efficient peroxiporin.

6.3.6. Effect of AQP-mediated H₂O₂ transport on cell migration

To investigate whether AQP5-mediated H₂O₂ transport underlies signal transduction in pancreatic cancer progression, we evaluated the rate of cell migration before and upon cell treatment with extracellular H₂O₂ of BxPC3 cells silenced for AQP3 (siAQP3), for AQP5 (siAQP5), and control cells transfected with a non-targeting construct (siCtrl).

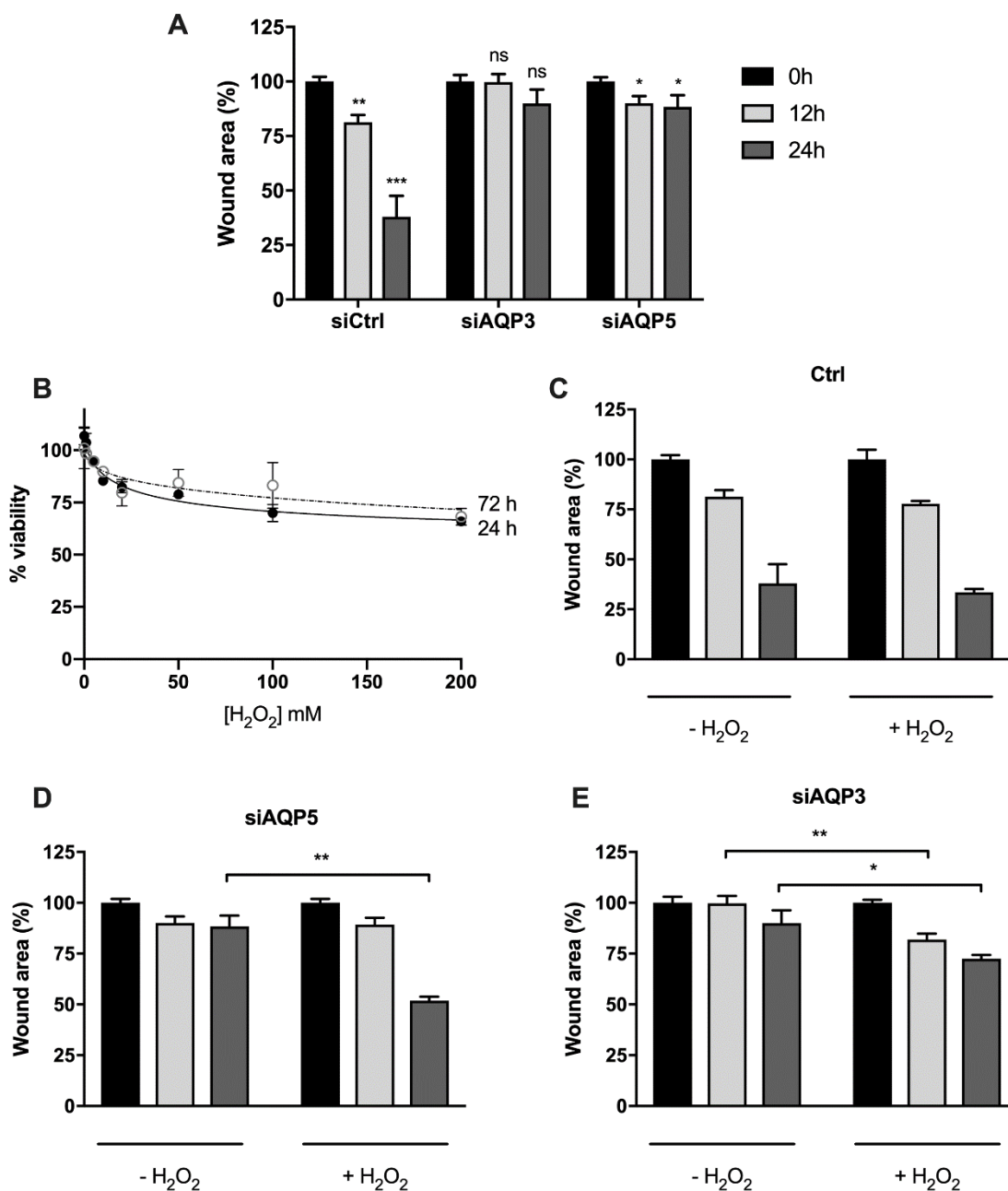


Figure II.6.7. Cell migration of BxPC3 cells upon oxidative stress. (A) Cell migration of cells transiently transfected with corresponding siRNA (control, AQP3 and AQP5) in normal

media. Significance levels: ns, non significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. time 0. **(B)** Cell viability determined by MTT assay after cell exposure to a range of H₂O₂ concentrations for 24h and 72h. **(C)** Cell migration of control cells, **(D)** cells silenced for AQP5 and **(E)** cells silenced for AQP3, before and after addition of 100 μ M H₂O₂. Wound repair was monitored at 12h and 24h under light microscopy and cell migration was normalized to original wound area at time 0. Results are mean $\pm$ SEM of triplicate experiments. Significance levels: * $p < 0.05$, ** $p < 0.01$ vs. non-treated cells.

Studies on monolayers of BxPC3 control cells (siCtrl) showed a partial recovery of wound area after 12 h (around 20%; $p < 0.01$) and significant recovery after 24 h (around 60%; $p < 0.001$) (Fig. II.6.7.A). However, silencing AQP3 completely impaired cell migration and silencing AQP5 substantially reduced cell migration and wound closure compared to control cells. These results are consistent with previous reports in AQP3-null mice, breast cancer and sarcoma cells [30, 166, 167] and indicate that AQP3 and AQP5 play a role in the regulation of cell migration in pancreatic cancer cells representing a promising target for treatment of human pancreatic adenocarcinoma.

Previous to the oxidative migration assay, we assessed the effect of external H₂O₂ stimulus on the viability of BxPC3 cells. As depicted, addition of H₂O₂ up to 200 μ M induced a maximal 30% loss of cell viability (Fig. II.6.7.B). Thus, in subsequent experiments we used 100 μ M H₂O₂, assuring > 70% cell viability for migration assays.

The contribution of each AQP to cell migration under oxidative stress was investigated in control cells (Fig. II.6.7.B), in siAQP3 (Fig II.6.7.D) or in siAQP5 cells (Fig II.6.7.E) before and after treatment with 100 μ M H₂O₂. Downregulation of AQP5 or AQP3 gene expression had a strong impact on cell migration of non-treated cells, that even at 24 h were not able to decrease the wound area when compared to control cells (Fig. 7D and E). However, in siAQP5 cells that still express AQP3, oxidative treatment induced a significant recovery at 24 h (50% wound area), similar to control cells, possibly due to H₂O₂ diffusion via AQP3 as previously reported for breast cancer cells [30].

When siAQP3 cells were treated with H₂O₂, the wound recovery was detected earlier at 12 h although at lesser extent, showing the positive effect of H₂O₂ diffusion via AQP5 on cell migration. These data demonstrate for the first time that AQP5 expression and peroxiporin activity in pancreatic cancer cells is critical for cell migration and tumor spread.

6.4. Discussion

In biological systems, ROS are generated endogenously by mitochondrial respiratory chain and oxidase enzymes or in response to extracellular stimuli. ROS products, including H₂O₂, contribute to oxidative stress and can lead to initiation and progression of several chronic diseases, like atherosclerosis, diabetes, neurodegeneration and tumorigenesis. However, at low concentration ROS can regulate signaling pathways and physiological processes, including cell growth, differentiation, and migration. Recent studies showed that some mammalian AQPs can channel H₂O₂ across cell plasma membrane and reported their involvement in signaling cascades and tumorigenesis [161]. AQP3-mediated H₂O₂ transport has been linked to cancer cell migration, explaining its overexpression in cancer tissues. Rat AQP5 has been characterized as peroxiporin, but studies with human isoform were lacking. Knowing that AQP5 is also highly expressed in human tumors [90], we investigated AQP5 functional regulation and its ability to transport H₂O₂ that may account for a role in tumor progression.

Here we report that human AQP5 facilitates H₂O₂ uptake in hAQP5-transformed yeast cells, an ability also detected in a cultured pancreatic adenocarcinoma cell line. It is worth mentioning that AQP5 was found overexpressed in pancreatic adenocarcinoma biopsies of patients compared with matched normal pancreas tissues, being correlated with tumor stage and aggressiveness[160].

We found that AQP5 expression increases yeast sensitivity to oxidative damage after a long-term insult (48h), but renders cells more resistant to short-term oxidative stress, evidencing the positive contribution of AQP5-mediated H₂O₂ diffusion for cell growth and survival. Mutagenesis studies demonstrated that while phosphorylation of S156 at the cytoplasmic end does not affect permeability, residue His173 located in the selective filter is crucial for water permeability and possibly

interacts with phosphorylated S183 for permeability regulation resulting in blockage of the pore. This mechanism of gating might be involved in the fine-tuning of cell sensitivity/resistance to oxidative external conditions, where NOX produced H₂O₂ is taken up via AQPs triggering signaling cascades and inducing cell proliferation and migration. In fact, both AQP5 and AQP3 showed measurable peroxiporin activity in pancreatic cancer cells, with AQP5 showing higher efficiency than AQP3. Moreover, both AQP5 and AQP3 revealed crucial for cell migration, as shown here for BxPC3 cells whose cell migration was drastically reduced when these peroxiporins were silenced.

The recovery of the migration rate by external oxidative stimulus demonstrates that when AQP3 or AQP5 are downregulated, signaling events triggered by H₂O₂ are blocked by the permeability barrier imposed by biomembranes, and additional H₂O₂ is needed to force cell migration. It was reported that during normal conditions (eustress) the gradient between extracellular and intracellular H₂O₂ is higher than 200-fold [168-170]; the presence of aquaporins will decrease this gradient, favoring H₂O₂ permeability and prompting cellular processes like proliferation and migration [7]. The observation that silenced-AQP3 or -AQP5 cells recover cell migration rate by treatment with H₂O₂ perfectly agrees with this notion.

Altogether, our findings demonstrate that AQP5 has an important role in the pathogenesis of cancer. By allowing a dynamic fine-tuning of intracellular H₂O₂ to activate signaling networks related to cell survival and proliferation, AQP5 can regulate cellular resistance to oxidative stress as well as facilitate cancer cell migration and represents a promising target for the development of cancer therapies.

7. References

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Chapter Three ||

Aquaporin gene regulation in human cancer cells under oxidative stress

This chapter is adapted from following manuscript in preparation:

- Rodrigues, C., Milkovic, L., Tartaro Bujak, I., Tomljanovic, M., Soveral, G. and Čipak Gašparović, A., Lipid profile and aquaporin expression under oxidative stress in breast cancer cells of different malignancy (accepted)

According to the World Health Organization, cancer is the second leading cause of death globally, accounting for 8.8 million deaths in 2015 and 9.6 million people worldwide died from cancer in 2018. Despite the advances attained for cancer treatment, in many cases it is not totally successful and increases the chance of recurrence. This fact leads to a great number of surviving cancer patients which require ongoing palliative care or support living with a chronic condition with negative impact on the quality of life of individuals [1]. Early diagnosis of cancer may lead to a more effective treatment and greater chance of surviving, less morbidity and less extended and aggressive treatments [2]. Tumor biomarkers have been used for diagnosis; however the results showed poor accuracy and efficacy and it is fundamental identify new and better biomarkers.

Aquaporins were found in more than 20 cancer types [3] and their expression has been correlated with tumor type, grade and aggressiveness [3]. Furthermore, several studies carried in cancer cell lines and knockout mice models showed that AQPs may be involved in tumor biology, including in tumor cell proliferation, migration and angiogenesis rendering these membrane channels promising diagnostic and pharmacological targets in anti-cancer treatment [4]. This section will summarize the AQP expression in the most relevant tumor types (breast, colon and pancreas) and will give an overview of AQPs physiological functions in tumor biology like tumor cell migration, tumor proliferation and angiogenesis

1. Aquaporin expression in human tumor tissues

Human aquaporins are distributed in different organs, tissues and subcellular organelles [5] and a complex pattern of AQP expression is commonly found in the cells which require rapid water and/or glycerol movement, such as observed in epithelial and endothelial cells, erythrocytes, adipocytes and other type of cells [6]. As reviewed in Table III.1, the overall

perspective indicates that AQPs are overexpressed in cancer cells when compared to normal cells even though a few studies reported reduced AQP expression [7-9]. However, results of downregulation should be carefully interpreted because a full screen of AQPs expression was not performed and the possibility of upregulation of others isoforms as a compensatory mechanism has not been taken into account. Additionally, several isoforms that are not normally expressed in healthy tissue were found expressed in tumor tissues [3].

Regarding aquaporin expression in cancers, the lack of tumor-specific expression as well as the complex pattern of AQPs expression in a single tissue have delayed the discovery and development of specific AQPs inhibitors for anti-cancer therapies. Notwithstanding these difficulties, aquaporins are considered promising drug targets for cancer treatment.

Table III.1. Expression of AQP isoforms in different human tumors and pathophysiological functions.

Tumor type	Aquaporins	Pathophysiological function	References
Breast	AQP1 ↑ AQP3 ↑ AQP5 ↑ AQP4 ↓	Proliferation, migration, metastasis and prognostic marker	[8, 10-13]
Colorectal	AQP1 ↑ AQP3 ↑ AQP5 ↑ AQP8 ↓	Proliferation, metastasis, prognostic marker, drug resistance	[9, 14-19]
Pancreatic ductal adenocarcinoma	AQP3 ↑ AQP5 ↑	Proliferation, tumor grade and prognostic marker	[9, 20]

1.1. Breast cancer

A few studies have reported the expression of AQPs in breast cancer, mainly AQP1, AQP3 and AQP5 that were found upregulated [8]. AQP1 is mainly detected in the cytoplasm of cancer cells of invasive breast cancer patients [21]. Clinical evidences suggest that upregulation of AQP1 is associated with a poor prognosis of patients with breast cancer and cytoplasmic expression of AQP1 was an independent prognostic factor [13, 22]. Also, it was reported that estrogen can induce AQP1 expression in a time- and dose-dependent fashion mediated through activation of estrogen response element (ERE) motif in the promoter region of the Aqp1 gene, resulting in cell proliferation, migration, invasion and tubule formation [23]. This data suggests the involvement of AQP1 in estrogen-induced angiogenesis by stimulation of endothelial cells via estrogen receptors and therefore AQP1 upregulation enhances tumor cell migration and invasion [24]. Similarly, ERE motif was identified in the promoter of Aqp3 gene and can be activated to upregulate AQP3 expression [10]. Overexpression of AQP3 was observed in ER-positive breast cancer tissues obtained from premenopausal patients when compared to those obtained from postmenopausal patients and is associated with tumor histological grade and lymph node metastasis [10]. The potential role of AQP3 in angiogenesis and invasion was suggested to be in part through regulation of the expression of molecules related to epithelial-mesenchymal transition (EMT) and the reorganization of actin-cytoskeleton which result in tumor cell migration and invasion [10]. In addition, AQP3 has been shown to be required for breast cancer cell migration by a mechanism involving AQP3-facilitated H_2O_2 transport influencing downstream signaling [25]. AQP3 overexpression in early breast cancer patients was shown to be associated with the worst prognosis in patients with HER2^{NEU}-overexpressing phenotype [26].

In benign tumors, AQP5 was mainly observed at the apical domains of ductal epithelial cells but apical polarity of AQP5 was lost in invasive ductal

carcinoma [11]. AQP1 and AQP5 overexpression are proposed independent prognostic markers of survival for breast cancer patients [12, 13].

1.2. Colorectal cancer

As in breast cancer, AQP1, AQP3 and AQP5 are also overexpressed in colorectal cancer [17]. AQP1 and AQP5 were found upregulated in the early stage and maintained through late stage of colon cancer development [17]. Overexpression of these two isoforms were also observed in metastatic lesions in the liver [17] and suggests that AQPs play important role in initiating and development of carcinogenesis. AQP3 and AQP5 expression in colorectal carcinoma were related to differentiation, lymph node and distant metastasis [14, 16, 18]. Upregulation of AQP1 in HT20 colon cancer cells increased water permeability and enhanced cell migration reverted by AQP1 inhibition [27]. In addition, the data suggested that AQP1 induced cell migration and metastasis in colon cancer cells in response to relocalization of actin protein and activation of RhoA and Rac [27].

Also, the migration of colon cancer cells can be impaired by AQP3-specific inhibitor as well as EGFR pathway inhibitors [16]. In human epidermoid carcinoma A431 and lung cancer H1666 cell lines, AQP3 knockdown suppressed the transport into the cells of extracellular H₂O₂ produced in response to EGF [28] and suggests a similar mechanism for colon cancer migration impairment when cells are incubated with EGFR pathway inhibitors. Expression of AQP5 expression in HCT 116 colon cancer cells promotes cell proliferation via Ras/ERK/Rb pathway [15]. AQP3 and AQP5 are proposed biomarkers for colorectal cancer and their overexpression are correlated with poor survival [16, 18, 29]. In addition, downregulation or blocking of AQP1, AQP3 and AQP5 function provided a new therapeutic strategy in colon cancer.

1.3. Pancreatic cancer

In a study performed by our group, AQP3 and AQP5 were found overexpressed in pancreatic ductal adenocarcinoma tissues [20]. AQP3 was detected at the plasma membrane of ductal cells while AQP5 was observed in the apical membrane of intercalated and intralobular ductal as well as in infiltrative cancer cells in duodenum [20]. Although the molecular mechanism remains unclear, the results suggested that AQP3 and AQP5 are important for pancreatic carcinogenesis. AQP3 may induce cancer cell migration and proliferation by glycerol uptake and AQP5 facilitates water permeation at the edge of cancer cells and may induce cell migration. Interestingly, both AQPs are able to transport H_2O_2 that can also activate signaling pathways related to migration and proliferation, like PI3K and ERK signaling pathways [30]. Pancreatic cancer is one of the most aggressive tumor types with poor prognosis and the 5-year survival rate is around 7%. This study revealed that AQP5 may be considered a sensitive and specific marker for early stages of pancreatic adenocarcinoma while AQP3 expression may be related with late and more aggressive stages of the disease [20].

2. Pathophysiological roles of aquaporin in tumor biology

Out of thirteen isoforms found expressed in normal human tissues, seven isoforms (AQP1, AQP3, AQP4, AQP5, AQP7, AQP8 and AQP9) were observed dysregulated in tumors [31]. The abnormal expression of these AQPs in tumors contributes to tumor-associated edema, tumor cell proliferation and migration, and tumor angiogenesis and metastasis [3]. Genetically modified mouse models have significantly contributed to understanding cancer biology. This section summarizes the pathophysiological functions of AQPs in cancer cells that were mainly derived from experiments comparing wild-type vs AQP knockout mice models.

2.1. Tissue water balance

The primary function of aquaporins is to transport water across cell membranes in response to osmotic gradients to maintain cellular homeostasis. In humans, AQPs are largely expressed in tissues associated with maintenance of water-based homeostasis such as in kidney [32], central nervous system [33], airways [34] and salivary glands [35]. In such case, aquaporins play an important role in mammalian physiology related to tissue water balance including transepithelial fluid transport, brain water balance, skin hydration and cell migration and proliferation, among others (reviewed in [6]). Therefore, it is not surprising that AQPs dysregulation has been associated with the onset of several pathologies [36] such as cerebral ischemia [37], glaucoma [38], nephrogenic diabetes insipidus [39] and cancer [3].

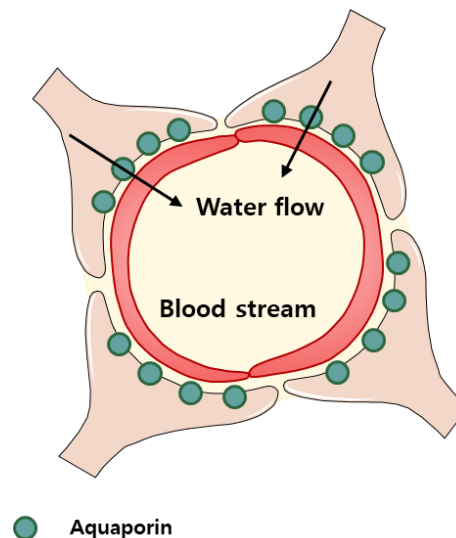


Figure III.1.1. Proposed mechanism for edema elimination in brain tumor. AQP4 is highly localized in the endfeet of astrocytes and can quickly mediate water efflux or influx in response to endothelial-driven osmotic gradients. In edematous brain tumors, AQP4 overexpression increases water efflux at the astrocyte endfoot into bloodstream and may facilitate brain edema clearance.

It is now clear that the maintenance of water balance through AQPs is important in human tissues. In central nervous system, AQP4 is the major isoform expressed and is important to edema fluid formation/clearance [40].

Recent evidence showed that AQP1 [41] and AQP4 [42] are overexpressed in astrocytes of edematous brain tumors, such as glioblastomas, compared to normal brain tissue. Also, a positive correlation between overexpression of AQPs and the presence of brain tumor edema was observed and suggests a pathophysiologic role in brain edema [41, 42]. Studies developed in AQP4 knockout mice showed increased edema formation around brain melanoma [43] and proposed that AQP4 may facilitate elimination of brain edema fluid (Fig III.1.1.). Although it is not fully understood if AQP4 upregulation is a cause for brain edema or a side effect aimed at eliminating fluid excess resulting from capillary leakage, both AQPs are interesting targets for development of AQP modulators and represent a novel pharmacological target for design of anti-brain tumor edema agents.

2.2. Cell migration and metastasis

At present, the involvement of AQPs in cancer development is well established even if the molecular mechanisms remains unclear. The lack of specific inhibitors led to investigate AQP function in knockout mice and several studies indicated that AQPs are important for cell migration, including malignant cells. AQP overexpression observed in tumor tissues suggests that these proteins enhance tumor cell migration and angiogenesis towards a chemotactic stimulus and ultimately participate in tumor growth, development, invasion and metastatic process [3]. Mechanistically, it has been hypothesized that AQP polarization at the front edge of migrating cells induces rapid changes in cell shape and volume driven by osmotic gradients and promotes lamellipodium formation stabilized by actin polymerization as depicted in Fig. III.1.2. [31, 44]. The formation of a flattened protrusion at the migrating cell leading edge (lamellipodium) enhances the ability to migrate to confined micro-spaces by squeezing through the extracellular matrix [44].

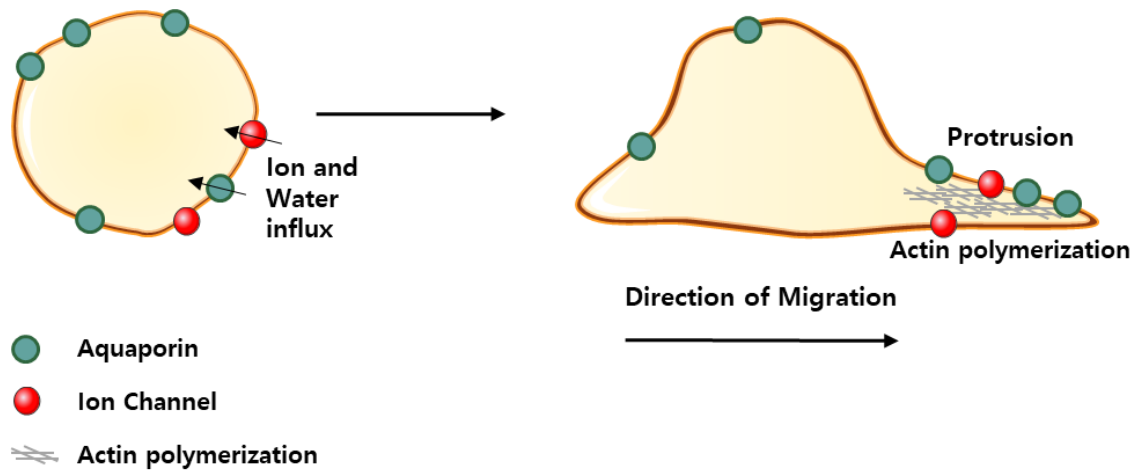


Figure III.1.2. Cancer cell migration. During migration, increased ion transport at the leading edge causes rapid changes in osmolality and drives water flux. AQP polarization facilitates rapid water transport increasing local hydrostatic pressure and lead to cell membrane expansion, which forms a protrusion. Local swelling is followed by actin polymerization which stabilizes lamellipodium formation and ultimately supports cell movement.

Both *in vitro* and *in vivo* evidences support that AQP1 is important for tumor cell infiltration into surrounding tissue and to the metastatic process [45]. B16F10 melanoma and 4T1 breast cancer cells were transfected with AQP1 and showed increased cell migration [45], as observed in glioblastoma [46]. Alveolar wall infiltration and lung metastasis were observed in mice injected with AQP1-expressing tumor cells [45]. Also, HT20 colon cancer cells transfected with AQP1 were injected in mice and the results showed that AQP1 enhanced cell migration and increase lung extravasation and spread [27]. Similar results were obtained in melanoma cells implanted subcutaneously in AQP1-null mice in which cell migration was greatly reduced in AQP1-deficient cells [47]. Next, histological examination of these tumors was performed and the results indicated an impairment of tumor angiogenesis in AQP1-null mice when compared to control [47]. In lung cancer cells, AQP1 can promote lung cancer cell proliferation [48] and in pulmonary adenocarcinoma it was observed a positively correlation between AQP1 overexpression and increase of metastasis ratios [49].

In vitro studies, performed in breast cancer cell lines, showed that AQP3 is required for cancer cell migration [25, 50]. Also, studies where AQP3-overexpressing and AQP3 knockdown cells were injected into the tail veins of mice showed that cell migration was dependent on AQP3 expression level and that AQP3 knockdown abrogates the number of metastatic cells [25]. In pancreatic cancer cells, AQP3 expression is induced by EGF in a dose- and time-dependent manner by EGFR/ERK signaling pathway and promotes cell migration [51]. Similar results were obtained in gastric cancer cells [52] and it was also observed that AQP3 regulates matrix metalloproteinases expression, important proteins for cancer migration, via PI3K/Akt signaling pathway [53]. Studies developed in skin and lung cancer cells showed that AQP3-mediated intracellular H₂O₂ uptake, produced in response to EGF, drives a downstream signaling activation by phosphorylation of ERK and Akt that promotes cancer cell progression [28]. These data strongly suggest that AQP3 is an important player in activation of signaling pathways that control cell migration and proliferation.

Interestingly, AQP5 upregulation is associated with lymph node metastases of several cancers, and it was hypothesized that this isoform may contribute to cancer cell migration from the primary tumor and promotes cell invasiveness and metastasis [54]. Studies performed *in vitro* using either AQP5-overexpressing or AQP5 knockdown cancer cells, including lung [55], gastric [56], breast [11] and glioma [57], showed that cell migration and invasion properties are positively correlated to AQP5 level expression. Moreover, the results obtained both in breast and in glioma cancer cells suggest that AQP5-induced cancer cell invasion and metastasis may be related to its direct or indirect interaction with ERK1/2 pathway, known to be important in cancer metastasis and invasiveness. [11, 57]. Phosphorylation of serine-156 of AQP5 seems to be important to cancer cell migration and proliferation [54] but further experiments are required to unveil the fully molecular mechanism.

2.3. Cell proliferation

In addition to the extensive data demonstrating that AQPs are involved in cancer cell migration, there is also evidence that AQPs are important for cancer cell proliferation. Cell proliferation is closely associated with AQP-mediated cell volume changes in response to osmotic gradients driven by ion fluxes [58]. Furthermore, AQPs have been linked to cell proliferation by facilitating glycerol and hydrogen peroxide transport (Fig. III.1.3.). Glycerol is an important intermediate of energy metabolism, largely metabolized to glycerol-3-phosphate [59] and ultimately is used to ATP production required for cellular biosynthesis and consequently for cell division [59]. AQP3 knockdown keratinocytes showed reduction of glycerol and ATP content along with impaired proliferation rate when compared to normal cells [60]. Hydrogen peroxide generated by NOX proteins and transported by AQPs through plasma membrane may activate signaling pathways and stimulate cell proliferation [61].

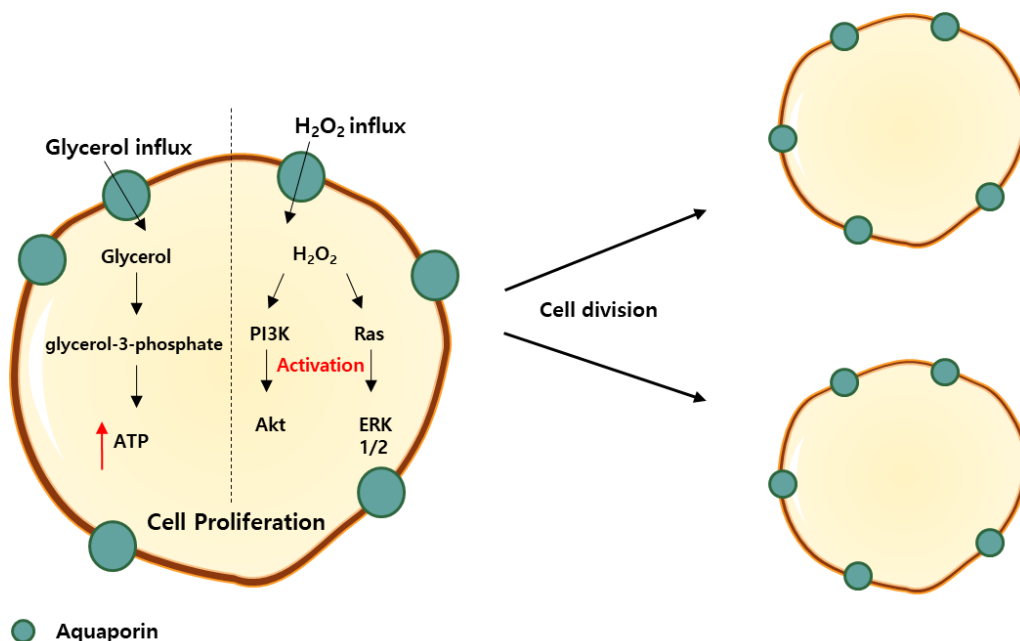


Figure III.1.3. Cancer cell proliferation. Aquaporins facilitate glycerol and hydrogen peroxide influx and promote cell proliferation. Glycerol (left) is essential for cellular biosynthesis as backbone in phospholipid synthesis and/or as an intermediate for ATP product. Hydrogen peroxide can activate intracellular signaling pathways promoting the transcription of genes involved in cancer cell proliferation.

Several studies highlight the direct or indirect involvement of AQPs in cancer cell proliferation. AQP3-null mice are more resistant to the development of skin cancer [62]. Glycerol supplementation restored intracellular ATP content and cellular proliferation and directly implicates AQP3 function in skin tumor development [62]. Downregulation of AQP3 also suppressed cell proliferation in breast cancer cells [50] and in non-small cell lung cancer cells [63]. In colon cancer cells, lack of AQP5 expression is associated with impairment of cell proliferation [15]. A recent study suggested that serine/threonine phosphorylation of AQP5 induces cell proliferation via Ras/ERK/Rb pathway [15]. Also in lung cancer [55] and in soft sarcoma [64], AQP5 expression enhanced cell proliferation by activation of Ras/ERK/Rb signaling pathway. In leukemia cells, proliferation was promoted by AQP8-mediated H₂O₂ transport that leads to PI3K and p38 MAPK activation [65].

Interestingly, both AQP3 and AQP5 transport H₂O₂ and it is well known that this small molecule can act as a second messenger activating signaling pathways associated with cell migration, proliferation, and differentiation. Thus, it will be interesting to further investigate if AQP-mediated hydrogen peroxide transport can activate these downstream signaling pathways in cancer cells and induce cell proliferation.

2.4. Cell adhesion

Interaction between aquaporins and adhesion molecules may indicate that AQPs promote cell-matrix adhesion important for tumor progression and development. Suppression of AQP3 in esophageal and oral cancer cells strongly inhibited cell-adhesion along with reduction in integrin $\alpha 5$ and $\beta 1$, both adhesion molecules [66]. In glioblastoma cells, both orthodox aquaporins AQP1 and AQP4 are expressed but only AQP4 induces cell-matrix adhesion [46]. It will be interesting to further investigate if other AQPs overexpressed in cancer also contribute to cell adhesion and to unveil the underlying molecular

mechanism.

Since cancer cells are highly proliferative and metabolic, H_2O_2 production near the tumor is considerably higher than in normal cells, and its uptake via AQPs may activate signaling cascades that control proliferation. In addition to AQPs expression, the lipid profile and steady-state peroxidation of cancer cells may also influence their ability to proliferate and migrate and modulate resistance to oxidative conditions. Thus, we investigated the interplay between fatty acid content and AQP modulation of breast cancer cells with different levels of aggressiveness, and correlate with the cell sensitivity to oxidative stress. In addition, we evaluated the expression profile of AQPs in pancreatic tumor biopsies from human patients, aiming at correlating *in situ* AQP expression level with tumor aggressiveness and identifying new potential biomarkers.

3. Material and methods

3.1.1. Cell culture and treatments

Human breast and colon cancer cell lines used to develop the experiments presented in Chapter 3 are described in Table III.2.1. and in Table III.2.2., respectively. Human breast and colon cancer cell lines were kindly provided by collaborators and cultured in DMEM (Lonza Biowhittaker) medium containing 1% penicillin-streptomycin solution (Lonza Biowhittaker) and 10% heat-inactivated FBS (Gibco Invitrogen) at 37°C in 5% CO_2 . For specific experiments, cells were treated with 100 μM H_2O_2 for 24h at 37°C in 5% CO_2 .

Table III.2.1. Characterization of human breast cancer lines used in Chapter 3 [67].
Abbreviations: ER, estrogen receptor; HER2^{NEU}, human epidermal growth factor receptor 2; PR, progesterone receptor.

Breast cancer cell line	General information	Immunoprofile	Other features
MCF-7	Breast adenocarcinoma derived from metastatic site	Hormone responsive (estrogen and progesterone receptor positive) ER ⁺ , PR ^{+/-} , HER2 ^{NEU-}	Highly differentiated and relatively less aggressive behavior (better prognosis)
SKBR3	Breast adenocarcinoma derived from metastatic site	Human epidermal growth factor receptor 2 positive ER ⁻ , PR ⁻ , HER2 ^{NEU+}	Moderately differentiated
SUM159	Breast anaplastic carcinoma	Triple-negative ER ⁻ , PR ⁻ , HER2 ^{NEU-}	Poorly differentiated and most aggressive behavior (worst prognosis)

Table III.2.2. Characterization of human colon cancer lines used in Chapter 3 [68, 69].

Colon cancer cell line	General information	Other features
Caco2	Colorectal adenocarcinoma	Enterocyte-like phenotype and exhibits properties associated with the physical and metabolic barrier of the intestinal epithelium
HT29	Colorectal adenocarcinoma derived from primary tumor	Moderately differentiated and lower expression of the EMT signature (less aggressive behavior)
HCT116	Colorectal carcinoma	Poorly differentiated and

	derived from primary tumor	strong expression of the EMT signature (more aggressive behavior and high propensity for local invasion)
SW620	Colorectal adenocarcinoma derived from lymph node metastasis	Strong expression of the EMT signature (more aggressive behavior and high propensity for local invasion)

3.2. Patients and clinical samples

Pancreatic tumor samples and matched adjacent non-neoplastic samples were obtained from 25 patients who underwent curative surgery for pancreatic cancer at Hospital Curry Cabral, Centro Hospitalar de Lisboa Central, Lisbon, Portugal, between November 2012 and February 2013. Tissues were obtained and used in a manner compliant with the Declaration of Helsinki, as revised in 1983. Immediately after collection, samples were preserved in RNA stabilization solution (RNAlater, Sigma) and stored at -20°C for RNA extraction. This study was approved by the ethics committee of Centro Hospitalar de Lisboa Central, and all participants signed an informed consent form.

3.3. RNA isolation and Real Time RT-PCR

Human cancer cell lines — Total RNA was isolated according to manufacturer's instructions. The total RNA concentration was determined using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies), and the quality was checked on the agarose gel. cDNA was obtained from 1 µg of total RNA and the reverse transcription was carried out with oligo dT23 primer (Sigma Aldrich) using MultiScribe reverse transcriptase (Applied Biosystems).

Patients — Tissue samples were stored in RNALater (Sigma) at -20°C until RNA extraction. Total RNA was extracted from 25 pairs of frozen samples by homogenization with a power homogenizer in TRIzol Reagent (Invitrogen), according to the manufacturer's protocol. Contaminating DNA was removed

using DNase I recombinant RNase-free (Roche Diagnostics), following the manufacturer's protocol. Extracted RNA was quantified with Nanodrop™ 2000c spectrophotometer (Thermo Fisher Scientific). For template cDNA synthesis, 1 µg of total RNA was reverse transcribed in a 20-µL final volume using random hexamers primers (Roche Applied Science) and 200 units of M-MLV reverse transcriptase (Invitrogen) as previously described [70].

Real-time PCR reactions were carried out using a CFX96 Real-Time System C1000 (BioRad), the TaqMan Universal PCR Master Mix (Applied Biosystems Thermo Fisher Scientific) and the following specific TaqMan pre-designed gene expression primers: AQP1 (Hs01028916_m1), AQP3 (Hs01105469_g1), AQP5 (Hs00387048_m1), ACTB (Hs99999903_m1) and GAPDH (Hs99999905_m1) (Applied Biosystems). The cDNA was amplified at the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

For human cancer cell lines, the relative quantification of gene expression was determined using the $2^{-\Delta C_t}$ method (adapted from [71]). Using this method, we obtained the fold variation in AQP gene expression normalized to an endogenous control (β -actin or GAPDH). For tumor biopsies, the mRNA relative expression level was determined using the $2^{-\Delta\Delta C_t}$ method [71]. Using this method, we obtained the fold changes in AQP gene expression normalized to β -actin, and relative to the calibrator (adjacent non-neoplastic). The expression level (EL) in tumor tissues was scored on a scale of four grades: 0, no difference; 1, weak expression; 2, moderate expression and 3, strong expression. All samples were run in triplicate and the average values were calculated.

3.4. Cell viability (MTT)

The cell viability was measured by MTT EZ4U assay (Biomedica) according to manufacturer's instructions. Briefly, 24 h after treatment cells were incubated with the colourless solution, which is oxidized in mitochondria of living cells forming yellow product, and the colour intensity is measured at 450 nm, with 620 nm as a reference wavelength.

3.5. Intracellular ROS analysis

Intracellular ROS was measured using the 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Fluka). This is non-fluorescent dye which is deacetylated in the cells and after oxidation with intracellular ROS turns to fluorescent product. For the ROS measurement, cells were seeded as described above, left for 24 h to attach and then they were incubated for 1 h with DCFH-DA. After removing the excess of the dye, cells were washed once and were treated with H_2O_2 in a range of concentrations. Fluorescence intensity was measured for 1 h for every 10 min in order to follow the kinetics of H_2O_2 cellular uptake.

3.6. Fatty acid analysis

Cells were treated as described above, and at the end of treatment they were trypsinized and dry pellet was stored at $-80^{\circ}C$ till analysis. Lipids were isolated according to modification of the Folch method [72]. Briefly, 5 mL of chloroform was added to the samples and then mixed thoroughly. The aqueous solution of $MgCl_2$ (1.5 mL; 0.034%, w/v) was added to the samples, then were vortexed and centrifuged. The upper aqueous phase was removed and 2 M solution of KCl in methanol (2.5 mL; 4:1, v/v) was added, then samples vortexed and centrifuged. The aqueous phase was removed, and chloroform/methanol solution (2.5 mL; 2:1, v/v) was added, then samples were vortexed and centrifuged. Hydrophobic phase was collected and transferred

to a new tube, and solvent was removed by evaporation in the nitrogen gas stream. Dry residues were stored at -80°C until further analysis.

To form fatty acid methyl esters, 0.5 M solution of KOH in methanol [73] was added to the lipid extracts for 10 min at RT. Fatty acid methyl was then extracted with n-hexane, and analysed by gas chromatography (GC). GC analysis of total fatty acids was performed by using Varian 450-GC equipped with a flame ionization detector. Stabilwax column (crossbond carbowax polyethylene glycol, 60 m × 0.25 mm) was used as a stationary phase with helium as a carrier gas. The heating was carried out at a temperature of 150°C for 1 min followed by an increase of 5°C/min up to 250°C. The methyl esters were identified by comparison with the retention times of commercially available standards.

3.7. Nrf2 analysis

Cells were grown and treated in 6-well plates as described above. For protein analysis, cells were harvested in RIPA Buffer. Next, protein concentrations were measured according to Bradford [74]. Aliquots containing 25 µg protein were separated by SDS-PAGE on 12,5% resolving gel. Proteins were transferred to nitrocellulose membrane (Roti-NC 0,2 µm; Carl Roth). The membrane was blocked with 2% non-fat dry milk and, after removing the blocking solution, membrane was incubated with anti-Nrf2 antibody (Cell Signaling). After washing, membrane was incubated with Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling) (1:2000 dilution in blocking buffer; CST). Signal was visualized with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (TFS) and the chemiluminescence was detected using the Alliance 4.7 Digital Imaging System (Uvitec, Cambridge, UK). Signals were quantified using the ImageJ software [75].

3.8. Statistical analysis

Results were expressed as mean $\pm$ SEM of n individual experiments. For *in vitro* work, statistical analysis between groups was performed by Student's t-test. Statistically significant outcomes are represented as $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***); ns is not significant. Statistical analysis was performed using the Graph Prism 5 software (GraphPad Software).

4. Lipid profile and aquaporin expression under oxidative stress in breast cancer cells of different malignancy

4.1. Abstract

Breast cancer is the major cause of tumor-associated mortality in women worldwide, with prognosis depending on the early discovery of the disease as well as of type of breast cancer diagnosed. Among many factors, lipids have a role in breast cancer malignancy either as protecting from peroxidation or participating in cellular processes. Also, aquaporins are membrane channels found aberrantly expressed in cancer tissues that were correlated with tumor aggressiveness, cancer progression and metastasis. However, the differences in lipid profile and aquaporin expression between cell types of different malignant potential have never been investigated.

Here, we selected three breast cancer cell lines representing the three major breast cancer types (hormone positive, HER2^{NEU} positive and triple negative) and analyzed their lipid profile and steady state lipid hydroperoxide levels to correlate with cell sensitivity to oxidative stress. Additionally, the expression profiles of AQP1, AQP3 and AQP5 and Nrf2 transcription factor were evaluated in the three cell types, before and after an oxidative stress challenge.

We found that the lipid profile is dependent on the cell type, having the HER2^{NEU}-positive cells the lowest level of PUFA and alpha linolenic acid, which is associated with antitumorigenic effects, whereas the triple negative show the highest. However in triple negative cancer cells, a lower level of the antioxidant transcription factor Nrf2 may be responsible for a higher sensitivity to hydrogen peroxide challenge. Interestingly, HER2^{NEU} positive cell showed the highest increase in intracellular ROS after oxidative challenge, concomitant with a significantly higher level of AQP1, AQP3 and AQP5 expression compared to the other cell types, being AQP3 always the most expressed isoform. AQP3

gene expression was stimulated by hydrogen peroxide treatment in hormone positive and HER2^{NEU} cells, together with Nrf2 expression, but was downregulated in triple negative cells that showed instead upregulation of AQP1 and AQP5. The lipid profile and AQP gene expression after oxidative challenge of these particularly aggressive cell types may represent metabolic reprogramming of cancer cells and reflect a role in adaptation to stress further contributing to therapy resistance.

4.2. Introduction

Breast cancer is the major cause of tumor-associated mortality in women worldwide. The prognosis depends on the early discovery of the disease as well as of the diagnosis. Generally, breast cancer is grouped in subtypes according to therapy, in hormone positive (estrogen and progesterone positive tumor) which are treated by hormones, HER2^{NEU}-positive (positive for the human epidermal growth factor receptor 2, HER2^{NEU}) and is treated with trastuzumab, and triple negative tumors, which are negative for estrogen, progesterone and HER2 receptor and are particularly aggressive, with a higher propensity for metastasis [76]. Although several biomarkers and criteria are in place to assist diagnosis and prognosis after surgery, it is imperative to find new markers and therapeutic targets and to develop new and better therapies able to bypass tumor adaptation.

Malignant cells commonly have different metabolic requirements than normal cells. Malignant cells tend to rely on glycolysis and anaerobic metabolism, require increased amount of substrates and are highly dependent on the movement of water and ions across cell membranes for the formation of lamellipodia (invadopodia) for cell migration [31]. Clinical and preclinical studies showed that aquaporins (AQPs), membrane channel proteins that mediate fluxes of water and glycerol and in some cases hydrogen peroxide (H₂O₂) across cell membranes [77-81], are aberrantly expressed in cancer tissues, and positively correlate with tumor aggressiveness, cancer progression

and metastasis [3, 31, 82]. Overexpression of AQPs was detected in tumor cells of different origins, being associated with tumor formation, angiogenesis, cell migration and proliferation, suggesting that AQPs might be novel targets of diagnostic and prognostic value and their modulation could be explored for anti-cancer treatment [83-86].

In particular, AQP1, AQP3 and AQP5 are abundantly expressed in diverse tumors and are involved in cell proliferation and migration by mechanisms that include changes in cellular shape and enriched ATP production during cell proliferation and differentiation [11, 24, 47, 62, 83, 87]. In addition, the ability of AQP3 and AQP5 to facilitate H₂O₂ diffusion through cell membranes [80, 81] may explain their role in activation of signaling cascades via H₂O₂-mediated signaling with impact in cell proliferation and resistance to oxidative stress [28, 81, 88].

A few studies have reported the expression of AQPs in breast cancer, mainly AQP1, AQP3 and AQP5, that were found higher expressed in tumors than in normal adjacent tissues [8]. While AQP3 overexpression in early breast cancer patients was shown to be associated with worse prognosis in patients with HER2^{NEU}-overexpressing phenotype [26], AQP1 and AQP5 were reported independent prognostic markers of survival for breast cancer patients [12, 13, 22]. In addition, AQP1 overexpression can be induced by estrogen in a time- and dose-dependent fashion through activation of the ERE motif in the promoter region of the Aqp1 gene, resulting in cell proliferation, migration, invasion and tubule formation [23]. These data suggest the involvement of AQP1 in estrogen-induced angiogenesis by stimulation of endothelial cells via estrogen receptors, resulting in enhancement of tumor cell migration and invasion [24]. Similarly, the ERE motif was identified in the promoter of Aqp3 gene and can be activated to upregulate AQP3 expression [10]. Overexpression of AQP3 was observed in ER-positive breast cancer tissues obtained from premenopausal patients when compared to those obtained from

postmenopausal patients and is associated with tumor histological grade and lymph node metastasis. The role of AQP3 in angiogenesis and invasion was suggested to involve regulation of expression of epithelial-mesenchymal transition (EMT) molecules and reorganization of actin-cytoskeleton, controlling tumor cell migration and invasion [10]. In addition, AQP3 expression was reported to affect breast cancer cell migration during metastasis via binding of the chemokine CXCL12 to its receptor CXCR4 [25]. Signaling via the CXCL12/CXCR4 axis induces Nox activation and extracellular H₂O₂ production, which is then rapidly transported intracellularly via AQP3. The localization of an AQP3-Nox2 complex at the leading edge of polarized breast cancer cells reinforce the idea that AQP3-mediated H₂O₂ transport is required for directional cell migration. Moreover, H₂O₂ transported by AQP3 promotes PTEN/PTP1B oxidation and activates the PI3K/Akt pathway, inducing cell proliferation and migration.

In benign breast tumors, AQP5 was mainly observed at the apical domains of ductal epithelial cells but this apical polarity was lost in invasive ductal carcinomas [11]. Silencing AQP5 in an ER-positive breast cancer cell line (MCF7) significantly reduced cell proliferation and migration strongly suggesting that AQP5 may play a role in the cell growth and metastasis of human breast cancer [11]. AQP5 upregulation was closely associated with cellular differentiation, lymph node invasion and breast tumor stage [8]. A significant AQP5 upregulation found in tumors from early breast cancer patients indicates it may be used as a prognostic marker [12]. Interestingly, AQP5-mediated H₂O₂ diffusion was demonstrated to modulate cell survival in oxidative stress conditions [81].

H₂O₂ is a reactive oxygen species (ROS) that besides the recognized harmful role acts as an important signaling molecule in regulation of cellular redox homeostasis [89]. ROS-signaling is attained through reversible oxidation of thiol residues within proteins, where the intracellular levels of anti-oxidant

molecules are crucial to maintain the redox balance. H_2O_2 regulates Nrf2 transcription factor, one of the major antioxidative transcription factors located in cytoplasm in complex with Keap1 [90]. Keap1 oxidation by H_2O_2 activates Nrf2 that translocates to nucleus and starts the transcription of antioxidative genes [90]. Yet if H_2O_2 overwhelms the capacity of the cell to cope with stress, oxidation of all intracellular molecules occurs. One of the most vulnerable molecules are lipids, especially their fatty acid moiety, which undergo peroxidation. Polyunsaturated fatty acids (PUFA) are substrate for ROS and are prone to peroxidation, leaving a variety of reactive aldehydes as end-products of these reactions [91]. Among these reactive aldehydes 4-hydroxynonenal (HNE), malondialdehyde (MDA) and acrolein are the most studied. HNE was shown to modulate signaling pathways and consequently modulate cellular processes leading to proliferation differentiation or apoptosis [92] and interestingly, it also affects aquaporin activity [93, 94]. As the produced amount of reactive aldehydes depends on their substrate, i.e. PUFA, it is not surprising that tumor cells can change fatty acid content in order to modulate sensitivity to lipid peroxidation or as a response to the diseased state [95]. Additionally, fatty acids that are substrate for HNE are omega6-PUFA, the ones that are substrates for prostaglandin production (e.g. arachidonic acid) [96] and therefore, modulation of PUFA membrane content may affect important cellular signaling pathways. Moreover, decreasing the PUFA content may also affect sensitivity to oxidative stress and lipid peroxidation.

In this study, we aimed to evaluate fatty acid content of breast cancer cell lines of different malignancy and correlate with the cell sensitivity to oxidative stress. Moreover, knowing that cell sensitivity to H_2O_2 largely depends on cell type, and that AQPs are aberrantly expressed in many tumors, particularly in the most aggressive, the effect of H_2O_2 on AQP1, AQP3 and AQP5 expression profile was investigated in breast cell lines representing different breast cancer malignancies, together with their response to oxidative

stress and reaction to Nrf2 transcription factor. We hypothesize that variation of expression of specific AQPs can determine the cell sensitivity to oxidative stress and may represent a target for anticancer treatments.

4.3. Results and discussion

4.3.1. Lipid profile and steady state lipid hydroperoxide levels

Among risk factors for breast cancer, obesity is certainly one of the most mentioned, which correlates with type of the cancer in pre- or post-menopausal age [97]. These data indicate the specific role of lipids in breast cancer malignancy, acting as active participants in cellular processes, such as arachidonic acid, which is precursor for prostaglandin synthesis [96]. In addition, fatty acids, especially polyunsaturated fatty acids (PUFA), are prone to ROS attack resulting in reactive aldehyde production, which in turn act as second messengers of free radicals [91]. In order to investigate role of lipids/fatty acids in breast cancer malignancy, we have selected three breast cancer cell lines representing the three major breast cancer types: hormone positive (MCF7), HER2^{NEU} positive (SkBr3) and triple negative (SUM159). In these cells, we first analyzed their lipid profile and steady state lipid hydroperoxide (LOOH) levels (Fig. III.4.1.).

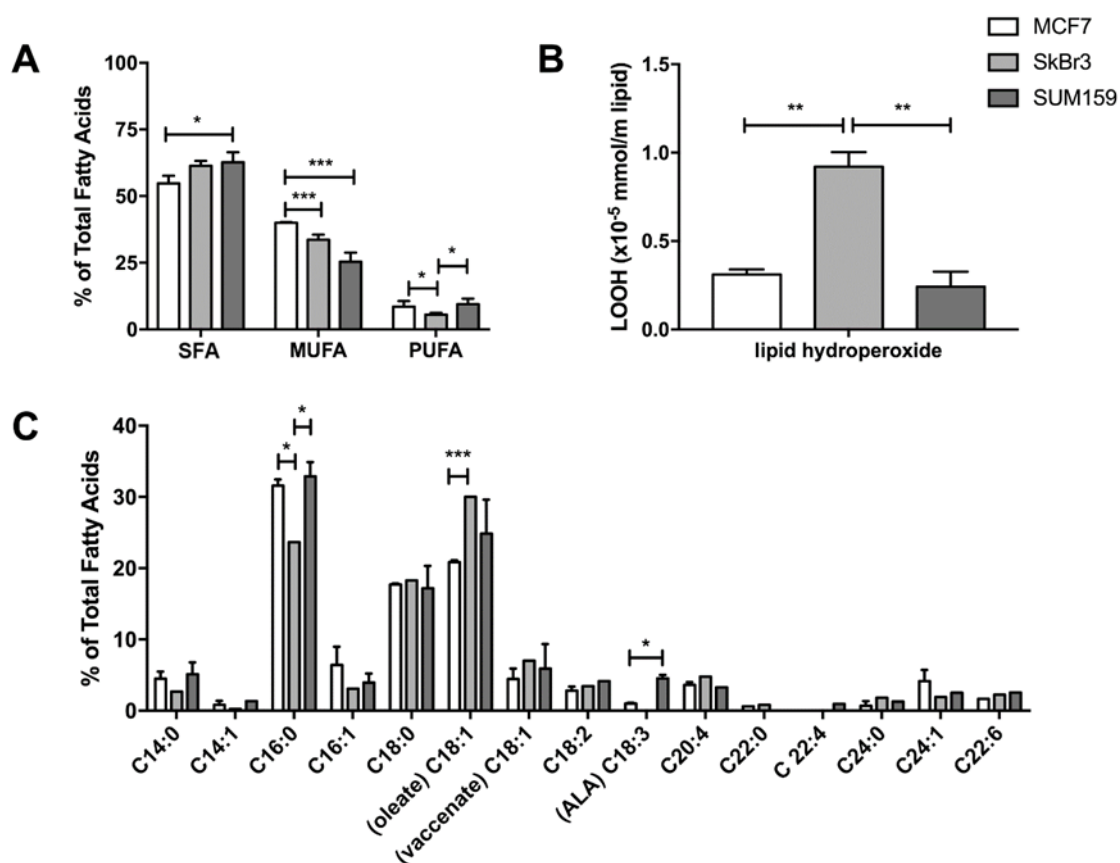


Figure III.4.1. Lipid profile and steady state lipid hydroperoxide levels. (A) Composition of total fatty acids (%) of MCF7, SkBr3 and SUM159 cells; (B) Level of lipid hydroperoxide (LOOH) in MCF7, SkBr3 and SUM159 cells; (C) Specific analysis of individual fatty acids (%) in MCF7, SkBr3 and SUM159 cells. **Abbreviation: ALA, alpha-linolenic acid.** Data are the mean $\pm$ SEM of three independent experiments. [Data was obtained by Ivana Tartaro Bujak and Branka Mihaljević].

The lipid profile showed that the MCF7 cell line has the lowest content of saturated fatty acids (SFA), while SUM159 has the highest (Fig. III.4.1.A). The content of monounsaturated fatty acids (MUFA) showed opposing tendency, while MCF7 had the highest levels, SUM159 had the lowest levels of MUFA. Although elevation of SFA indicates a potential protective mechanism against oxidative stress, due to lower levels of substrate for peroxidation, this was not accompanied by a decrease in PUFA, as there were no significant differences between MCF7 and SUM159 cells, while SkBr3 had significantly lower levels of these fatty acids ($p < 0.05$). Yet, specific analysis of individual PUFA showed a

significantly higher level of α -linolenic acid (ALA) in SUM159. ALA is an essential omega3-PUFA, which is used to synthesize eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). ALA is associated with antitumorigenic effects in mice fed with ALA rich diet [98] and was reported to be toxic for MCF7 cells causing cell cycle arrest and apoptosis through mitochondrial membrane depolarization [99]. In MCF7 cells it was shown that exogenously added ALA decreased growth via estrogen receptor-mediated signaling [100]. No doubt that in cancer cells metabolic reprogramming occurs, and is one of the hallmarks of cancer. This reprogramming also includes reprogramming of the lipid metabolism, but it is still uncertain if the changes are due to fatty acid uptake or de novo synthesis [101]. It was shown that fatty acid content changed in red blood cells membrane in cancer patients [102], indicating that fatty acid content is involved in cancer growth/development, but the precise role is not clarified. In the context of ALA, the differences between cell types of different malignant potential have never been investigated.

The slight differences observed in MUFA and PUFA content in the three tested cell lines prompted us to additionally measure their lipid hydroperoxides (LOOH). LOOH are intermediate products in peroxidation, and this is the step that can multiply the lipid peroxidation cascade. Therefore, we investigated if the slight and non-significant differences between the cell lines could be multiplied in this reaction step. These results showed that SkBr3 cells, a HER2^{NEU} positive cell line, had significantly higher content of steady state LOOH. Since this unexpected result did not correlate with the lower PUFA content in SkBr3 (Fig. III.4.1.B), we proceeded to investigate if the observed differences in fatty acid content and LOOH levels in steady state could influence cells sensitivity to H₂O₂.

4.3.2. Cell viability

Thus, we evaluated cell viability upon H_2O_2 challenge by MTT assay (Fig. III.4.2). H_2O_2 is a small molecule produced intracellularly under physiological conditions in controlled, as well as uncontrolled manner. When H_2O_2 is produced by the cell itself, in physiological conditions, it contributes to redox signaling [89].

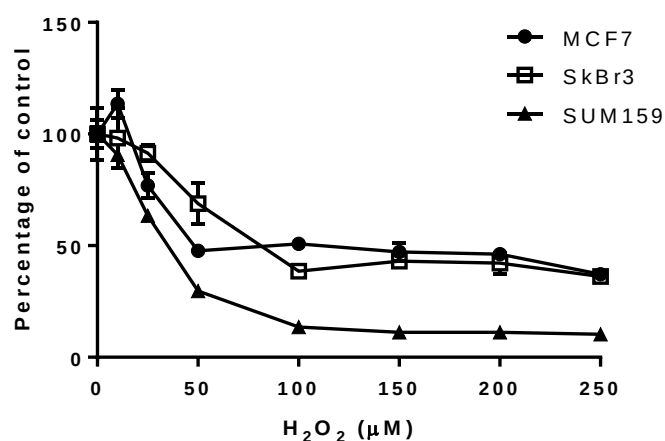


Figure III.4.2. Cell viability upon oxidative stress. Cell viability of MCF7, SkBr3 and SUM159 cell lines were determined by MTT assay after exposure to a range of H_2O_2 concentrations for 24h. Data are the mean $\pm$ SEM of three independent experiments. [Data was obtained by Ana Čipak Gašparović].

Interestingly, the hormone positive MCF7 and the HER2^{NEU} positive SkBr3 cell lines showed similar sensitivity, while the triple negative line SUM159 was more sensitive to H_2O_2 challenge. This difference in sensitivity cannot be explained by the fatty acid content analysis, where SUM159 and SkBr3 cells show similar SFA content. SFA do not contain double bonds that are substrates for ROS attack and are therefore more resistant to peroxidation. As a consequence, it is supposed that cells with higher SFA content could be more resistant to oxidative stressors. However, this is not entirely true, as evidenced by SkBr3 cell line, which had the highest LOOH content but showed higher resistance to H_2O_2 than SUM159. A possible explanation is that SkBr3 cells

may have an adequate antioxidative defense to cope with the (per)oxidation challenge.

4.3.3. Hydrogen peroxide influx and Nrf2 expression

As PUFA content and H_2O_2 sensitivity did not show correlation, we then investigated if differences in H_2O_2 sensitivity were due to differences in H_2O_2 influx between these cell lines. As the viability curves showed plateau at 100 μM H_2O_2 and above, this was the highest concentration used for influx measurements. According to the eustress theory, an extracellular concentration of 100 μM H_2O_2 corresponds to intracellular 1 μM H_2O_2 , which may trigger both effects, oxidative eustress and distress [103]. Oxidative eustress is term for positive low levels of oxidative stress, turning on adaptive mechanisms without damaging the cells. On the other hand, distress refers to higher levels of stress leading to pathological adaptation with cellular damage resulting in different diseases [103]. Which of these effects will occur in a certain cell line/type depends on the cell gene expression profile of the antioxidative defense system, but also on energy metabolism; in addition, the expression of AQPs in cell membranes fine-tuning H_2O_2 fluxes, may also dictate the cell response to oxidative stress.

Therefore, we first analyzed the levels of ROS accumulation after 60 minutes H_2O_2 treatment in the three cell lines (Fig. III.4.3.A). As expected, the most pronounced differences between ROS levels were found when cells were treated with 100 μM H_2O_2 . Since this concentration induced a significantly different level of ROS between the three cell lines and was the maximal inhibitory concentration of cell viability after 24h (Fig. III.4.2), we followed the rate of ROS accumulation after a challenge with 100 μM H_2O_2 . Interestingly, SkBr3 cells showed the highest increase in intracellular ROS, and SUM159 had the lowest, indicating that this is not the only factor that accounts or the difference in sensitivity.

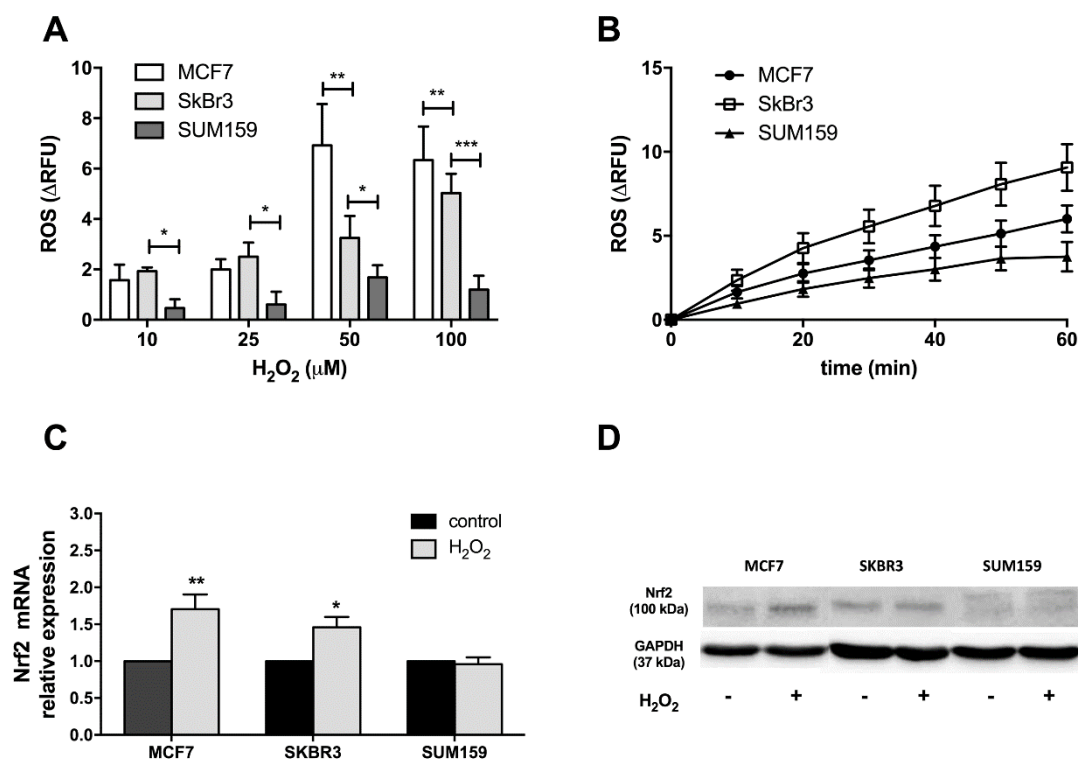


Figure III.4.3. Antioxidant defense response upon oxidative stress. (A) Endpoint of ROS intracellular accumulation. Cells were incubated with a range of H₂O₂ concentrations (10 μM to 100 μM) and ROS accumulation was recorded over 60min. Data was normalized with basal levels of intracellular ROS; (B) Representative signals of fluorescence output after addition of 100 μM H₂O₂; (C) Nrf2 gene expression of treated (100 μM H₂O₂) vs. non-treated cells, normalized to GAPDH; (D) Nrf2 protein expression of cells treated (+) and non-treated (-) with 100 μM H₂O₂ (representative blots). [Data was obtained by Ana Čipak Gašparović and Lidija Milkovic].

Considering that the antioxidative defense system may be in part responsible for cell resistance to oxidative stress, we then analyzed the expression of the antioxidant transcription factor Nrf2 under oxidative stress conditions (Fig. III.4.3.C and D). As expected, after exposure to H₂O₂ the cell lines MCF7 and SkBr3 increased their Nrf2 levels, in line with the Nrf2 role in the state of disturbed cellular redox balance [90]. On the other hand, the triple negative cell line, SUM159 had levels of Nrf2 lower than those of MCF7 and SkBr3 that were not changed upon H₂O₂ treatment. The lower levels of Nrf2 and the lack of reactivity to H₂O₂ may explain the higher sensitivity to H₂O₂ of

SUM159 cells. Nrf2 targets a variety of antioxidative defense genes including Glutamate-cysteine ligase, catalytic subunit and modifier subunit (GSH synthesis), thioredoxin reductase 1, peroxiredoxin 1, Nqo1 and glutathione-S-transferase family [104], meaning that low levels of Nrf2 consequentially lead to low levels of antioxidative defense. The failure to increase Nrf2 levels after H₂O₂ challenge may be due to inability of its steady state inhibitor Keap1 to release Nrf2 upon sensing oxidative conditions. When cells are not under oxidative stress, Nrf2 is in complex with Keap1-Cul3 –ubiquitinaseE2 complex, and is being ubiquitinated for subsequent peroxisomal degradation [90]. In stress conditions, Keap1 cystein moieties are oxidized by stressor, leading to release of Nrf2 and activation of antioxidative gene transcription. In SUM159 cells under oxidative stress, lack of Nrf2 response indicates that this pathway is probably downregulated.

4.3.4. AQP modulation under oxidative stress

In order to investigate the influence of H₂O₂ influx on the three breast cancer cell lines, the expression profile of the three AQPs isoforms most expressed in breast cancer tissues, namely AQP1, AQP5 and AQP3 [105], was investigated. The expression analysis revealed that the level of expression of these AQPs is significantly different between the three cells lines, being much higher in the HER2 positive cell line SkBr3 (Fig. III.4.4). Moreover, in all the cell lines AQP3 was the aquaporin most expressed, followed by lower levels of AQP1 and AQP5. This result is not surprising, since AQP3 has been reported as an important player in cancer biology and its expression is correlated with the epidermal growth factor receptor (EGFR) in several types of cancer [106].

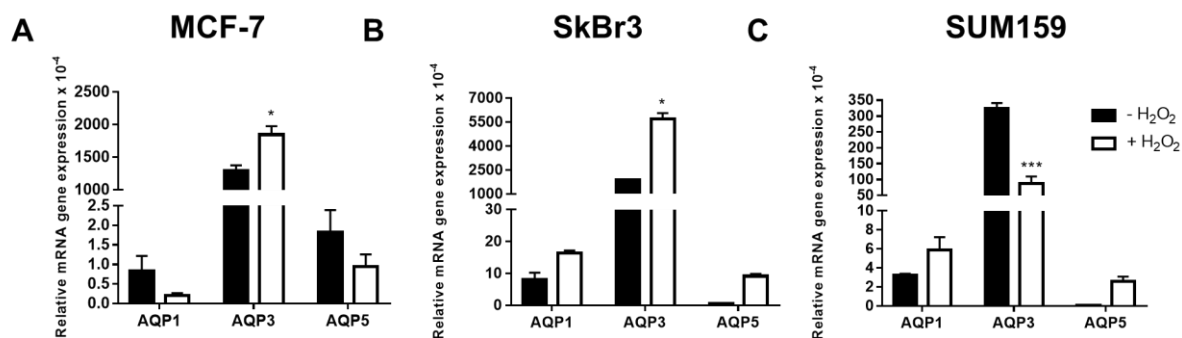


Figure III.4.4. Effect of H₂O₂ in AQP1, AQP3 and AQP5 expression. AQP1, AQP3 and AQP5 mRNA expression level **(A)** in MCF7 cell line; **(B)** SkBr3 cell line and **(C)** SUM159 cell line. The effect of H₂O₂ was achieved by culturing the cells in the presence of 100μM H₂O₂ for 24 h at 37 °C in 5% CO₂. Data represent mean ± SEM from three independent experiments. *, *p* < 0.05 and ***, *p* < 0.001 versus non-treated cells.

Interestingly, AQP3 expression was stimulated by H₂O₂ treatment in MCF7 and in SkBr3 cells, and followed the pattern of Nrf2 expression. Yet, unlike MCF7 and SkBr3 cells, in SUM159 cells H₂O₂ treatment downregulated AQP3 expression. The mechanism by which AQP3 contributes to cancer malignancy could be due to regulation of H₂O₂ influx, which is enhanced after CXCL12 stimulation in MCF7 cells [25]. A previous study with the epidermoid carcinoma cell line A-431 showed that AQP3 co-precipitated and co-localized with EGFR, and its regulation of H₂O₂ influx was imperative for EGF/EGFR signaling, including Erk and Akt activation [28]. Our results show that SkBr3, the HER2 positive cell line having high expression of EGFR, had the highest level of AQP3, in agreement with the above reported relation between EGFR and AQP3. In addition, the fact that AQP3 also transports glycerol that can be used as energy source in the glycolytic pathway or as building unit for phospholipids thus contributing to cancer growth, cannot be disregarded.

Regarding AQP1 and AQP5, while in the least malignant cell line MCF7 their expression decreased after H₂O₂ treatment, the opposite is observed for the more aggressive SkBr3 and SUM159 cells. The upregulation of AQP gene expression after H₂O₂ challenge in these particularly aggressive cell types may

reflect their role in adaptation to stress further contributing to therapy resistance.

In conclusion, breast cancer cells with different malignancy show distinct lipid steady state profiles and sensitivity to oxidative stress. Their altered pattern of aquaporin gene expression triggered by oxidative stress brings evidence to the involvement of these membrane proteins in cancer aggressiveness. The interplay of cellular antioxidative defense factors such as Nrf2 with specific AQPs in the progression of breast cancer deserves further investigation.

5. Expression of AQP1, AQP3 and AQP5 in colon cancer cell lines

5.1. Introduction

Colorectal cancer is the third prevailing cancer worldwide, and survival depends seriously on the stage of diagnosis [107]. Despite of radical surgery and chemotherapy, the recurrence and metastasis after treatment have been recognized as the leading causes of the poor outcome of colorectal cancer patients [16]. Therefore is urgent to understand the molecular mechanisms underlying colorectal cancer initiation and progression as well as identify new biomarkers and molecular targets for drug development.

Previous studies have shown that alterations of the expression and localization of AQP1, AQP3, AQP5 and AQP8 in the colon may suggest their role in tumor initiation, progression and development [108] due to their recognized physiological roles in cellular mechanisms including in cell migration, proliferation and angiogenesis, as mentioned above. In colon cancer cell lines, upregulation of AQP1, AQP3 and AQP5 was found, and expression of AQP1 and AQP5 was observed in the early stage of colorectal cancer development [17]. A study developed in human colorectal carcinoma cell line HCT116 observed that AQP3 upregulation is induced by human epidermal growth factor through the PI3K/AKT pathway and enhances colorectal cell migration [16]. Additionally, AQP3 and AQP5 expression in colorectal carcinoma were related to differentiation, lymph node and distant metastasis [14, 16, 18]. Considering these results, we can speculate that AQPs play an important role in initiating and development of carcinogenesis and can be promising drug targets and potential biomarkers for colorectal cancer.

Moreover, recent evidences showed a novel role for AQP3-mediated H_2O_2 transport in mechanism of breast cancer cell migration [25]. So far, in colon cancer cells remains to elucidate if AQP-mediated H_2O_2 can be involved

in tumorigenesis and even if H_2O_2 can regulate AQP expression to promote cell migration and proliferation. This work aimed to investigate the expression of AQP1, AQP3 and AQP5 in four colon cancer cell lines and to examine the gene regulation of AQPs upon H_2O_2 treatment.

5.2. Results

Caco2 cancer cell line shows enterocyte-like phenotype and exhibits resemblance with the barrier of the intestinal epithelium. As depicted Fig. III.5.1.A we found that AQP3 expression is 500-fold higher than AQP1 in Caco2 cells. Interestingly, when Caco2 cells were incubated with 100 μM H_2O_2 , AQP3 remained at same level of expression but AQP1 and AQP5 was significantly increased ($p < 0.05$ for AQP5). Comparing HT29 and HCT116 cells, we can observe a drastic decrease of AQP expression along with loss of differentiation. Interestingly, of four colon cancer cell lines tested, moderately differentiated HT29 cells showed higher expression of AQP5 than AQP3. Since, AQP3 is the isoform most overexpressed in all types of cancer, this result suggests that AQP5 can be used as potential specific biomarker for early colon cancer prognosis, although further analysis in different cancer tissues is required. HCT116 cells have a strong expression of epithelial-mesenchymal transition (EMT) signature and cells are poorly differentiated. When incubated with 100 μM H_2O_2 , these cells showed a significant increase of AQP expression ($p < 0.01$ for AQP1, $p < 0.005$ for AQP3). These data suggest a potential regulation mechanism of AQPs under oxidative stress on poorly differentiated cells that can contribute to cell proliferation and invasion. Finally, SW620 cells are similar to HCT116 cells in relation to EMT signature and aggressiveness. SW620 cells have higher level of AQP expression than HCT116 cells, which can be explained by metastatic potential displayed by SW620 cells enhanced by overexpression of AQPs. A similar trend was observed when SW620 cells were incubated with 100 μM H_2O_2 although with no statistically significant difference.

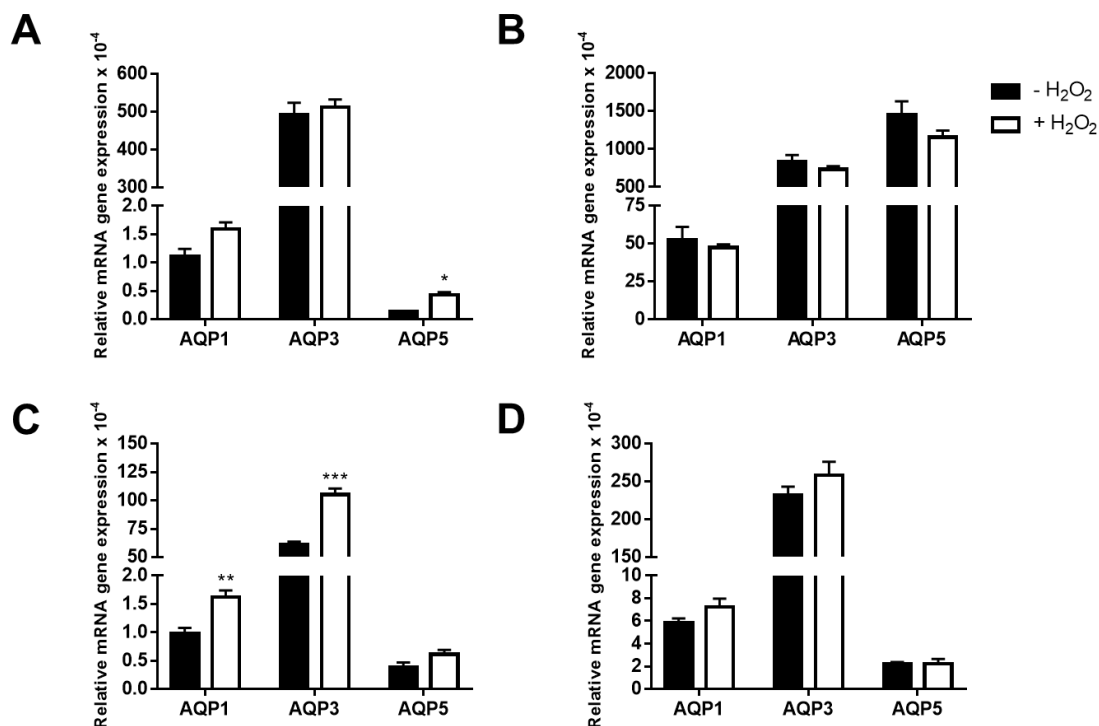


Figure III.5.1. Effect of H₂O₂ on AQP expression in colon cancer cell lines. AQP1, AQP3 and AQP5 mRNA expression level in (A) Caco2 cell line; (B) HT29 cell line; (C) HCT116 cell line and (D) SW620 cell line. The effect of H₂O₂ was achieved by culturing the cells in the presence of 100μM H₂O₂ for 24 h 37°C in 5% CO₂. Data represent mean ± SEM from three independent experiments. Significance levels: * $p < 0.05$; ** $p < 0.01$ and ***, $p < 0.001$ versus non-treated cells.

These results suggest a potential regulation mechanism of AQPs at the level of mRNA transcription under oxidative stress conditions. However, these data need to be further investigated to infer AQP importance on colon cancer progression and invasion.

6. Expression of AQP1, AQP3 and AQP5 in human pancreatic

ductal adenocarcinoma biopsies

6.1. Introduction

Pancreatic cancer is the seventh leading cause of cancer mortality in the world characterized by poor prognosis and high aggressiveness. Therefore, is imperative to find new biomarkers and potential drug targets as well as elucidate the molecular mechanism of pancreatic tumor progression.

It is still to be unveiled the underlying molecular mechanism of AQPs in pancreatic cancer initiation and progression. However, our group revealed that AQP3 and AQP5 are overexpressed in human pancreatic ductal adenocarcinoma tissues [20]. AQP3 was detected at the plasma membrane of ductal cells while AQP5 was observed in the apical membrane of intercalated and intralobular ductal as well as in infiltrative cancer cells in the duodenum [20]. The results suggested that AQP3 and AQP5 are important for pancreatic carcinogenesis. While AQP3 may induce cancer cell migration and proliferation by glycerol uptake, AQP5 may induce cell migration by facilitating water permeation at the edge of cancer cells. Interestingly, both AQPs are able to transport H_2O_2 that can also activate signaling pathways related to migration and proliferation, like PI3K and ERK signaling pathways [30]. Pancreatic cancer is one of the most aggressive tumor types with poor prognosis and the overall 5-year survival rate is around 7%. Our study revealed that AQP5 was a sensitive and specific marker for early stages of pancreatic adenocarcinoma while AQP3 expression may be related with late and more aggressive stages of the disease [20].

Therefore, here we aimed to investigate the mRNA transcription level of AQP1, AQP3 and AQP5 in tumor biopsies surgically removed from pancreatic cancer patients.

6.2. Results

We evaluated the expression of AQP1, AQP3 and AQP5 in pancreatic cancer biopsies from 23 patients and compared to matched adjacent non-neoplastic samples (Table III.6.1.). The samples obtained are very heterogeneous regarding tumor type. However, we obtained samples from eight patients with pancreatic ductal adenocarcinoma (PDA) with different degrees of differentiation that correlate with tumor aggressiveness (Patient 3, 6, 11, 14, 15, 17, 20, and 21). Moderately differentiated PDA samples did not show any alteration in AQP expression. However, loss of PDA differentiation shows an increase in AQP5 expression and a marked increase in AQP1 and AQP3 expression.

Although these data strongly suggest a potential role for AQPs in pancreatic cancer prognosis, a larger number of samples is required to achieve a statistically significant difference. In addition, it should be interesting to evaluate markers of cell proliferation and tumorigenesis that may correlate with level of AQP expression.

Table III.6.1. AQP expression in pancreas tumor samples in relation to matched adjacent non-neoplastic samples. Data represent mean $\pm$ SEM from three replicate experiments. **Abbreviations:** F, female; M, male; IPMN, intraductal papillary-mucinous; AQP, aquaporin; **NA: not available; Expression level (EL) score:** 0, no difference; 1, weak expression; 2, moderate expression and 3, strong expression.

Patient	Gender	Tumor type	Histological differentiation	Survival (months)	AQP1	AQP3	AQP5
1	M	Ampullary	NA	73,6	0	0	0
2	M	Renal tumor pancreatic metastasis	NA	72,5	2	2	0
3	F	Ductal adenocarcinoma	Poorly	72,9	2	2	1
4	M	Ampullary	Moderately	73,4	0	0	0
5	M	Distal Cholangiocarcinoma	Moderately	19,7	0	0	0
6	M	Ductal adenocarcinoma	Poorly	11,4	2	2	1
8	F	IPMN (noninvasive)	NA	72,9	3	0	3
9	F	IPMN (invasive)	NA	72,6	0	0	0
10	F	Pancreatic endocrine neoplasia	NA	56,6	2	3	0
11	F	Ductal adenocarcinoma	Moderately	57,1	0	0	0
12	M	Distal Cholangiocarcinoma	Moderately	5,3	0	0	0
13	F	IPMN (noninvasive)	NA	71,7	0	0	0
14	F	Ductal adenocarcinoma	Moderately	15,9	0	0	0
15	M	Ductal adenocarcinoma	NA	71,5	0	0	0
16	F	Ampullary	Moderately	35,0	1	2	1
17	F	Ductal adenocarcinoma	Poorly	3,1	1	2	0
18	M	Ampullary	Moderately	71,0	0	0	0
19	M	Pancreatic neuroendocrine tumors	NA	69,9	2	2	1

Patient	Gender	Tumor Type	Histological differentiation	Survival (months)	AQP1	AQP3	AQP5
20	M	Ductal adenocarcinoma	Poorly	8,9	2	2	1
21	F	Ductal adenocarcinoma	Moderately	7,2	1	1	2
22	M	Distal Cholangiocarcinoma	Moderately	69,6	1	1	2
23	F	IPMN (invasive)	Highly	61,8	3	2	1

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Chapter Four ||

General conclusions and future perspectives

Aquaporins contribution to organs/cells homeostasis relies on their undisputable role in permeating water and solutes across cell membranes, as well as on unexpected functions that are now gradually becoming known. Progressively, new insights into the molecular mechanisms responsible for AQP expression, trafficking and function enlighten their physiological role in cellular processes including renal water absorption, exocrine fluid secretion, epidermal hydration, neuro-homeostasis and fat metabolism. Mutations and/or abnormal expression of AQPs have been associated with human chronic diseases like nephrogenic diabetes insipidus, epilepsy and cancer, among others. Regarding cancer, recent studies showed an irregular level expression of AQPs and their participation in pathological mechanisms like cell volume regulation, energy metabolism, cell migration, adhesion, proliferation and differentiation. In addition, it is well known that cancer cells are frequently characterized by increased levels of ROS related to a constitutive activation of signaling pathways and hence influencing a wide variety of cellular pathways linked to proliferation, differentiation, tissue repair and inflammation. Hydrogen peroxide is a key signaling mediator in redox biology and recent evidences showed that AQP3-mediated H_2O_2 is essential for breast cancer cell migration. Emerging evidences showed that not only permeation of water and glycerol but also hydrogen peroxide through AQPs play an important role in tumor initiation, progression and development as well as represent a novel therapeutic targets and prognostic biomarkers for cancer treatment.

In the first part of this thesis (Subchapter 2.3), we hypothesized that the accumulation of lipid peroxidation product HNE could affect aquaporin function with an outcome in oxidative stress response. Peroxidation of polyunsaturated fatty acids (PUFAs) results in the production of great variety of reactive aldehydes, including HNE that play an active role in cellular signaling. HNE activates most of the pathways triggered by oxidative stress, by binding to redox sensitive molecules and HNE-protein adducts were

detected in numerous pathologies like cancer. Therefore, we used two yeast models with increased levels of membrane PUFAs and yeast aquaporin AQY1 overexpression to evaluate their interplay in cell's oxidative status. Our data showed that increased levels of membrane PUFAs triggered AQY1 expression, and its activity is impaired by HNE. Moreover, AQY1 expression increased cellular sensitivity to oxidative stress by facilitating H₂O₂ influx and had no effect on the cellular antioxidant GSH levels and catalase activity. These results strongly suggest that aquaporins are important players in oxidative stress response and could contribute to regulation of cellular processes by regulation of H₂O₂ influx.

Then, the work developed contributed to expand the knowledge concerning AQP5 function and physiological effect in tumorigenesis as well as unveil potential regulatory mechanisms (Subchapter 2.4 and 2.5). First, we aimed at characterizing the function of mammalian (rat and human) AQP5 as potential H₂O₂ channel. In addition, we also investigated the role of AQP5 in resistance to oxidative stress and the importance AQP5-mediated H₂O₂ transport in cancer cell migration. For this purpose, we used a yeast heterologous expression system silenced on endogenous AQPs to characterize AQP function and to ascertain regulation mechanisms. The yeast *Saccharomyces cerevisiae* is a widely used eukaryotic model to study proteins in general due to the broad library of strains available, their easy and inexpensive genetic manipulation when compared to mammalian cell cultures. Beyond these advantages, yeast models possess high functional homology to higher eukaryotes including mammals and can endure severe external environments that allows setting several experimental conditions that could not be applied to mammalian cells. Using this approach, we demonstrated that PKA phosphorylation significantly increases AQP5 permeability and this activation is dependent on pH. Moreover, a gating mechanism regulating human AQP5 activity has been proposed by molecular dynamics simulations

in which it is suggested that AQP5 channel could change between a closed and open state, and when it is open can exhibit a wide and narrow conformation decided by the side chain orientation of His173 and the proximity to Ser183. Site-directed mutation of these residues markedly altered the water transport activity validating the gating mechanism proposed by molecular dynamics simulations for AQP5. Our *in vitro* studies suggest a proximity of S183 to H173, mainly supported by the negative charge of phosphate group that restricts water permeability. In addition, we showed for the first time that both rat and human AQP5 could function as peroxiporins facilitating bidirectional transmembrane H_2O_2 transport. Moreover, we observed AQP5-mediated transport in pancreatic cancer cells (BxPC3). AQP5 expression rendered the yeast cells more resistant to short-term oxidative stress and AQP5-facilitated H_2O_2 transport promoted cancer cell migration. It was already reported that AQP5 plays an important role in tumor migration, proliferation and metastasis. Our results elucidate the underlying mechanisms by which AQP5 enhances cell migration and propose a functional involvement of peroxiporins in dynamic fine-tuning of intracellular H_2O_2 and activation of signaling pathways related to cell growth and proliferation.

In the second part of the work, we evaluated a potential regulation mechanism of AQPs at the level of mRNA transcription in oxidative stress conditions. AQP1, AQP3 and AQP5 are found overexpressed in several tumor types and play important roles in tumor biology. Since it is now known that AQP-facilitated H_2O_2 transport contributes to breast and pancreatic cancer cell migration, we used breast and colon cancer cells to screen the expression profile of AQP1, AQP3 and AQP5 and regulation of their expression in oxidative stress conditions. Finally, biopsies from normal and tumoral tissues were analyzed aiming to correlate *in situ* AQP expression levels with disease stage and tumor aggressiveness to unveil their potential as new pancreatic cancer biomarkers.

The results obtained with cancer cell lines point to a possible regulation mechanism at the level of mRNA transcription in oxidative stress conditions and implies their role in adaptation to stress, which could further lead to therapy resistance. Yet, it remains to elucidate if AQP upregulation in oxidative stress conditions improves H₂O₂ uptake and examine which are the signaling pathways that are activated in such conditions responsible for cell survival, growth and proliferation. The results obtained with tumor biopsies revealed a different pattern of AQP1, AQP3 and AQP5 profiles according to the histological differentiation of pancreatic ductal adenocarcinoma and strongly suggest their potential as prognostic biomarkers for pancreatic cancer. However, a larger number of samples are required to achieve a statistically significant difference and the variation of AQP expression should be further correlated with markers of cell proliferation and tumorigenesis.

Although some particular questions remained to be addressed, at the end of the work here described and discussed we gave new evidences of the yeast AQY1 interplay with cellular peroxidation products, and of AQP5 function as peroxiporin and its physiological impact in cancer cell migration and proliferation. In addition, two distinct regulation mechanisms were described in this work: a gating mechanism for AQP5 function, and a regulatory mechanism at mRNA level depending on oxidative stress status in breast and colon cancer cells. Finally, we showed that AQP1, AQP3 and AQP5 may be potential biomarkers for pancreatic ductal adenocarcinoma diagnosis and prognosis.

Overall, this thesis provides new insights on the importance of AQPs function and regulation for cancer progression and development. Based on these results, future studies investigating activation of signaling pathways dependent on AQP-mediated H₂O₂ transport may help elucidate the molecular mechanism by which AQPs participate in cancer biology.

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