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Specific aquaporins facilitate Nox-produced hydrogen peroxide transport through plasma membrane in leukaemia cells

Francesco Vieceli Dalla Sega [a,*], Laura Zambonin [a], Diana Fiorentini [a], Benedetta Rizzo [b], Cristiana Caliceti [c], Laura Landi, Silvana Hrelia [b,1], Cecilia Prata [a,1]

[a] Department of Pharmacy and Biotechnology, University of Bologna, Italy
[b] Department for Life Quality Studies, University of Bologna, Italy
[c] Department of Cardiology and Laboratory for Technologies of Advanced Therapies (LTTA Center), University Hospital of Ferrara and Maria Cecilia Hospital, GVM Care&Research, E.S: Health Science Foundation, Cotignola, Italy

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ABSTRACT

In the last decade, the generation and the role of reactive oxygen species (ROS), particularly hydrogen peroxide, in cell signalling transduction pathways have been intensively studied, and it is now clear that an increase of ROS level affects cellular growth and proliferation pathways related to cancer development. Hydrogen peroxide (H_2O_2) has been long thought to permeate biological membranes by simple diffusion since recent evidence challenged this notion disclosing the role of aquaporin water channels (AQP) in mediating H_2O_2 transport across plasma membranes. We previously demonstrated that NAD(P)H oxidase (Nox)-generated ROS sustain glucose uptake and cellular proliferation in leukaemia cells. The aim of this study was to assess whether specific AQP isoforms can channel Nox-produced H_2O_2 across the plasma membrane of leukaemia cells affecting downstream pathways linked to cell proliferation. In this work, we demonstrate that AQP inhibition caused a decrease in intracellular ROS accumulation in leukaemia cells both when H_2O_2 was produced by Nox enzymes and when it was exogenously added. Furthermore, AQP8 overexpression or silencing resulted to modulate VEGF capacity of triggering an H_2O_2 intracellular level increase or decrease, respectively. Finally, we report that AQP8 is capable of increasing H_2O_2-induced phosphorylation of both PI3K and p38 MAPK and that AQP8 expression affected positively cell proliferation. Taken together, the results here reported indicate that AQP8 is able to modulate H_2O_2 transport through the plasma membrane affecting redox signalling linked to leukaemia cell proliferation.

1. Introduction

Reactive oxygen species (ROS) can act as messengers in cell signalling pathways, and it is now firmly established that an increase in intracellular ROS level supports growth and proliferation, contributing to cancer development [1–3]. NAD(P)H oxidases (Nox) are a family of membrane-associated enzymatic complexes that transport electrons across membranes. Members of the Nox family generate ROS by transferring electrons from NAD(P)H inside the cell to molecular oxygen across the membrane resulting in the generation of superoxide anion or hydrogen peroxide (H_2O_2) outside the cells [4]. Among ROS, H_2O_2 is thought to be the main effectors in redox signalling due to its relative stability and capacity to diffuse from the sources of production. Over the last decade, H_2O_2 enzymatic production and downstream targets have been intensely studied [5]. Nevertheless, is still poorly understood how H_2O_2 production is spatially and temporally regulated to permit redox signalling over oxidative stress pathways. In fact, H_2O_2 has been long thought to permeate biological membranes by simple diffusion, since recent evidence challenged this notion disclosing the role of aquaporin water channels (AQP) in mediating H_2O_2 transport across the plasma membrane [6]. AQP are a family of integral membrane proteins, found in all domains of life, that form pores appearing to be designed for the selective passage of water and glycerol [7]. Seminal studies have shown that specific AQP isoforms are capable of funnelling H_2O_2 across the plasma membrane when expressed in yeast or plant cells [8,9]. Furthermore, it has been recently reported that AQP1 is able to channel H_2O_2 in the rat smooth muscle cell line rASMC mediating downstream redox pathways [10]. It has also been shown that AQP3 and AQP8 can modulate H_2O_2 intracellular level and are engaged in H_2O_2-mediated signalling in several human cell lines such as HEK 239, HT29 and HeLa [11].

Recently, some AQP isoforms have also been studied for the ability to promote cell migration and proliferation and are emerging as new effectors in cancer biology [12,13]. Interestingly, AQP1 and AQP3 appear to be involved in cancer proliferation: AQP1 facilitates tumour angiogenesis and endothelial cell migration [14]; AQP3 is implicated in skin tumourigenesis and in colorectal carcinoma cell migration [15,16].

* Corresponding author at: Department of Pharmacy and Biotechnology, Alma Mater Studiorum, University of Bologna, Via Irnerio, 48 – 40126 Bologna, Italy. Tel.: +39 051 2091222; fax: +39 051 2091234.
E-mail address: francesco.vieceli2@unibo.it (F. Vieceli Dalla Sega).
[1] These authors contributed equally.

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However, the proposed mechanisms do not contemplate a role played by AQP in H_2O_2 transport. Intriguingly, the three AQP isoforms reported to channel H_2O_2 are also widely expressed in blood cells: AQP1 was discovered in the erythrocyte plasma membrane representing one of the most abundant proteins [7]; AQP3 is an aquaglyceroporin expressed in lymphocytes and erythrocytes. Both AQP1 and AQP3 expression in red cells have been exploited for the determination of blood group: AQP1 is the antigen of the Colton antigen system [17], whereas AQP3 is the antigen of the Gill blood group [18]. Moreover, it has been recently shown that AQP3 and AQP8 are expressed in primitive erythroid cells, while AQP1 and AQP9 are specifically expressed in adult mouse erythroid cells [19].

We have previously reported that Nox enzymes are a major source of ROS in various leukaemia cell lines and that Nox-derived ROS are required for proliferation of acute leukaemia cells being directly involved in maintaining the high glucose uptake essential for cellular growth [20,21]. In particular, we demonstrated that Nox-derived ROS are involved in early signalling events such as the autophosphorylation of VEGF receptor-2 and in the modulation of glucose uptake mediated by Glut1, a cellular activity strictly bound to VEGF-induced leukaemia cell proliferation [22]. Therefore, the aim of the present study was to assess whether AQP isoforms are a potential way through which H_2O_2, produced by Nox enzymes, is channelled across plasma membrane to act as signal molecule contributing to the aberrant proliferation of leukaemia cells. As an *in vitro* model, the human acute leukaemia B1647 cell line, constitutively VEGF producing [23], was chosen. The attention was focused on AQP1, AQP3 and AQP8 owing to their reported capacity to channel H_2O_2 and their broad expression in blood cells.

2. Material and methods

2.1. Reagents

Hank's Balanced Salt Solution (HBSS), H_2O_2, $AgNO_3$, phloretin, 2,7-dichlorodihydrofluorescin diacetate (DCFH-DA), diphenyleneiodonium chloride (DPI), 2-deoxy-glucose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue, Igepal CA-630, sodium orthovanadate, phenylmethanesulfonyl fluoride (PMSF), N-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), protease inhibitor cocktail, Laemmli sample buffer containing 2-mercaptoethanol, were obtained from Sigma–Aldrich (USA). Human serum, penicillin/streptomycin, were purchased from PAA (PAA Laboratories GmbH, Cölbe. Germany). Nitrocellulose membranes and Amersham ECL Plus Western Blotting Detection Reagents were from GE-Healthcare. Anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase were obtained from Sigma–Aldrich. PageRuler Prestained protein ladder was from Fermentas-Thermo Fisher Scientific (Rockford, USA). Primary antibodies: anti-AQP1 (# TA502357—Origene), anti-AQP3 (# SC20811—Santa Cruz), anti-AQP8 (# WH0000343—Sigma–Aldrich), anti phospho-PI3 kinase (# PA5-17387—Thermo Scientific), anti-PI3 kinase (# 06–195—Millipore), anti-phospho-p38-MAP kinase (# 9211—Cell Signalling), anti-p-38-MAP kinase (# 9212—Cell Signalling), anti-ß-actin (# A5441—Sigma–Aldrich). Peroxyfluor-1 (PF1) was synthesized according to [24] and gently provided by Stefano Lena (University of Bologna).

All the other chemicals and solvents were of the highest analytical grade.

2.2. Cell culture

B1647 erythro-megakaryocytic cell line, established from bone marrow of a patient with acute myelogenous leukaemia, is maintained in IMDM supplemented with 5% heat-inactivated human serum (HS) as previously reported [20]. Cells were cultured in a humidified 37 °C, 5% CO_2 incubator.

2.3. Viability assays

Cell viability was evaluated by trypan blue exclusion test and/or by MTT assay. The reduction of tetrazolium salts is, indeed, widely accepted as a reliable way to examine cell viability/proliferation [25]. Cells (2×10^4) were incubated with 0.5 mg/mL MTT for 2 h at 37 °C. At the end of the incubation, purple formazan salt crystals were formed and dissolved by adding the solubilization solution (10% SDS, 0.01 M HCl), then the plates were incubated overnight in humidified atmosphere (37 °C, 5% CO_2). The absorbance at 570 nm was measured in a multiwell plate reader (Wallac Victor^2, Perkin-Elmer).

2.4. Measurement of intracellular ROS levels

To evaluate intracellular ROS levels, 1×10^6 cells/mL were washed twice in HBSS and incubated with 5 μM DCFH-DA for 20 min at 37 °C. DCFH-DA is a small nonpolar and nonfluorescent molecule that diffuses into the cells, where it is enzymatically deacetylated by intracellular esterases to a polar nonfluorescent compound, that is oxidized to the highly green fluorescent 2,7-dichlorofluorescein (DCF). DCF fluorescence was measured using a multiwell plate reader (Wallac Victor2, PerkinElmer) at excitation and emission wavelengths of 485 nm and 535 nm, respectively [26]. For the PF1 assay, cells (1×10^6 cells/mL) were washed twice in HBSS and incubated with 10 μM PF1 for 20 min at 37 °C, in the dark. Then, the cells were washed three times in HBSS and pre-treated with 5 μM $AgNO_3$; after 10 min, 100 μM H_2O_2 was added to the cells, and fluorescence of the oxidized probe was measured after 10 min on a multiwell plate reader (Wallac Victor^2, Perkin Elmer) at excitation and emission of 485 nm and 535 nm, respectively.

2.5. Glucose transport assay

Cells were suspended in PBS in the presence or absence of 5 μM $AgNO_3$, and glucose uptake was assayed as previously reported [22]. Briefly, 0.5 mL cell suspension (2×10^6 cells) in PBS buffer, pH 7.2 (glucose-free), was treated with 2-deoxy-D-[2,6-^{3}H] glucose (15 kBq/assay) and 1 mM unlabeled 2-deoxy-D-glucose. After 1 min incubation at 37 °C, the uptake was stopped by adding phloretin (0.3 mM final concentration). The uptake was linear up to 3 min. Transported 2-deoxy-D-glucose was <20% of the extracellular sugar; therefore, glucose transport assay could be considered in zero-trans conditions [27]. Sample radioactivity was measured by liquid scintillation counting.

2.6. Western blotting analysis of AQP isoforms

Cells were lysed in an ice-cold lysis buffer (1% Igepal, 150 mM NaCl, 50 mM Tris–Cl, 5 mM EDTA, 0.1 mM PMSF, 0.1 mM TLCK, 0.1 mM TPCK, 1 mM orthovanadate and protease inhibitor cocktail, pH 8.0) for 15 min, and then the lysates were centrifugated at 12,000 ×*g* for 15 min in a cooled centrifuge. The protein concentration of the cleared lysates was determined by the Bio-Rad Bradford protein assay (Bio-Rad Laboratories s.r.l., Milan, Italy). Proteins (10 μg *per* lane) were electrophoretically separated on 10% SDS-polyacrylamide gel using a Mini-Protean II apparatus (Bio-Rad Laboratories) and transferred to supported nitrocellulose membrane at 100 V for 90 min. Non-specific binding to membrane was blocked incubating in Tris-Buffered Saline (TBS)/Tween, pH 8.0, containing 5% non-fat dried milk for 1 h at room temperature. Then, the nitrocellulose membranes were incubated overnight at 4 °C with primary antibodies (anti-AQP1, AQP3, AQP8, P-PI3K, total PI3K, P-p38 MAPK or total p38 MAPK). Blots were washed with TBS/Tween and incubated for 45 min at room temperature with HRP-conjugated secondary antibodies in TBS/Tween containing 5% non-fat dried milk. Membranes were washed with TBS/Tween and developed using Western Blotting Luminol Reagent. Image acquisition and relative densitometric analysis were performed by Image Lab software (Bio-Rad Laboratories).

2.7. Cell transfection

B1647 cells were nucleofected with Cell Line Nucleofector™ Kit V (Amaxa Biosystems, Cologne, Germany) with Program T-019 following the manufacturer's instructions; plasmid vectors pCMV6-XL4 or pCMV6-XL5 for the overexpression of AQP1 (NM_198098), AQP3 (NM_004925) and AQP8 (NM_001169) were obtained from Origene (Rockville, USA). siRNA against AQP3 (Duplex seguence: GAGCAGAU CUGAGUGGGCA[dT][dT]; UGCCCACUCAGAUCUGCUC[dT][dT]), AQP8 (Duplex seguence: CUGCUCAUUAGGUGCUUCA[dT][dT]; UGAAGCAC CUAAUGAGCAG[dT][dT]) and scrambled were obtained from Sigma-Aldrich (USA). Overexpression was accomplished with 1 μg of plasmidic DNA; RNA silencing was obtained with 300 nm siRNA.

Subsequently, cells were immediately suspended in complete medium and incubated in a humidified 37 °C/5% CO_2 incubator. After 24 h or 48 h, cells were used for the experiments: evaluation of AQP1, AQP3, and AQP8 expression by Western blotting, transfection efficiency and cell viability assays.

2.8. RT-PCR

Total RNA extraction was performed using RNA MiniPrep kit (Zymo Research, Freiburg, Germany) according to the manufacturer's recommendations. After extraction, the RNA concentration was determined with NanoVue spectrophotometer (GE HealthCare). Retrotranscription was executed with Promega I-script cDNA kit (Promega, Madison, USA). RNA amplification was performed with Intron I-taq DNA polymerase (Intron Bioetechnology, Korea). PCR products were amplified by using specific primers (Origene): AQP3 (Forward Sequence: CCGTGACCTTTGCCATGTGCTT; Reverse Sequence: TTGTCG GCGAAGTGCCAGATTG; amplicon of 145 pb) or AQP8 (Forward Sequence: TTCTCCATCGGCTTTGCCGTCA; Reverse Sequence: CAGCCAGT AGATCCAGTGGAAG; amplicon of 135 pb). RT-PCR was carried out using Access RT-PCR Systems (Promega).The reaction mixtures were kept for 45 min at 45 °C, 2 min at 94 °C, then cycled 35 times through a programme of 30 s at 94 °C, 1 min at 56 °C and 1 min at 72 °C; finally, the reaction was incubated for an extra 7 min at 68 °C. After RT-PCR, the DNA products were electrophoresed on 2% agarose gel and stained with GelRed (Biotium, Hayward, USA).

2.9. Cell proliferation assay

24 h after transfection, 2×10^5 cells/mL were washed twice in HBSS and incubated with 100 μM H_2O_2 for 30 min at 37 °C. Cells were then washed and maintained in IMDM supplemented with 5% heat-inactivated HS. After 24 h from the treatment with H_2O_2, cell count and viability were determined by Guava EasyCyte Mini flow cytometry (Guava Technologies, USA) with Guava ViaCount reagent (Guava Technologies), according to the manufacturer's recommendations, as previously reported [28]. Briefly, cells were mixed with an adequate volume of Guava ViaCount Reagent and allowed to stain for at least 5 min at room temperature. The Guava ViaCount Reagent provides absolute cell count and viability data based on the differential permeability of DNA-binding dyes and the analysis of forward scatter. The fluorescence of each dye is resolved operationally to allow the quantitative assessment of both viable and non-viable cells present in a suspension.

2.10. Statistical analysis

Each experiment was performed at least three times, and all values are represented as means ± SD. One-way ANOVA was used to compare differences among groups followed by Dunnett's or Bonferroni's test (Prism 5; GraphPad Software, USA). Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Effect of aquaporin inhibition on intracellular ROS level in leukaemia cells

To investigate a possible role exerted by natural levels of AQP in H_2O_2 transport, we evaluated the intracellular ROS level following AQP inhibition in the acute leukaemia cell line B1647. Silver ion is a potent inhibitor of aquaporins that acts by binding to cysteine or histidine residues in the region of the pore [29,30]. Preliminary experiments, such as MTT assay and trypan blue exclusion test, showed that treatment with $AgNO_3$ did not affect B1647 cell viability up to 10 μM for 2 h (data not shown). Thus, cells were treated with 5 μM $AgNO_3$ for 10 min and then exposed to 10 μM or 100 μM H_2O_2. Intracellular ROS level was evaluated by DCF fluorescence measurement. As shown in Fig. 1A, a significant fluorescence increase was observed when 10 μM or 100 μM H_2O_2 was added; cells pre-incubated with AQP inhibitor exhibited a 35% and 19% decrease in fluorescence compared to the cells spiked with H_2O_2 10 μM or 100 μM, respectively. Interestingly, cells treated with $AgNO_3$ also exhibited a 22% decrease in the basal intracellular ROS level compared to control cells (Fig. 1A). The role of extracellular H_2O_2 in triggering DCF fluorescence is confirmed by the observation that the treatment with extracellular catalase (5 mg/mL) abrogated the effect of AQP inhibition on the intracellular ROS level. In order to confirm that intracellular ROS level is mainly due to H_2O_2,

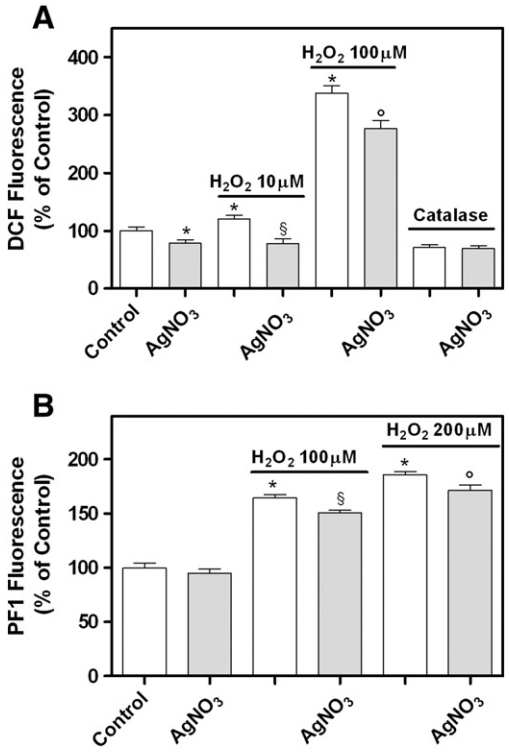

Fig. 1. Effect of $AgNO_3$ on intracellular ROS level after treatment with two different concentrations of H_2O_2 in B1647 cells. (A) Cells were incubated with 5 μM $AgNO_3$ and then treated with 10 or 100 μM H_2O_2. Intracellular ROS level was evaluated as DCF fluorescence as reported in Material and methods. In the experiment with 5 mg /mL catalase, the enzyme was added prior to $AgNO_3$ treatment. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: $^*p < 0.05$ significantly different from Control; $^§p < 0.05$ significantly different from cells treated with 10 μM H_2O_2; $^°p < 0.05$ significantly different from cells treated with 100 μM H_2O_2. (B) Cells were incubated with 5 μM $AgNO_3$ and then treated with 100 or 200 μM H_2O_2. Intracellular ROS level was evaluated as PF1 fluorescence as reported in Material and methods. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: $^*p < 0.05$ significantly different from Control; $^§P < 0.05$ significantly different from cells treated with 100 μM H_2O_2; $^°p < 0.05$ significantly different from cells treated with 200 μM H_2O_2.

H_2O_2 level was also measured by using PF1, a fluorescent boronate dye selective for H_2O_2 over similar ROS such as superoxide anion and hypochlorite ion [31]. After the treatment with $AgNO_3$, cells were incubated with two different H_2O_2 concentrations. As shown in Fig. 1B, a PF1 fluorescence increase of 64% and 86% compared to control cells was observed when 100 μM or 200 μM H_2O_2 was added; in contrast, following pre-incubation with AQP inhibitor, the intracellular H_2O_2 level increase was only 50% and 71% of the control cells, respectively. Unfortunately, although PF1 is a useful tool owing to its selectivity towards H_2O_2, it is not sensitive enough to evaluate possible changes in basal intracellular H_2O_2 level [32].

3.2. Correlation between Nox-generated ROS and AQPs in leukaemia cells

To investigate the possible role of Nox as a source of ROS putatively transported by AQPs in basal conditions, B1647 cells were pre-incubated with the Nox inhibitor DPI and then treated with $AgNO_3$. Intracellular ROS level was evaluated spectrofluorimetrically by means of DCFH-DA. As shown in Fig. 2A, intracellular ROS level decreased, as expected, after pre-treatment with 10 μM DPI for 30 min. Interestingly, both $AgNO_3$ and DPI treatment decreased intracellular ROS level, but their effect was not additive. We have previously demonstrated that Nox-derived ROS are required for cell proliferation being directly involved in maintaining the high glucose uptake essential for cellular growth; in particular, DPI treatment caused a decrease of the glucose uptake rate [22]. Here, the effect of AQP inhibition on glucose transport in B1647 cells was assessed measuring this uptake by means of a radioisotopic assay, following a 10 min treatment with 5 μM $AgNO_3$ (Fig. 2B). $AgNO_3$ caused a 32% decrease in glucose transport compared to the control cells. In agreement with data on intracellular ROS level, the simultaneous treatment with DPI and $AgNO_3$ did not cause an addictive effect on glucose uptake (Fig. 2B).

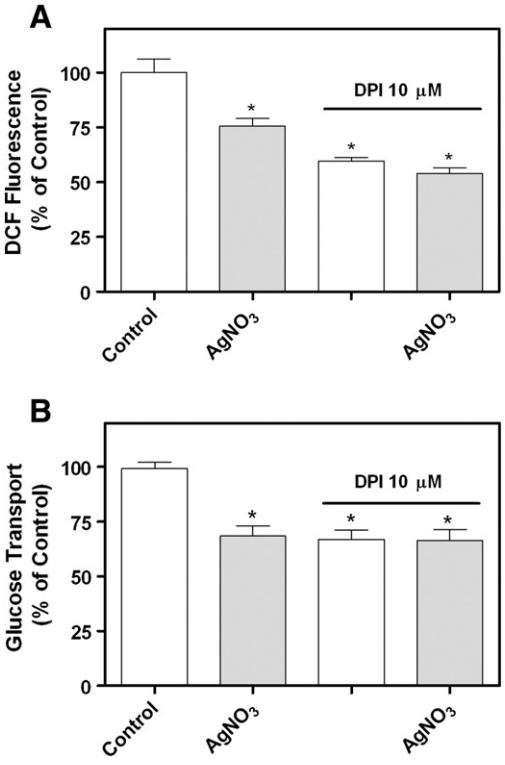

Fig. 2. Effect of AQP inhibition and/or DPI on intracellular ROS level and glucose transport in B1647 cells. (A) Cells were incubated with 5 μM $AgNO_3$ and then treated with 10 μM DPI. Intracellular ROS level was evaluated as DCF fluorescence as reported in Material and methods. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test: $^*p < 0.05$ significantly different from Control. (B) Cells were incubated with $AgNO_3$ and then treated with 10 μM DPI. Glucose uptake was evaluated by means of a radioisotopic assay as reported in Material and methods. Data are expressed as % of Control and represent means ± SD of, at least, three independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test: $^*p < 0.05$ significantly different from Control.

3.3. Intracellular ROS level in cells with overexpressed or silenced AQP isoforms

To assess the involvement of the different AQP isoforms in facilitating H_2O_2 diffusion, cells were transfected by electroporation with different plasmids designed for the overexpression of AQP1, AQP3 or AQP8. Transfection efficiency, estimated as fluorescent/non-fluorescent cell ratio 24 h after the electroporation with a GFP vector, was about 70%. MTT assay and trypan blue exclusion test were performed 24 h and 48 h after transfection, and data showed no significant differences in the viability of the cells transfected with the three plasmidic vectors compared to the control cells electroporated without DNA (data not shown). Constitutive expression and overexpression of the target isoforms were established by Western blotting analysis using specific antibodies against AQP1, AQP3 or AQP8 (Fig. 3A). Densitometric analysis normalized by actin content shows that, when AQP3 is overexpressed, there is a 3.5-fold protein increase; AQP8 overexpression produces a 4.1-fold increase in the target protein (data not shown). Moreover, expression of constitutive AQP3 and AQP8 isoforms (but not AQP1 since

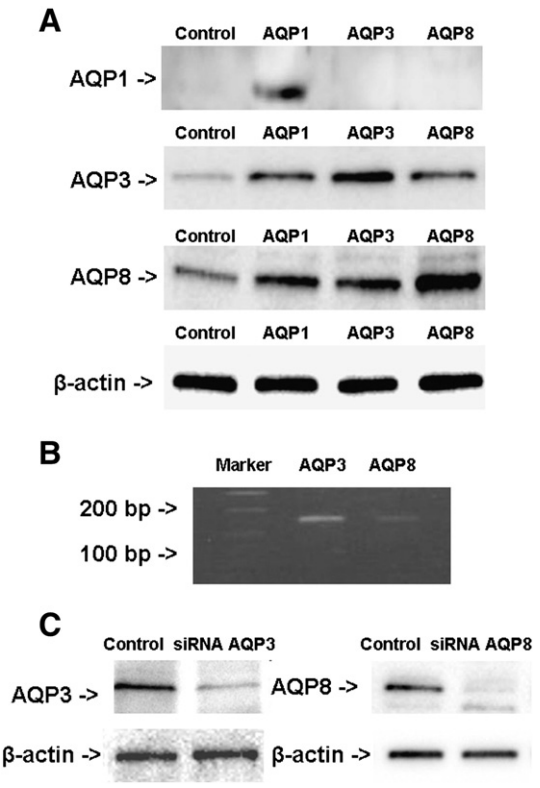

Fig. 3. Expression of AQP isoforms in B1647 cells. (A) B1647 cells were transfected by electroporation with plasmids designed for the overexpression of AQP1, AQP3, AQP8 or electroporated without DNA and lysed after 24 h. Overexpression of target isoforms was confirmed by Western blot with specific antibodies against AQP1, AQP3 or AQP8. The images show a representative experiment out of three independent analyses. (B) Constitutive expression of AQP3 and AQP8 was confirmed on mRNA extracted from B1647 cells by RT-PCR using specific primers as described in Material and methods. Amplicons of AQP3 and AQP8 were electrophoresed on agarose gel. The image shows a representative experiment out of three independent analyses. (C) B1647 cells were transfected by electroporation with siRNA against AQP3, AQP8 or a random RNA sequence (scrambled) as negative Control. Effect of RNA interference of the target isoforms was confirmed by Western blot with specific antibodies against AQP3 or AQP8. The images show a representative experiment out of three independent analyses.

the protein was not detectable by Western blotting) was confirmed by RT-PCR with specific primers for AQP3 and AQP8 (Fig. 3B).

Intracellular ROS level was measured 24 h after transfection, either in basal condition or following 100 μM H_2O_2 treatment. Fig. 4A shows that, when H_2O_2 was added exogenously to AQP8 or AQP3 overexpressing cells, intracellular ROS level was 36% and 13% higher than in control cells under similar experimental conditions, respectively. AQP8 overexpressing B1647 cells also exhibited an 18% increase in the basal intracellular ROS level compared to control cells (Fig. 4A). In contrast, AQP1 overexpressing cells showed similar intracellular ROS level compared to control cells both in basal condition or when exposed to exogenous H_2O_2. In order to confirm that ROS level is mainly due to H_2O_2, intracellular level of H_2O_2 was also measured by using PF1. After 24 h from the transfection, cells were spiked with 100 μM H_2O_2, and afterwards PF1 fluorescence level was detected. Fig. 4B shows a 15 % PF1 fluorescence increase when H_2O_2 is added to B1647 cells overexpressing AQP8 compared to control cells under the same experimental conditions. AQP3 overexpressing cells exhibited a slight increase in the intracellular H_2O_2 level compared to control cells spiked with H_2O_2. As shown in Fig. 4C the inhibition of AQP3 and AQP8 with siRNA caused a decrease in the intracellular ROS level when H_2O_2 was added, but only AQP8 siRNA was able to decrease basal ROS level. To corroborate the data obtained with $AgNO_3$ (Fig. 2A), AQP3 and APQ8 were also inhibited with specific siRNA. As shown in Fig. 4D, silencing AQP8 by siRNA caused a 30% decrease in the intracellular ROS content; moreover, the effect of DPI on the intracellular ROS level is attenuated compared to control cells. In contrast, AQP3 silencing does not modulate intracellular ROS level in basal conditions but only when cells are treated with H_2O_2.

3.4. Effect of AQP isoforms on VEGF-dependent intracellular ROS level modulation

After establishing that AQP3 and AQP8 isoforms are able to increase H_2O_2 permeability when overexpressed in B1647 cells, we turned our attention to the putative effect of AQP isoforms on VEGF-dependent ROS generation. Thus, AQP1, AQP3 or AQP8 overexpressing cells, and AQP3 or AQP8 silenced cells, were serum starved for 16 h in order to better evaluate the role of VEGF ruling out other growth factor effects. B1647 cells were then stimulated with 50 ng/mL VEGF for 30 min, and intracellular ROS level was detected and expressed as DCF fluorescence compared to control cells. VEGF treatment caused an intracellular ROS increase of similar extent (about 30% compared to untreated cells) in both control cells and in AQP1 and AQP3 overexpressing cells (Fig. 5A). In contrast, in the AQP8 overexpressing cells, the ROS level increment was about 60% of control cells; furthermore, this increase was strongly higher (compared to) than in VEGF-treated cells not subjected to AQP overexpression. Data obtained in AQP8 silenced cells showed a 34% decrease in intracellular ROS content triggered by VEGF highlighting the importance of APQ8 basal expression in modulating VEGF-induced ROS generation (Fig. 5B). These results directly demonstrate that AQP8 is able to enhance the VEGF-triggered H_2O_2 intracellular accumulation. In contrast,

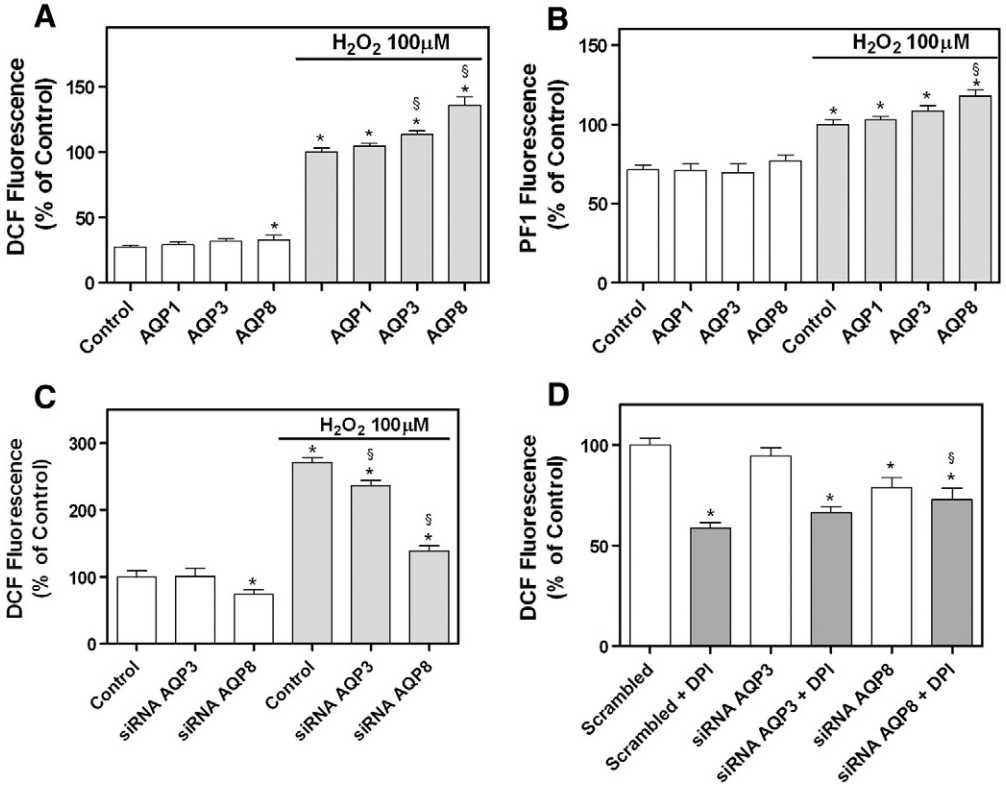

Fig. 4. Effect of AQP overexpression or inhibition by RNA interference on intracellular ROS level. B1647, cells were transfected by electroporation with plasmids designed for the overexpression of AQP1, AQP3, AQP8 or electroporated without DNA; or with specific siRNA against AQP3, AQP8 or a random RNA sequence (scrambled) as negative Control. (A) 24 h after transfection, cells overexpressing different AQP isoforms were treated with 100 μM H_2O_2, and then intracellular ROS level was evaluated as DCF fluorescence as reported in Material and methods. Data are expressed as % of Control and represent means ± SD of, at least, three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: $^*p < 0.05$ significantly different from Control; $^§p < 0.05$ significantly different from cells treated with 100 μM H_2O_2. (B) 24 h after transfection, cells overexpressing different AQP isoforms were treated with 100 μM H_2O_2, and then intracellular H_2O_2 level was evaluated as PF1 fluorescence. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: $^*p < 0.05$ significantly different from Control; $^§p < 0.05$ significantly different from cells treated with 100 μM H_2O_2. (C) 24 h after transfection with siRNA were treated with 100 μM H_2O_2, and then intracellular ROS level was evaluated as DCF fluorescence. Data are expressed as % of Control and represent means ± SD of, at least, three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: $^*p < 0.05$ significantly different from Control; $^§p < 0.05$ significantly different from cells treated with 100 μM H_2O_2. (D) 24 h after transfection with siRNA cells were treated with 10 μM DPI, and then intracellular ROS level was evaluated as DCF fluorescence. Data are expressed as % of Control and represent means ± SD of, at least, three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: $^*p < 0.05$ significantly different from Control; $^§p < 0.05$ significantly different from cells treated with 10 μM DPI.

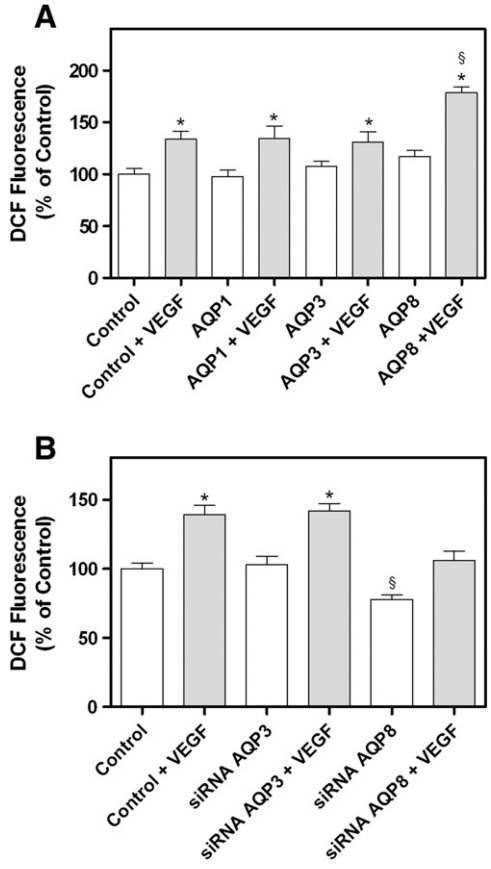

Fig. 5. Effect of VEGF on intracellular ROS level of AQP overexpressing or AQP silencing cells. (A) B1647 cells were transfected by electroporation with plasmids designed for the overexpression of AQP1, AQP3, AQP8 or electroporated without DNA; (B) cells were transfected by electroporation with specific siRNA against AQP3, AQP8 or a random RNA sequence (scrambled) as Control. (A) After 24 h from transfection control and overexpressing AQP1, AQP3 or AQP8 cells were serum starved for 16 h and then stimulated for 30 min with 50 ng/mL VEGF. Then, intracellular ROS level was evaluated as DCF fluorescence as reported in Material and methods. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: $^{*}p < 0.05$ significantly different from corresponding VEGF untreated cells; $^{§}p < 0.05$ significantly different from VEGF treated control cells. (B) After 24 h from transfection Control, AQP3 or AQP8 silenced cells were serum starved for 16 h and then stimulated for 30 min with 50 ng/mL VEGF. Then, intracellular ROS level was evaluated as DCF fluorescence. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: $^{*}p < 0.05$ significantly different from corresponding VEGF untreated cells; $^{§}p < 0.05$ significantly different from Control cells.

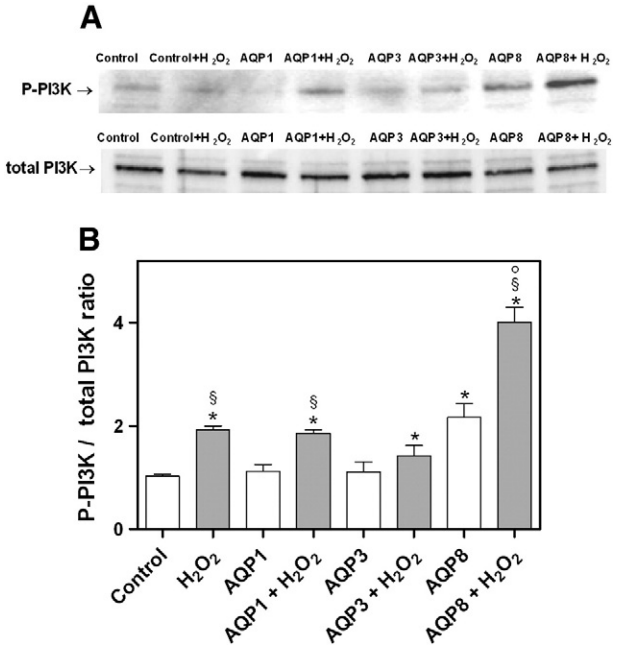

Fig. 6. Effect of AQP isoform overexpression on H_2O_2-mediated PI3K phosphorylation. B1647 cells were transfected by electroporation with plasmids designed for the overexpression of AQP1, AQP3, AQP8 or electroporated without DNA. After 24 h control and AQP1, AQP3 or AQP8 overexpressing cells were treated with 100 μM H_2O_2 for 30 min and immediately lysed. (A) Proteins form different lysates were probed for phospho-PI3K then stripped and re-probed for total PI3K. (B) Densitometric analysis of PI3K phosphorylation status is expressed as phospho-PI3K/total PI3K ratio. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: $^{*}p < 0.05$ significantly different from Control; $^{§}p < 0.05$ significantly different from corresponding H_2O_2 untreated cells; $^{°}p < 0.05$ significantly different from H_2O_2 treated cells.

AQP1 and AQP3 do not appear capable of amplifying VEGF-dependent intracellular ROS level increase.

3.5. Effect of AQP on H_2O_2 modulated pathways

To assess the involvement of some AQP isoforms in modulating H_2O_2-dependent redox signalling pathways, cells overexpressing AQP1, AQP3 or AQP8 were treated with 100 μM H_2O_2 for 30 min and then immediately lysed in the presence of phosphatase inhibitors. Western blotting analysis of phosphorylation status was performed by means of specific antibodies against phospo-PI3K and total PI3K or against phospho-p38 MAPK and total p38 MAPK. Fig. 6A shows that PI3K phosphorylation increased in all the H_2O_2-stimulated cells in comparison to the corresponding untreated cells at different extents. Data expressed as ratio of phosphorylated over total forms show that AQP8 overexpression caused a more than 2-fold increase of the PI3K phosphorylation in both basal condition and following H_2O_2 treatment (Fig. 6B). In contrast, AQP1 and AQP3 overexpressing cells do not exhibit significant changes in the PI3K phosphorylation status compared to control cells both in the absence or in the presence of H_2O_2 stimulation (Fig. 6B). Fig. 7A and B shows that also p38 MAPK phosphorylation increased in all the H_2O_2-stimulated cells in comparison to the corresponding untreated cells at different extents. Furthermore, densitometric analysis of Western blots revealed, as expected, that H_2O_2 caused an increase in the kinase phosphorylation level both in the AQP overexpressing cells and in the control cells. It is noteworthy that p38 MAPK phosphorylation level was strongly enhanced in AQP8 overexpressing cells in both basal condition and following H_2O_2 treatment. However, AQP1 and AQP3 do not appear capable of amplifying p38 MAPK phosphorylation induced by H_2O_2 compared to control cells. To corroborate the data on phosphorylation, the activity of PI3K and p38 MAPK was also evaluated analysing cell proliferation both in the cells overexpressing AQP isoforms and after silencing with siRNA against AQP3 or AQP8. As shown in Fig. 8, AQP8 overexpression increased cell proliferation in basal conditions or when cells were stimulated with H_2O_2; on the contrary, when AQP8 was silenced, a negative modulation of the cell proliferation was observed, and the effect of H_2O_2 stimulation was attenuated.

4. Discussion

Enzymatic production of H_2O_2 and its downstream targets have been intensely studied, and it is now clear that H_2O_2 produced by NAD(P)H oxidases can act as messenger in redox signalling. Nox enzymes generate ROS by transferring electrons from NAD(P)H inside the cell to molecular oxygen across the membrane resulting in the generation of superoxide anion or H_2O_2 outside the cells [4]. Intracellular ROS levels can be upregulated in leukaemia cells, and it has been previously established that Nox-generated ROS can trigger different downstream pro-survival pathways [33]. In particular, we previously demonstrated that acute leukaemia B1647 cells produce high level of ROS that sustains cellular growth and glucose uptake [20,22]. Even if

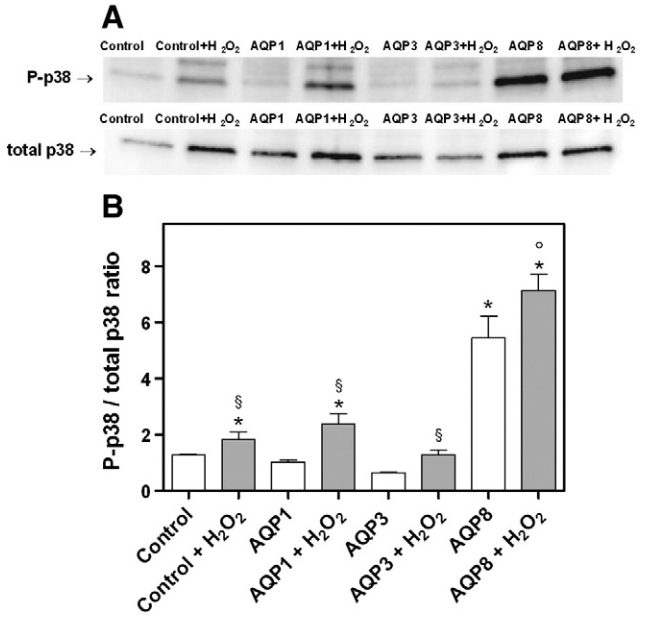

Fig. 7. Effect of AQP isoforms overexpression on H_2O_2-mediated p38-MAPK phosphorylation. B1647 cells were transfected by electroporation with plasmids designed for the overexpression of AQP1, AQP3, AQP8 or electroporated without DNA. After 24 h control and AQP1, AQP3 or AQP8 overexpressing cells were treated with 100 μM H_2O_2 for 30 min and immediately lysed. (A) Proteins from different lysates were probed for phospho-p38 MAPK then stripped and re-probed for total p38 MAPK. (B) Densitometric analysis of p38 MAPK phosphorylation status is expressed as phospho-p38 MAPK/total p38 MAPK ratio. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: $^{*}p < 0.05$ significantly different from Control; $^{\S}p < 0.05$ significantly different from corresponding H_2O_2 untreated cells; $^{\circ}p < 0.05$ significantly different from H_2O_2 treated cells.

H_2O_2 has been long thought to permeate biological membranes by simple diffusion, recent evidence challenged this concept revealing the role of some AQP isoforms in mediating H_2O_2 transport across cellular membranes. Interestingly, AQP1, AQP3 and AQP8, the three aquaporin isoforms so far reported to channel H_2O_2 in other models [8,11,34], are also widely expressed in blood cells [17–19].

In the present work, we demonstrated that specific AQP isoforms constitute a possible way through which Nox-derived H_2O_2 can enter

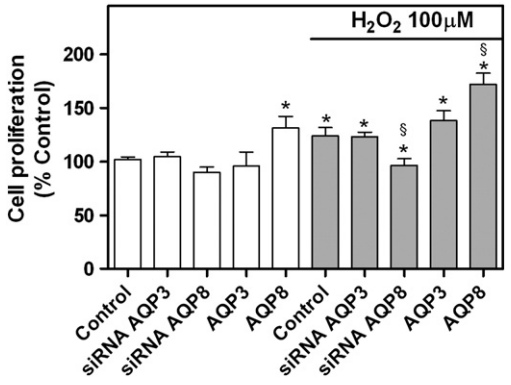

Fig. 8. Effect of AQP isoforms on cell proliferation. B1647 cells were transfected by electroporation with plasmids designed for the overexpression of AQP1, AQP3, AQP8 or electroporated without DNA; or with specific siRNA against AQP3, AQP8 or a random RNA sequence (scrambled) as Control. After 24 h, 2×10^5 cells were plated and treated with 100 μM H_2O_2 for 30 min and immediately tested for viability with Guava cytofluorimeter. After 24 h from the treatment with H_2O_2 cells were counted and tested again. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: $^{*}p < 0.05$ significantly different from Control; $^{\S}p < 0.05$ significantly different from H_2O_2 treated cells.

the cells to act as signal molecule contributing to the aberrant proliferation of leukaemia cells. Results here reported show that, in the acute leukaemia B1647 cell line, AQP inhibition is able to decrease H_2O_2 transport when H_2O_2 is exogenously added or is endogenously produced by Nox enzymes. In fact, inhibiting AQP channels by means of $AgNO_3$ caused a decrease in intracellular ROS level.

The lack of an additive effect on intracellular ROS level exerted by $AgNO_3$ and by the well-known Nox inhibitor DPI suggests that the two inhibitors act by preventing Nox-produced H_2O_2 to enter the cell in two different ways, *i.e.* either by blocking H_2O_2 production or transport. Data obtained by AQP8 silencing confirmed that DPI effect on ROS level is dependent, at least in part, on AQP8 basal expression.

As we previously demonstrated that Nox-derived ROS are required for maintaining the high glucose uptake essential for cellular proliferation [35], here we evaluated glucose transport in the absence or presence of AQP and/or Nox inhibitors. AQP inhibition is able to decrease glucose uptake in B1647 cells. In agreement with data on ROS level, the effects of DPI and $AgNO_3$ are not additive corroborating the hypothesis that AQPs play a pivotal role in mediating Nox-derived ROS signalling.

B1647 cells express, at least, AQP3 and AQP8, as shown in Fig. 3. Inhibition of constitutively expressed AQP is able to decrease intracellular H_2O_2 level both when H_2O_2 is exogenously added and when H_2O_2 is produced extracellularly by Nox. It has been demonstrated that the exposure of cells to external sources of H_2O_2 generates gradients across cell membranes depending on the intensity of the intracellular H_2O_2 consumption and on H_2O_2 membrane permeability [36]. Therefore, it appears likely that the inhibition of AQP by $AgNO_3$ decreases H_2O_2 membrane permeability: in this way, H_2O_2 produced by Nox outside the cells generates a lower concentration inside the cells. The role of extracellular H_2O_2 is also confirmed by the observation that treatment with extracellular catalase abrogated the effect of AQP inhibition on the intracellular ROS level (Fig. 1A).

In addition, the overexpression of the unorthodox AQP8 isoform and, to a minor extent, of the aquaglyceroporin AQP3 is able to increase intracellular H_2O_2 content when extracellular H_2O_2 was added. However, only AQP8 overexpressing cells show an increase in H_2O_2 intracellular level in basal condition. In contrast, AQP1 is not able to facilitate H_2O_2 diffusion when overexpressed in B1647 cells. Furthermore, AQP8 silencing is able to decrease ROS content both when H_2O_2 is added or in basal conditions, confirming the pivotal role of AQP8 basal expression in facilitating H_2O_2 diffusion across plasma membrane.

Since AQP3 and AQP8 isoforms were demonstrated to be able to increase H_2O_2 permeability, the putative effect of AQP isoforms on VEGF-dependent redox signalling was investigated. VEGF is not only a key growth factor related to angiogenic signalling but also a pivotal regulator of malignancy in leukaemia cells [37]. Moreover, Nox-derived ROS are involved in early signalling events such as the modulation of glucose uptake mediated by Glut1, a cellular activity strictly bound to VEGF-induced leukaemia cell proliferation [22]. Data here reported demonstrate that when AQP8 is overexpressed in B1647 cells, the ability of VEGF to trigger an increase of H_2O_2 intracellular level is enhanced; on the contrary, AQP1 or AQP3 overexpression is not able to increase VEGF-induced ROS accumulation. Results were also strengthened by data obtained in AQP8 silenced cells showing a decrease in intracellular ROS level triggered by VEGF. Moreover, the importance of APQ8 basal expression in modulating VEGF-induced ROS generation is confirmed, as VEGF induced a greater increase in control cells (scrambled) compared to AQP8 silenced cells (Fig. 5B).

H_2O_2 signalling is, at least in part, due to reversible oxidation of specific cysteine residues of target proteins including protein tyrosine phosphatases [38,39]. Recently, the identification of redox regulation of tyrosine kinase is becoming increasingly apparent [5]. Furthermore, it is known that Nox-produced H_2O_2 exerts its effect by directly and/or indirectly regulating specific downstream signalling protein phosphorylation [40]. Phosphoinositide 3-kinase signalling pathway

regulates survival, proliferation, growth, motility, and it is deregulated in numerous human cancers, including myeloid leukaemia [41]. Current evidence shows that PI3K signalling pathways are positively regulated by ROS through different mechanisms; for instance, the oxidative inhibition of PTEN, a natural PI3K negative modulator, can lead to increased PI3K phosphorylation [42]. Moreover, one major effector of redox signalling is p38 MAP kinase, a highly redox-sensitive enzyme activated by exogenous H_2O_2 that controls cellular responses like growth, apoptosis, and stress signals [43]. Interestingly, here we reported that AQP8 overexpression is able to increase the phosphorylation of both PI3K and p38 MAPK triggered by H_2O_2. AQP1 and AQP3 overexpression, instead, seems not able to provoke the same effect. The important role of AQP8 in modulating PI3K and p38 MAPK activity is also confirmed by data reported in Fig. 8 showing that AQP8 is crucial for cell proliferation pathways, in which the two kinases investigated are known to be involved.

NAD(P)H oxidases appear to be activated within discrete subcellular compartments, including membrane *caveolae* and lipid rafts. This localization facilitates a spatially confined ROS production, which, having redox-sensitive targets in proximity, may allow ROS to activate specific redox signalling events [44]. As it has been shown in other models that lipid raft domains contain both Nox enzymes and AQP8 [44–46], it is reasonable to hypothesize that membrane compartmentalization plays a role in explaining different behaviour of AQP3 and AQP8 in triggering redox pathways even if both isoforms can potentially facilitate H_2O_2 uptake.

5. Conclusions

In summary, this study indicates that some AQP isoforms (AQP8 and AQP3, in a lesser extent) are able to funnel H_2O_2 across the plasma membrane in the acute leukaemia B1647 cell line. Indeed, our data suggest that AQP8, but not AQP1, is able to transport Nox-generated H_2O_2 through cellular membranes affecting downstream redox signalling pathways mediated by H_2O_2. Recent reports demonstrate that AQP isoforms affect angiogenesis, cell migration and metastasis in a variety of cancer cells; however, the suggested mechanisms do not include H_2O_2-facilitated transport across plasma membrane by AQP [47]. Our findings, for the first time, ascribe to AQP (AQP8, in particular) an important role in H_2O_2-mediated redox signalling linked to leukaemia cell proliferation. Therefore, the development of new drugs targeting specific AQP isoforms might be an interesting novel anti-leukaemia strategy.

Acknowledgments

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