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Identification of a functional role for the protease-activated receptor-1 in hypoxic breast cancer cells

Antonella Naldini[a,*], *Irene Filippi*[a], *Camilla Ardinghi*[a], *Antonietta Silini*[b], *Raffaella Giavazzi*[b], *Fabio Carraro*[a]

[a]*Department of Physiology, University of Siena, Via Aldo Moro, 53100 Siena, Italy*
[b]*Department of Oncology, Mario Negri Institute for Pharmacological Research, Milano, Italy*

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ABSTRACT

Aberrant expression of the protease-activated receptor (PAR)-1 has been associated with tumour progression. Indeed, PAR-1 expression correlates with tumour invasiveness, as well as with cancer cell survival. As the tumour microenvironment is characterised by a low oxygen tension, we decided to investigate the role of PAR-1 in cancer cells exposed to a hypoxic microenvironment. In this study we show that hypoxia enhances PAR-1 expression in MDAMB231 breast cancer cells. We next provided evidence for a novel role of PAR-1 in protecting hypoxic breast cancer against cell death, since inhibition of PAR-1 by RNA interference resulted in a decreased cell survival. Finally, we found that treatment of hypoxic MDAMB231 cells with the specific PAR-1 agonist peptide (TRAP) resulted in a significant increase of cell survival and migration. The overall results identify for the first time a functional role for PAR-1 in the cellular responses of breast cancer to a hypoxic microenvironment.

1. Introduction

Thrombin is a multifunctional serine protease which promotes proliferation[1], survival[2] and chemotaxis[3,4] in several cell lines. Many of the cellular effects exerted by thrombin are mediated by the protease activated receptors (PARs), a family of G-protein-coupled receptors which show a unique mechanism of proteolytic activation.[5] Recently, there has been considerable interest in understanding the biological significance of PAR-1, the proto-typical thrombin receptor, in neoplastic diseases.[6–9]

The rapid growth and the disorganised vasculature of solid tumours results in altered blood flow and a hypoxic microenvironment.[10] Despite the negative effects of hypoxia on cellular proliferative responses, its ability to select for cells that are more resistant to cell death has been extensively established.[11,12] In the past, we have shown that PAR-1 is associated with cell survival.[13] More recently, PAR-1 has been described as a survival factor for melanoma cells[14] and another report has revealed that a hypoxic microenvironment may affect PAR-1 invasive capabilities.[15] However, despite intensive studies suggesting that the biological effects exerted by PAR-1 and hypoxia are intimately related, the functional role of PAR-1 in hypoxic cancer cells has yet to be reported. In this study we propose that PAR-1 plays a pivotal role in breast cancer cellular responses in a hypoxic microenvironment.

2. Materials and methods

2.1. Cell culture

The highly invasive breast cancer cell lines MDAMB231 (obtained from NCI-Frederick Cancer DCT tumour repository,

* *Corresponding author*: Tel.: +39 0577 234212; fax: +39 0577 234219.
E-mail address: Naldini@unisi.it (A. Naldini).
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Frederick, MD) and 8701-BC (kindly provided by Prof. I. Pucci-Minafra, University of Palermo, Italy)[16] were maintained in growth medium (RPMI supplemented with 10% FBS) (Euroclone, Devon, UK). A 2% (≈14 mmHg) O_2 environment was obtained using a water-jacketed incubator (Forma Scientific, Marietta, OH) that provides a customised and stable humidified environment through electronic control of CO_2 (5%), O_2 and temperature (37 °C), as previously described.[17] Such conditions resemble a mild hypoxic state similar to that observed in breast cancer tissues *in vivo*.[18] At the end of the experiments, cells were promptly lysed and analysed. The PAR-1 ligands, human α-thrombin (Enzyme Research Laboratories, Swansea, UK) and human PAR-1 agonist peptide (TRAP) (TFLLRN; Tocris Bioscience, Avonmouth, UK) were used at a concentration of 300 nM and 40 μM, respectively.

2.2. Cell survival

Cell survival was assessed by the LIVE/DEAD® Viability/Cytotoxicity Assay (Molecular Probes, Eugene, OR), which is based on the simultaneous determination of live and dead cells using calcein AM and Ethidium homodimer-1 as previously described.[19] Briefly, cells (5×10^3/well in a total volume of 100 μl of growth medium) were exposed to either 21% O_2 or 2% O_2 for 24 h. Some experiments were conducted in the presence of the human PAR-1 agonist peptide, TRAP, at a concentration of 40 μM. In this case the FBS concentration in the culture medium was reduced (1%) as previously described.[13,20] Immediately after, the LIVE/DEAD assay was performed and the percentage of alive cells was determined in accordance with the manufacturer's instructions. Fluorescence was measured using a microplate reader (FluostarOptima, BMG LABTECH, Offenburg, D) which provided excitation at 485 nm and emission at 520 nm (for live cells) and excitation at 530 nm and emission at 645 nm (for dead cells).

2.3. Migration assay

Breast cancer cell migration was evaluated using a chemotaxis microchamber technique as described previously[21], employing a modified Boyden 48 well micro chemotaxis chamber (Neuro Probe, Gaithersburg, MD) with 8 μm pore size polycarbonate polyvinylpyrrolidone-free Nucleopore filters.[22] A cellular suspension of 1×10^6 cells/ml in RPMI supplemented with 0.1% bovine serum albumin (BSA) was added to the upper chamber and a solution of medium alone or media supplemented with TRAP (40 μM) was added to the lower chamber. After overnight incubation under hypoxic conditions (2% O_2), cells on the upper surface of the filter were removed. Migrated cells on the lower surface were stained using DiffQuick (Merz-Dade, Düdingen, Switzerland) and photographed by an OLYMPUS IX81 Research Microscope with a 10× magnification. The images were processed with Soft Imaging System software (Olympus Company, Munster, Germany). The cells migrated in 10 high-power fields were counted and data were expressed as number of migrated cells/field. Each assay was done in quadruplicate.

2.4. RNA preparation and qRT-PCR

PAR-1, HIF-1α, Bax and Bcl-xL mRNA expression was determined by qRT-PCR using a MJ miniOpticon Cycler (Bio-Rad Laboratories, Hercules, CA), as previously described.[23] Briefly, cells (5×10^4 well/ml) were exposed to either 21% O_2 or 2% O_2 for 24 h, as described above. Total RNA was extracted using the TRI Reagent® (Ambion, Austin, TX). First-strand cDNA synthesis was performed using iScript™cDNA Synthesis Kit (Bio-Rad Laboratories). qRT-PCR was performed using iTaq™ SYBR Green Supermix with ROX (Bio-Rad Laboratories) and the specific primers were designed using the PRIMER3 program (available at http://frodo.wi.mit.edu). Data were quantitatively analysed on an MJ OpticonMonitor detection system (Bio-Rad Laboratories). Relative quantisation was done by using the ΔCT method by taking the difference (ΔCT) between the CT of β-actin and CT of each transcript, as previously described.[24]

2.5. Western blot analysis

For Western blot analysis, cells were incubated as described above and properly lysed. Aliquots (30 μg) of the extracted material were analysed by Western blotting using antibodies against p42/p44 MAPK, p38 MAPK, anti-Poly-(ADP-Ribose)-Polymerase (PARP) or β-actin (Cell Signaling, Danvers, MA), or HIF-1α (BD Biosciences, San Jose, CA), or PAR-1 (Immunotech, Marseille Cedex, France), as previously described.[25] Chemiluminescence was quantified using a ChemiDoc XRS apparatus and Quantity One software (Biorad Laboratories).

2.6. PAR-1 and HIF-1α inhibition by siRNA

siRNA sequences were selected according to Invitrogen (Stealth RNA system) for specific silencing of PAR-1 and HIF-1α. The siRNA target sequences correspond to nucleotides 961–982 of human PAR-1 mRNA and to nucleotides 1028–1049 of human HIF-1α mRNA. The negative controls were designed with the same GC ratio without any known target to the human genome. Cells were transfected with the Stealth RNAs at a concentration of 40 nM using Lipofectamine (Invitrogen, Paisley, UK) at 40% confluency of the cell monolayer. Cells were incubated for 48 h prior to PAR-1 or HIF-1α level determination. Following transfection, cells were exposed to normoxia or hypoxia for 24 h and analysed as described above.

3. Results

3.1. PAR-1 signalling in MDAMB231

Recent studies have shown that PAR-1 is expressed and functionally relevant in breast cancer cell invasion.[26] Here we investigated PAR-1 signalling in MDAMB231 cells by the evaluation of p42/p44 MAPK phosphorylation.[27] Fig. 1A shows that both thrombin and the PAR-1 agonist peptide, TRAP, caused a rapid phosphorylation of p42/p44 MAPK ($p < 0.05$, at 15 min). PAR-1 ligation is followed also by p38 phosphorylation in several cell types.[28] Fig. 1B shows that activation of PAR-1 by either thrombin or TRAP caused an evident p38

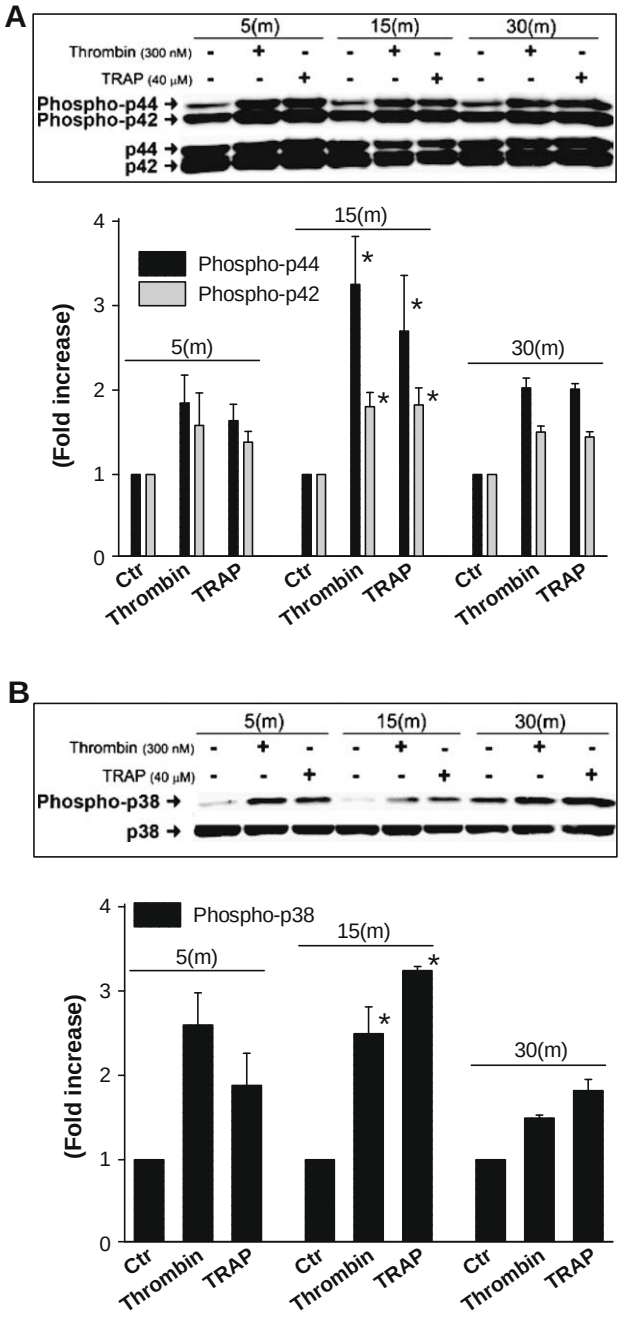

Fig. 1 – PAR-1 signalling in MDAMB231 breast cancer cells. MDAMB231 cells were treated with thrombin or TRAP, lysed at different time points. Western blot analysis was conducted using either anti-phospho-p42/p44 antibody and an anti-pan-p42/44 antibody (Panel A) or antiphospho-p38 and an anti-pan-p38 antibody (Panel B). A representative blot from three similar experiments is shown. Quantification was achieved by chemiluminescence. Results are mean ± SD of three experiments. *, significantly different from control (Ctr), $p<0.05$ by Student's t test.

phosphorylation ($p < 0.05$, at 15 min). These results suggest that PAR-1 is functionally relevant in MDAMB231 by activating pathways associated with cell proliferation (e.g. p42/p44 MAPK) and cell migration (e.g. p38).[29]

3.2. *Hypoxia enhances PAR-1 expression in breast cancer cells*

As the tumour microenvironment is characterised by a low oxygen tension[18], we investigated whether hypoxia affected PAR-1 expression. Fig. 2A shows that hypoxia induced a significant upregulation of PAR-1 mRNA in both MDAMB231 and 8701-BC breast cancer cell lines. In agreement with previous studies conducted on several cell lines[30], hypoxia did not affect HIF-1α mRNA levels. Parallel experiments were conducted to evaluate the accumulation of HIF-1α and to confirm PAR-1 expression at protein levels under hypoxic conditions. Fig. 2B shows that hypoxia markedly increased the protein levels of HIF-1α and PAR-1 in both breast cancer cell lines.

As exposure to hypoxia resulted in an overexpression of PAR-1 along with HIF-1α accumulation, we decided to clarify the role of HIF-1α in PAR-1 expression under normoxic and hypoxic conditions. To this end, we inhibited HIF-1α expression by RNA interference. MDAMB231 cells were transfected with stealth RNA oligonucleotides for human HIF-1α mRNA and exposed to either a normoxic or a hypoxic environment for 24 h. Cells transfected with control oligos were used as controls. As determined by qRT-PCR, inhibition of HIF-1α resulted in a significant downregulation of HIF-1α expression

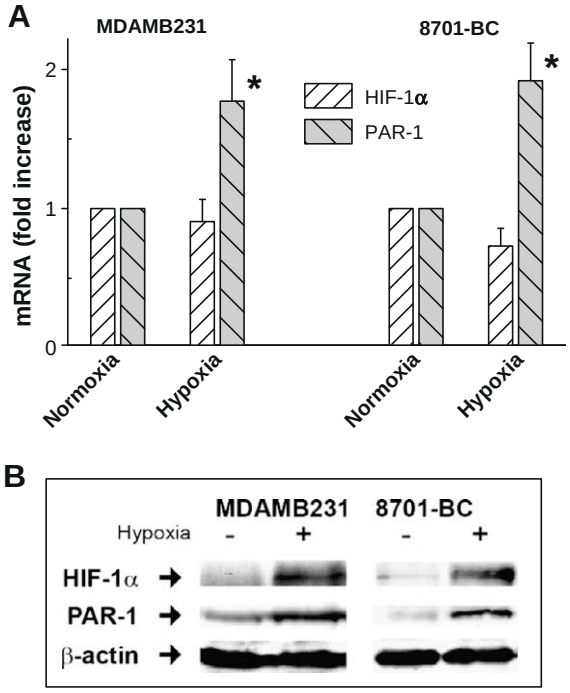

Fig. 2 – Hypoxia enhances PAR-1 expression. MDAMB231 and 8701-BC cells were incubated under normoxic and hypoxic conditions for 24 h. HIF-1α and PAR-1 mRNA expression was analysed by qRT-PCR (Panel A). Values (mean ± S.D.) refer to four independent experiments. *, significantly different from normoxic treatment; $p < 0.05$ by Student's t test. In parallel, Western blot analysis was conducted using anti-HIF-1α, anti-PAR-1, and anti-β-actin antibodies (Panel B). A representative blot from four similar experiments is shown.

(more than 80%), when compared with the controls (Fig. 3A). Such downregulation was similar in both normoxic and hypoxic conditions. Fig. 3B shows that silencing of HIF-1α significantly reduced PAR-1 mRNA expression in MDAMB231 cells exposed to hypoxia for 24 h, while under normoxia, siRNA for HIF-1α had no effect on the constitutive PAR-1 expression. As expected, after siRNA treatment, hypoxic incubation failed to induce the accumulation of HIF-1α protein in MDAMB231 cells (Fig. 3C), as determined by Western blot analysis. Accordingly with the mRNA results, HIF-1α siRNA inhibited the hypoxic induction of PAR-1. Conversely, PAR-1 protein was induced by hypoxic treatment when MDAMB231 cells were treated with the Stealth control. Thus, HIF-1α appears critically involved in hypoxic-induced PAR-1 expression in MDAMB231 breast cancer cells.

3.3. *Role of PAR-1 in MDAMB231 cellular responses in a hypoxic microenvironment*

The data presented above suggest a role for PAR-1 in hypoxic cellular responses. To establish such a role, we first inhibited PAR-1 expression by RNA interference and then determined functional responses in MDAMB231 cells. Fig. 4A shows that inhibition of PAR-1 resulted in a significant downregulation of PAR-1 mRNA expression (more than 80%) in MDAMB231 breast cancer cells. A striking PAR-1 downregulation was also observed in cell knock-down for PAR-1 and exposed to a hypoxic condition. PAR-1 inhibition by siRNA resulted in a similar downregulation of PAR-1 at protein levels (Fig. 4B).

Since PAR-1 protects cells from apoptosis[31,32], we set out to investigate whether PAR-1 expression could affect the

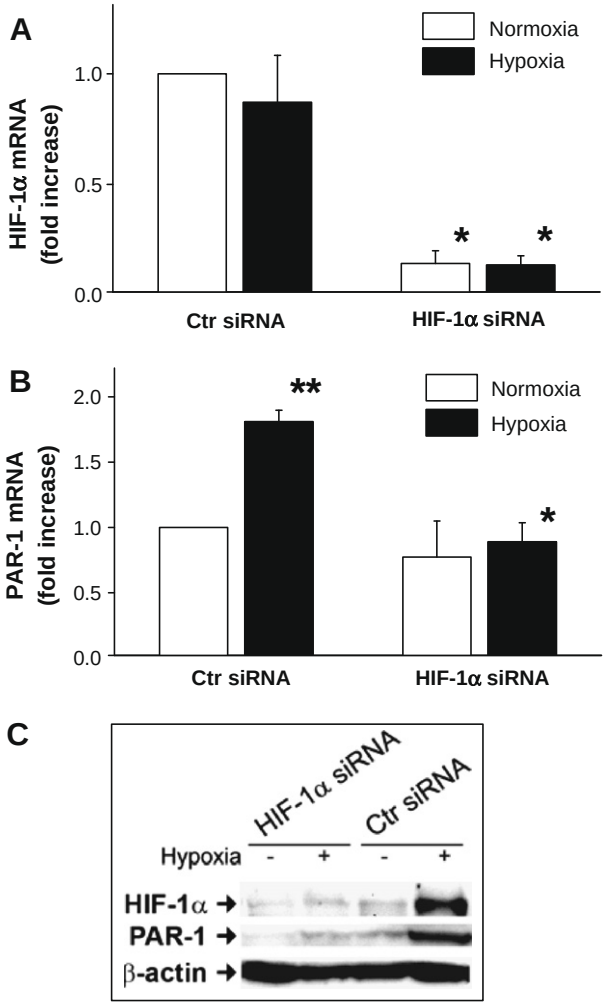

Fig. 3 – Role of HIF-1α in PAR-1 expression. MDAMB231 cells were treated either with Stealth oligos for HIF-1α (HIF-1α siRNA) or with Stealth control oligos (Ctr siRNA) and exposed to normoxia or to hypoxia for 24 h. HIF-1α (Panel A) and PAR-1 (Panel B) mRNA expression was examined by qRT-PCR. Values (mean ± S.D.) refer to three independent experiments. *, significantly different from control (Ctr siRNA), $p < 0.05$ by Student's *t* test; **, significantly different from normoxic treatment, $p < 0.05$ by Student's *t* test. In parallel, whole cell extracts were prepared and 30 μg of total cellular proteins were subjected to SDS-PAGE, followed by HIF-1α, PAR-1 and β-actin analysis by Western blot (Panel C). A representative blot from three similar experiments is shown.

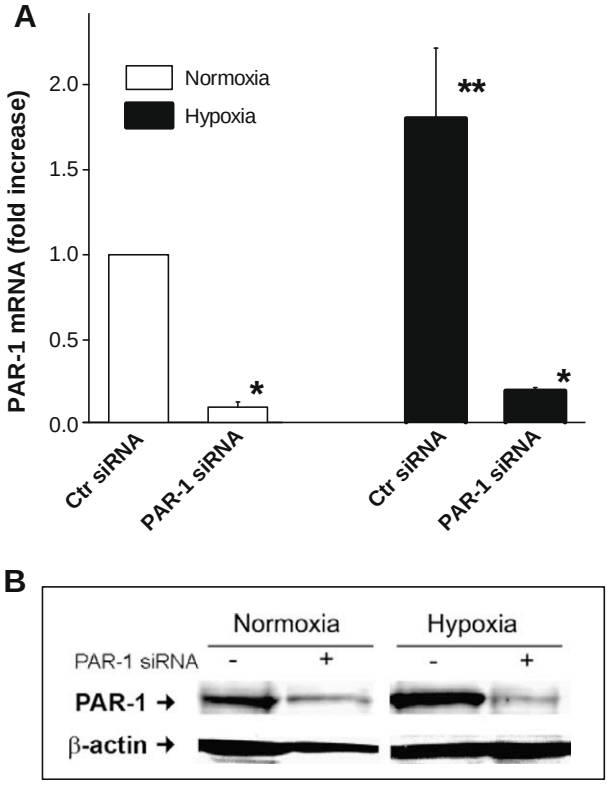

Fig. 4 – Inhibition of PAR-1 expression by siRNA. Cells were treated either with Stealth oligos for PAR-1 (PAR-1 siRNA) or with Stealth control oligos (Ctr siRNA). PAR-1 mRNA inhibition was quantified by qRT-PCR (Panel A). Values (mean ± S.D.) refer to four independent experiments. *, significantly different from controls (Ctr siRNA), $p < 0.05$ by Student's *t* test. **, significantly different from normoxic treatment, $p < 0.05$ by Student's *t* test. In parallel, Western blot analysis was conducted using anti-PAR-1 and β-actin antibodies (Panel B). A representative blot from three similar experiments is shown.

survival of hypoxic MDAMB231 cells. Cells transfected with siRNA against PAR-1 were subjected to a LIVE/DEAD assay in normoxic or hypoxic conditions. Fig. 5A shows that inhibition of PAR-1 expression by siRNA significantly reduced

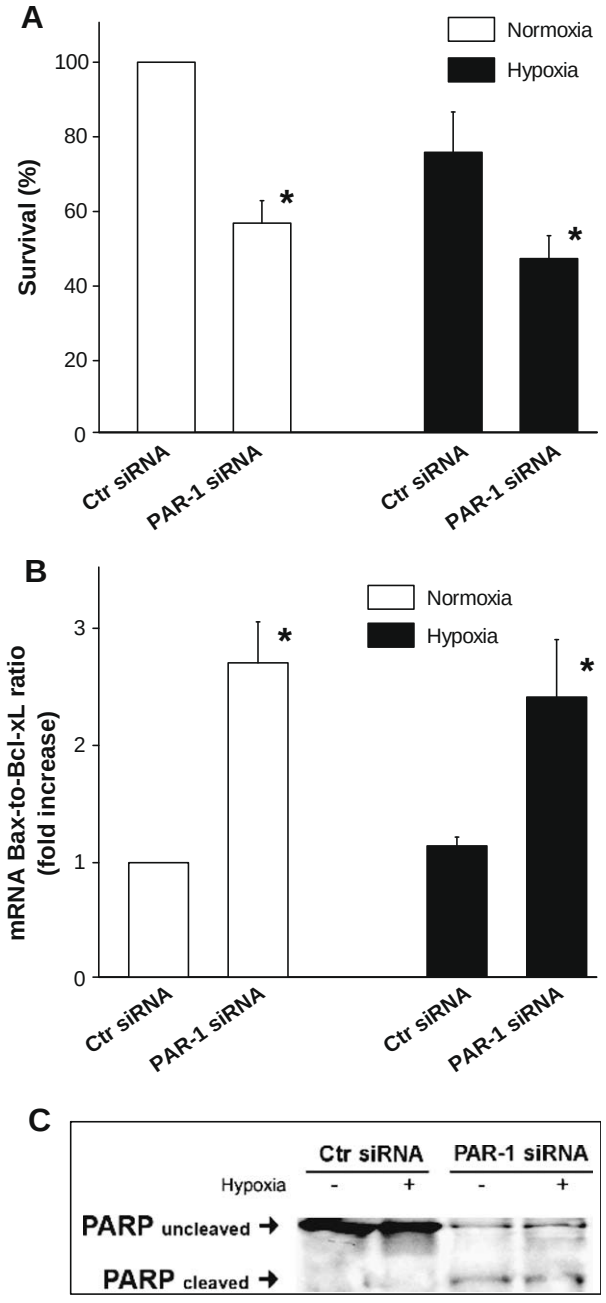

Fig. 5 – Inhibition of PAR-1 by siRNA affects cell survival and apoptosis. Transfected cells were cultured for 24 h under normoxic and hypoxic conditions. Cell survival was determined by LIVE/DEAD assay (Panel A). Bax to Bcl-xL ratio mRNA expression was determined by qRT-PCR (Panel B). Values (mean ± S.D.) refer to three independent experiments. *, significantly different from control (Ctr siRNA), $p < 0.05$ by Student's t test. Panel C: PARP (cleaved and uncleaved) protein expression was determined by Western blot (a representative blot from two similar experiments is shown).

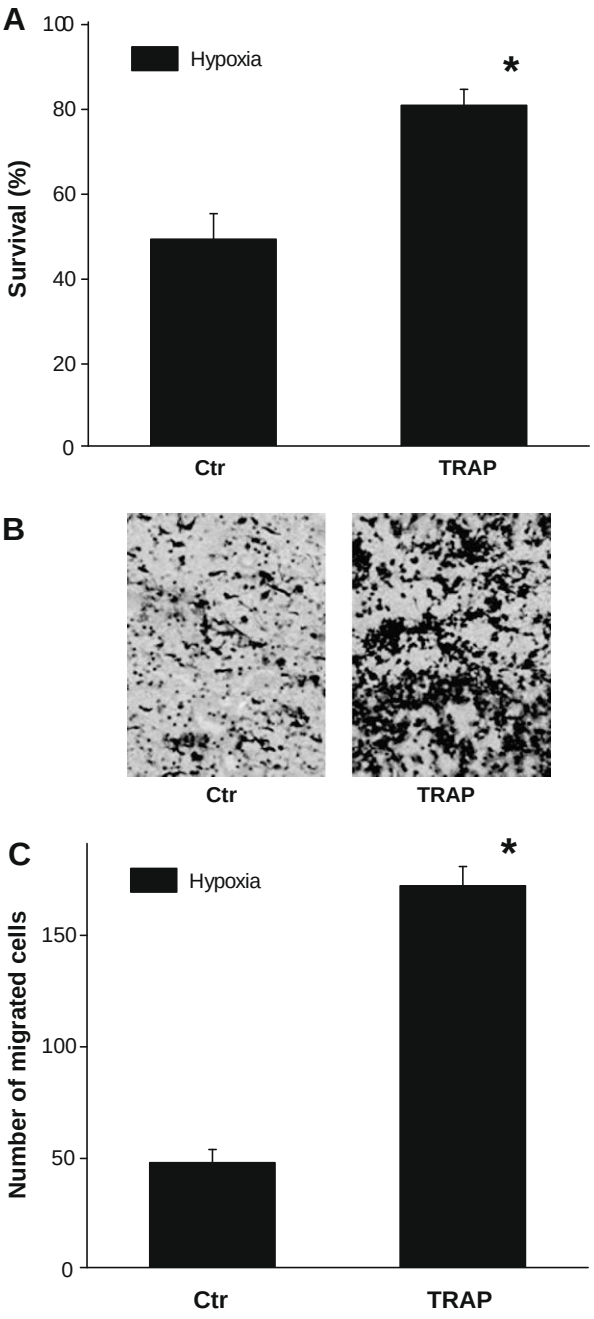

Fig. 6 – PAR-1 activation promotes cell survival and migration under hypoxic conditions. Cell survival and migration experiments were conducted in the absence (Ctr) or in the presence of 40 μM TRAP for 24 h under hypoxic conditions. Cell survival was determined by LIVE/DEAD assay (Panel A). Values (mean ± S.D.) refer to three independent experiments performed in triplicate. *, significantly different from control (Ctr), $p < 0.05$ by Student's t test. Cell migration was evaluated by chemotaxis microchamber technique. After overnight incubation under hypoxic conditions, migrated cells were photographed and counted. Panel B: photomicrographs of representative fields from the indicated conditions. Panel C: quantification of migration using cell counts of representative fields. Results are mean ± SD of four experiments. *, significantly different from control (Ctr), $p < 0.05$ by Student's t test.

MDAMB231 cell survival in normoxic condition when compared with cells transfected with control siRNA. More interestingly, MDAMB231 cell survival was also reduced under hypoxic conditions. Accordingly, siRNA against PAR-1 caused an increased ratio between the pro-apoptotic molecule Bax and the anti-apoptotic molecule Bcl-xL expression, as determined by qRT-PCR (Fig. 5B). Interestingly, such increase was similar in both normoxic and hypoxic conditions. In parallel, to further establish the protective role of PAR-1 against hypoxia-induced apoptosis, we performed additional experiments to address whether PARP cleavage was affected in MDAMB231 silenced against PAR-1. Fig. 5C shows a representative experiment where the inhibition of PAR-1 expression by siRNA upregulated PARP cleavage when compared with cells transfected with control siRNA. These findings thus suggest that PAR-1 may play an anti-apoptotic role in tumour cells even under hypoxic conditions, with important implications in tumour progression.

To further characterise the relevance of PAR-1 in the survival of hypoxic cancer cells, we determined the effect of the specific PAR-1 agonist peptide, TRAP, on MDAMB231 cell survival under hypoxia. Fig. 6A shows that exposure to hypoxia for 24 h resulted in a survival of about 50%, calculated as percentage of cell survival under normoxia. However, when cells were exposed to a hypoxic environment in the presence of 40 μM TRAP, a cell survival higher than 80% was observed. The increased cell survival was significantly different when compared with the untreated cells ($p < 0.05$). Interestingly, TRAP did not significantly affect cell survival under normoxic conditions (data not shown), suggesting that the mechanism of protection by TRAP is effective only in hypoxic cells.

Cell migration is another cellular function associated with tumour progression and hypoxia.[21] To study the relevance of PAR-1 in cell migration under hypoxic conditions the migration of MDAMB231 was assayed in response to TRAP used as chemoattractant. Fig. 6B–C shows that, after an overnight incubation under hypoxia, the number of migrated cells was significantly ($p < 0.05$) increased in the presence of TRAP.

The overall results indicate that PAR-1 expression is important for MDAMB231 cell functions, especially when cells are exposed to a hypoxic environment.

4. Discussion

In the present study we demonstrate for the first time that hypoxia enhances PAR-1 expression. We also show that PAR-1 expression is positively related to cell survival under hypoxic conditions. Furthermore, PAR-1 activation under hypoxic conditions promotes cell survival and migration.

PAR-1 activation has been associated with survival in several cell types.[1] Here we demonstrate that PAR-1 activation induces the phosphorylation of the mitogen-activated kinases, p42/p44 MAPK and of p38 MAPK in breast cancer cells. Interestingly, p42/p44 MAPK pathway activation by PAR-1 has been associated with endothelial cell proliferation and survival.[27] In our study, we observed, for the first time, phosphorylation of p42/p44 MAPK induced by PAR-1 also in breast cancer cells, with important implications for tumour cell proliferation and survival. In addition, p38 phosphorylation has been extensively associated with migratory activities of breast cancer cells.[33] Thus, our results suggest that p42/44 MAPK and p38 activation by PAR-1 may promote cell survival and migration in breast cancer, with important implications in tumour progression.

Despite the fact that hypoxia represents a microenvironmental stress, tumour cells survive and proliferate in a hypoxic microenvironment.[11] Here we show that PAR-1 is overexpressed and functionally relevant in hypoxic MDAMB231 breast cancer cells. Thus, we propose that PAR-1 activation could protect breast cancer cells from cell death in a hypoxic microenvironment. This hypothesis is strengthened by the fact that inhibition of HIF-1α with short interfering RNA resulted in a significant reduction of PAR-1 expression in MDAMB231 cells. Indeed, it is well documented that hypoxia, a common feature of the tumour microenvironment, results in adaptationally appropriate alterations of gene expression through HIF-1α to overcome any shortage of oxygen. Thus, the transcription of genes such as PAR-1, which may be controlled, at least in part, by HIF-1α could promote cell survival in breast cancer cells. Accordingly, PAR-1 has been recently described as a survival factor in melanoma cells in normoxia.[14] Here we report that PAR-1 is involved in MDAMB231 cell survival not only under normoxic conditions, but, more importantly, under hypoxic conditions. In addition, the increased cell survival, observed when hypoxic parental cells were treated with TRAP, strongly supports the hypothesis that PAR-1 is important for MDAMB231 cell survival under hypoxic conditions.

PAR-1 elicits tumour progression as it has been identified as a promoter of tumour invasiveness.[26] Here we show that PAR-1 activation by TRAP under hypoxia resulted in a significant enhancement of cell migratory activities. Since regulation of cell migration is a critical step for tumour invasiveness, our observations suggest that hypoxic conditions could exert a direct or permissive role on the PAR-1 invasive potential. Accordingly, a recent study shows that low oxygen levels were inefficient in inducing a spontaneous invasive phenotype in colon cancer cells, while, under hypoxia, a PAR-1 agonist peptide promoted this response.[15]

All together these data provide an additional molecular explanation for PAR-1 overexpression in cancer cells. The peculiarity that PAR-1 is involved in survival and migratory cues in tumour cells may be important for developing novel cancer therapeutic approaches targeting PAR-1.

Conflict of interest statement

None declared.

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