Toll-Iike Receptor-3 Activation Enhances Malignant Traits in Human Breast Cancer Cells Through Hypoxia-inducible Factor-1α

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Abstract. *Background/Aim: Hypoxia-inducible factor 1 (HIF1) inhibitors have been proposed as therapeutic agents for several tumor types. HIF1α is induced by hypoxia and by pathogens in normoxia through toll-like receptors (TLRs). The TLR3 activator polyinosinic:polycytidylic acid [poly(I:C)] induces apoptosis in various types of cancer but not in the most aggressive breast cancer cell lines. We hypothesized that the failure of TLR3 stimulation to induce apoptosis in these cells might be due to an elevated HIF1α level and this link might be exploited. Materials and Methods: Poly(I:C)-induced signaling pathway and expression of HIF1α and HIF1α targets were studied in MDA MB-231 and MCF-7 breast cancer cell lines by western blot. Flow cytometry was used for apoptotic responses and vasculogenic mimicry as bioassay. Results: Poly(I:C) increased expression of HIF1α and its targets BCL2 apoptosis regulator and c-MYC. Moreover, using pharmacological or genetic HIF1 inhibition, reduction of poly(I:C)-induced expression of HIF1α was paralleled by lowering of c-MYC and increased sensitivity to poly(I:C)-induced apoptosis, demonstrating the crucial role of this factor. We provide the first evidence in breast cancer cells that TLR3 stimulation induces HIF1α-dependent vasculogenic mimicry. By using specific inhibitors, we identified a signaling cascade upstream of HIF1α induction. Conclusion: Combined treatment with poly(I:C) and HIF1 inhibitors deserves consideration as an effective strategy in breast cancer therapy.*

This article is freely accessible online.

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Key Words: HIF1α, vasculogenic mimicry, HIF1 inhibitors, apoptosis, acriflavine.

Breast cancer is the most common invasive cancer in women and is associated with a high mortality index in the female population worldwide; indeed, this cancer represents the second cause of cancer death in women (1). Most patients with advanced breast cancer die primarily as a result of distant metastases. The rapid growth of tumor cells leads to hypoxia in the tumor microenvironment [about 1% oxygen (O_2)], which can further promote breast cancer development and metastasis (2, 3). Hypoxia results from the altered balance between the supply and consumption of O_2 and is defined as an oxygen level lower than the approximate 7% (normoxia) observed in normal and well-vascularized tissues (4). Cells respond to the reduction of O_2 through the activation of hypoxia-inducible factor-1 (HIF1), a key mediator of the cellular response to hypoxia. HIF1 is a heterodimeric basic-helix-loop-helix-transcription factor consisting of two proteins: an oxygen-regulated subunit HIF1α (or its parologs HIF2α and HIF3α) and a constitutively expressed subunit HIF1β (5). HIF1α protein has a short half-life ($t_{1/2}$=5 min) and its levels are regulated by the oxygen-dependent enzyme proline hydroxylase. In normoxia, HIF1α level is low because it is hydroxylated by prolyl hydroxylase domain proteins, which are responsible for the binding of the tumor suppressor von Hippel-Lindau complex, resulting in proteasomal degradation of HIF1α (5). During hypoxia, this enzyme is inhibited and the stabilized HIF1α is translocated to the nucleus, where, after dimerization with HIF1β and recruitment of several coactivators, it forms the HIF1 complex that binds specific regions of promoters, named hypoxia-responsive elements (6), activating the transcription of HIF1 target genes. These genes are

involved in various crucial mechanisms of breast cancer biology, such as stem cell maintenance, metabolic reprogramming, angiogenesis, epithelial mesenchymal transition, metastasis and resistance to radiation and chemotherapy (2, 7). Interestingly, using HIF1α as a marker for hypoxia, approximately 25-40% of all invasive breast cancers have been shown to exhibit hypoxic areas (4). Although HIF1α protein is induced by hypoxic conditions, there are other stimuli capable of strongly increasing the formation of the HIF1 complex in normoxia and of modulating the transcription of hypoxic genes (8). These non-hypoxic stimuli include cytokines, such as tumor necrosis factor-α, interleukin-1β and interferon-α, reactive nitrogen- or oxygen-derived radicals, growth factors, such as insulin-like growth factor-1 (9-12). Moreover, a variety of bacterial and viral components have been described as activating HIF1α in normoxia by triggering their specific toll-like receptors (TLRs) (13, 14). TLRs are a group of trans-membrane proteins that bind pathogen-associated molecular patterns conserved in bacteria, viruses and parasites (15). Most studies have shown that TLRs expressed on immune cells enhance the effects of tumor vaccines or of chemotherapy by improving antitumor immune responses, reviewed in (16), while expression of TLRs on tumor cells is reported to induce tumor progression by enhancing anti-apoptotic and pro-survival signals, the production of tumor-promoting cytokines, angiogenesis and invasiveness (17).

Intriguingly, stimulation of TLR3 expressed in several breast tumor cell lines has been shown to trigger a pro-apoptotic signal cascade leading to the inhibition of tumor growth (18, 19). Moreover, the highly expressed TLR3, TLR4 and TLR9 in human breast cancer may represent therapeutic targets because their levels of expression have clinical interest as indicators of tumor aggressiveness (20). Conversely, it has been recently shown that under stimulation of TLR3 two double-negative breast cancer cells lines acquire a cancer stem cell phenotype *in vitro* and *in vivo* (21), and inhibition of TLR3 signaling in combination with tamoxifen may increase the effectiveness of current treatments of breast cancer (22). Accordingly, our group previously demonstrated that stimulation of TLR3 expression induced apoptosis in the androgen-dependent LNCaP prostate cancer cell line *in vitro* (23) and *in vivo* (24), whereas it induced angiogenesis and apoptosis resistance through HIF1 complex activation in the more aggressive androgen-independent PC3 and DU-145 prostate cancer cells (25).

In this study, we aimed to examine whether TLR3 stimulation by its specific agonist polyinosinic:polycytidylic acid [poly(I:C)] induced HIF1α accumulation in breast cancer cell lines insensitive to TLR3-induced apoptosis and differing in hormone-resistance, and whether HIF1 activity correlated with protumor responses. The signal transduction triggered by TLR3 agonist involved in HIF1α-induced up-regulation was also investigated.

Besides induction of protumor and anti-apoptotic genes, hypoxia and subsequent HIF1 overexpression in tumor cells can promote a phenomenon involved in tumor progression called vasculogenic mimicry (VM) (26). VM, a strategy developed by solid tumors to grow and spread, is the formation of vessel-like channels that are not formed from pre-existing vessels, do not involve endothelial cells and hence are not true blood vessels but mimic their role, allowing for blood flow and oxygenation. Hypoxic conditions not only induce VM formation but also favor tumor metastasis and increase the risk of resistance to chemotherapy in breast cancer (27). After the first observations in melanoma (28, 29), the presence of VM has been described in other types of human cancer such as renal cell carcinoma (30), colorectal cancer (31), prostate cancer (32), glioblastoma (33), ovarian carcinoma (34), gastric adenocarcinoma (35), hepatocellular carcinoma (36), laryngeal squamous cell carcinoma (37) and breast cancer (38). Although microenvironmental factors and various proteins are recognized to modulate VM, the mechanism that regulates it remains unclear. In a model of triple-negative breast cancer cells (TNBC) clinically characterized by fast growth, high invasive ability and no available targeted treatment, it was suggested that HIF1 might play a key role in the biological mechanisms of this malignancy (39). Considering the multiple roles of HIF1 complex in tumor progression and metastasis, in the present study we investigated the possible causal link between HIF1α and VM in TNBC. Our data from both genetic and pharmacological approaches show that HIF1α activation is necessary for the formation of VM in this highly metastatic malignancy. The present experimental evidence brings support to the proposed use of HIF1α inhibitors in combined therapy for breast cancer with other agents that further promote tumor hypoxia, such as angiogenesis inhibitors, or in combination with conventional chemotherapy or ionizing radiation (40, 41).

Materials and Methods

Cell cultures and reagents. Breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO USA), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM glutamine (Sigma-Aldrich) and 100 mM penicillin/streptomycin (Sigma-Aldrich). For all the experiments cells were serum-starved for 18 hours and then treated with high molecular weight polyI:C at 25 μg/ml (InvivoGen, San Diego, CA USA) in FBS-free medium. HIF inhibitor acriflavine and nuclear factor κB (NF-κB) inhibitor BAY 11-7082 were from Sigma-Aldrich, and extracellular-regulated kinase (ERK) inhibitor UO126 was from Calbiochem (San Diego, CA, USA). Matrigel was from BD Biosciences (San Diego, CA, USA).

Transfection assay. One day after plating (8×10^4 cells/ml), cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) plus 10 nM siRNA targeting HIF1α (Qiagen, Venlo, the Netherlands) or the relative control siRNA (scramble), overnight. The next day, the cells were treated with poly(I:C) at 25 μg/ml in FBS-free medium for different times depending on the assay performed.

Western blot analysis and preparation of nuclear extracts. Cells were washed with cold PBS and lysates were prepared in cell lysis buffer (Cell Signaling, Danvers, MA, USA), 1% sodium dodecylsulphate-polyacrylamide (Sigma-Aldrich) and 1 mM Phenylmethanesulfonyl fluoride solution (Sigma-Aldrich). Preparation of nuclear extracts to detect nuclear HIF1α was performed as described elsewhere (25). Protein concentration was determined by bicinchoninic acid Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions and then equal amounts of proteins (25 μg) were subjected to dodecylsulphate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (GE Healthcare Life Science, Vienna, Austria). Membranes were saturated with 5% non-fat dry milk, incubated with the primary antibody at 4°C overnight and subsequently with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Membranes were then washed with T-TBS and developed using the chemiluminescence system (ECL Advance; Amersham Biosciences, San Diego, CA, USA). Anti-HIF1α was from BD Bioscences (San Diego, CA, USA); anti-c-MYC and anti-BCL2 apoptosis regulator (BCL2) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against phospho-NF-κB p65, TLR3, survivin and phospho-ERK were from Cell Signaling; anti-β-actin and anti-α-tubulin were from Sigma; anti-proliferating cell nuclear antigen (PCNA) was from Abcam (San Francisco, CA, USA). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (Bio-Rad, Hercules, CA, USA). The intensity of western blot bands was quantified by Chemidoc Gel Imaging System Image Lab 5.2.1. software (Bio-Rad) from three independent experiments.

VM assay. Firstly, 24-well plates were coated with cold matrigel (150 μl/well) and incubated at 37°C for 1 h until the gel solidified. Prior to seeding, MDA-MB-231 cells had been treated in suspension with 25 μg/ml poly(I:C) or 100 μM CoCl$_2$ (Sigma-Aldrich) for 3 h. In HIF1α-inhibition experiments, the cells were pretreated with 5 μM acriflavine for 8 h, prior to the addition of 25 μg/ml poly(I:C). Moreover, we pre-treated cells with 5 μM acriflavine for 8 h before the addition of 100 μM CoCl$_2$, then we removed the medium and added 25 μg/ml poly(I:C) to the cells. To test the inhibition of VM after genetic down-regulation of HIF1α, we transfected MDA-MB-231 cells with Lipofectamine 2000 (Invitrogen) plus 10 nM siRNA targeting HIF1α (Qiagen, Venlo, the Netherlands) or control siRNA (scramble) overnight. The formation of vessel-like channels was analyzed 16 h after plating. Images were then captured under an Eclipse TS100 ELWD 0.3/=D75 T1-SNCP inverted microscope (Nikon, Tokyo, Japan). The vessel-like channels were counted as the total number of closed polygons in random microscope fields. Eight fields/well were assessed and three experiments in triplicate were performed.

Apoptosis assays. To detect apoptotic cells using propidium iodide (PI) staining, cells were untreated or treated with 5 μM acriflavine for 8 h and then with/without 25 μg/ml poly(I:C) for 48 and 72 h and were then harvested by trypsin, washed with cold phosphate-buffered saline (PBS)-5% FBS and fixed in 70% ethanol for 24 h. After washing with PBS, cells were incubated with 1 μg/ml PI (Sigma) for

3 h at room temperature, and then examined in a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA, USA). When cell DNA content was lower than 2N (sub-G$_1$ cell population), cells were considered apoptotic. For annexin V staining, the cells were detached with trypsin, washed with PBS and then stained with annexin V-fluorescein isothiocyanate in a buffer containing PI. Annexin V-positive PI-negative cells were considered early apoptotic, while annexin V-positive PI-positive cells were considered late apoptotic.

Statistical analysis. All data are presented as the mean±SEM resulting from at least three independent experiments. Student's *t*-test was used for statistical comparison between means where applicable. *p*-Values of 0.05 or less were considered significant.

Results

TLR3 stimulation induces HIF1α and regulates HIF1α target proteins up in human breast cancer cell lines insensitive to TLR3-induced apoptosis. We examined if TLR3 stimulation by its specific agonist poly(I:C) modulated the expression of HIF1α protein in two breast cancer cell lines: MDA-MB231, a TNBC cell line (lacking estrogen, progesterone and epidermal growth factor receptors) and MCF-7, a triple-positive breast cancer cell line (estrogen- and progesterone-dependent), which are known to be insensitive to poly(I:C)-induced apoptosis (19). The cells were treated with 25 μg/ml poly(I:C) and the level of HIF1α was evaluated in total cell lysates by western blot. In MDA-MB-231 and MCF-7 cells increased levels of HIF1α were observed after poly(I:C) treatment up to 24 h (Figure 1A and B). Since activated HIF1α rapidly moves into the nucleus, we evaluated HIF1α expression in nuclear extracts in poly(I:C)-treated MDA-MB-231 cells. Our data demonstrate HIF1α nuclear translocation starting from 16 h poly(I:C) treatment (Figure 1C).

Interestingly, we previously reported that poly(I:C) induced an increase in expression of TLR3 in prostate cancer cells (42), which prompted us to examine this effect in breast cancer. Figure 1D shows that in MDA-MB-231 cells, TLR3 was significantly up-regulated upon poly(I:C) stimulation after 16 and 24 h.

Moreover, as the oncogene c-*MYC* and the anti-apoptotic genes BCL2 and survivin are known to be putative targets of HIF1α (43-45), MDA-MB-231 and MCF-7 cells were treated with poly(I:C) for different times and the expression of these proteins was evaluated by western blot. A significant increase in c-MYC was observed at 16 and 24 h both in total cell lysates (Figure 2A) and in the nuclear extracts (Figure 2B); in MCF-7 total lysates, the c-MYC expression level peaked at 4 h (Figure 2C).

In addition, BCL2 protein expression was enhanced after 16 h and 24 h poly(I:C) treatment (Figure 3A) in MDA-MB-231 cells but not in MCF-7 cells (Figure 3C). Conversely, expression of survivin, a unique member of the inhibitor of apoptosis protein family, was significantly increased in MCF-7 cells treated with poly(I:C) (Figure 3D) but not in MDA-MB-
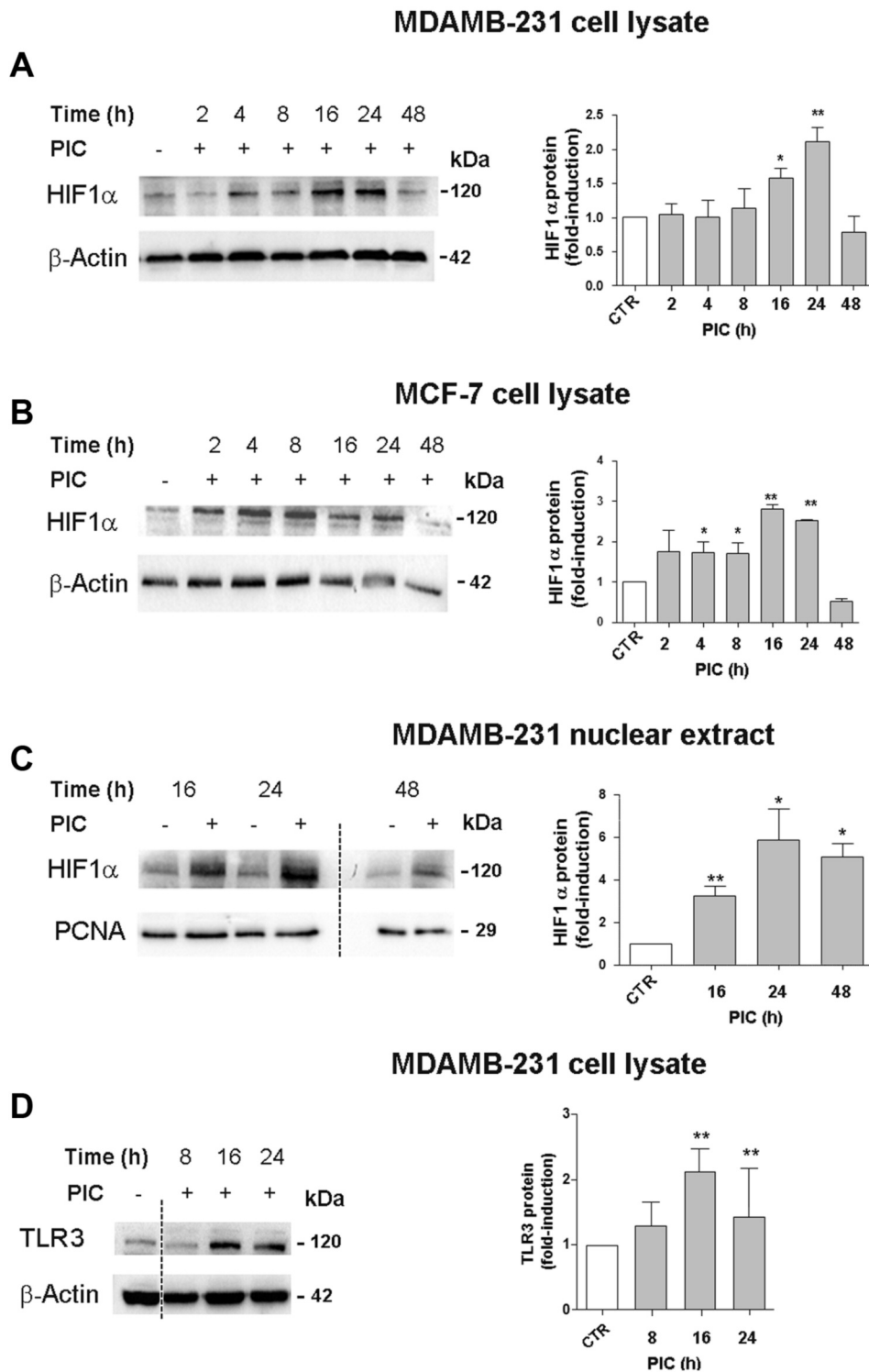
Figure 1. *Effect of polyinosinic:polycytidylic acid (PIC) on hypoxia-inducible factor 1α (HIF1α) accumulation in MDA-MB-231 and MCF-7 cells. Cells were stimulated with PIC for the indicated times and whole-cell extracts were analyzed by western blot (left panel) and quantified (right panel) for HIF1α (A, B, C). Western blots and quantification of toll-like receptor-3 (TLR3) in nuclear extracts from MDA-MB-231 cells treated with PIC are shown in D. The histograms represent the densitometric analysis of three separate experiments with the value of untreated cells set as 1. β-Actin and proliferating cell nuclear antigen (PCNA) were used to control for equal loading of proteins or whole and nuclear extracts, respectively. Significantly different from the control (CTR) at *p<0.05, and **p<0.01.*
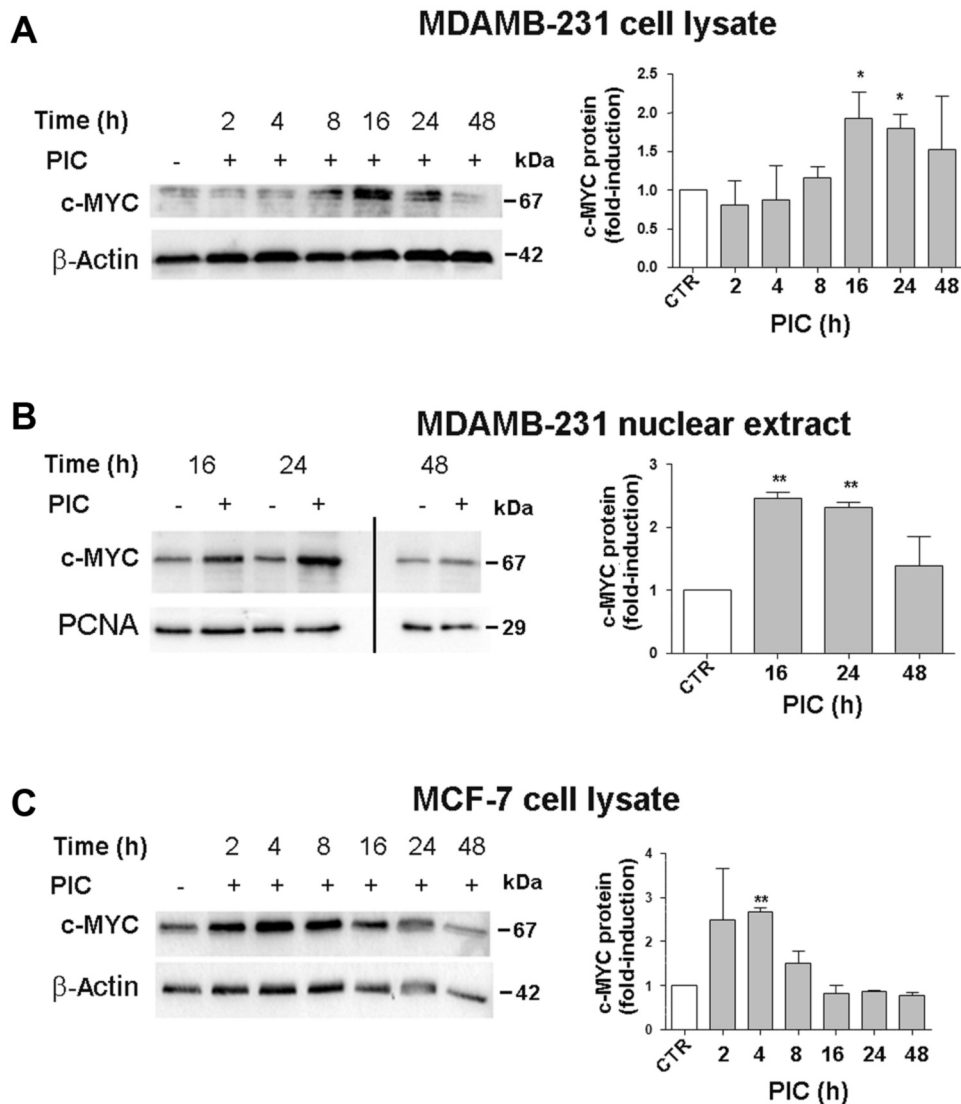
Figure 2. *Effect of polyinosinic:polycytidylic acid (PIC) on c-MYC expression in MDA-MB-231 and MCF-7 cells. Cells were stimulated with PIC for the indicated times and whole-cell extracts of MDA-MB-231 (A) and MCF-7 cells (C) were analyzed by western blot for c-MYC. Western blots and quantification of c-MYC in nuclear extracts from MDA-MB-231 cells treated with PIC are shown in B. The histograms represent the densitometric analysis of three separate experiments with the value of untreated cells set as 1. β-Actin and proliferating cell nuclear antigen (PCNA) were used to control for equal loading of proteins of whole-cell and nuclear extracts, respectively. Significantly different from the control (CTR) at *p<0.05, and **p<0.01.*

231 cells (Figure 3B). These results suggest that up-regulation of HIF1α protein induces overexpression of anti-apoptotic and pro-tumor proteins after poly(I:C) stimulation in human breast cancer cell lines insensitive to poly(I:C)-induced apoptosis.

HIF1α inhibition promotes sensitivity to poly(I:C)-induced apoptosis and reduces up-regulation of c-MYC and TLR3 proteins in MDA-MB-231 cell line. Experimental evidence on different cell types reported an anti-apoptotic role for HIF1α (25, 46, 47), therefore, given the low sensitivity of MDA-MB-231 cells to poly(I:C)-induced apoptosis (48),

through pharmacological HIF1α inhibition, we investigated whether HIF1α plays a role in resistance to this apoptotic stimulus in these cells. MDA-MB-231 cells were pre-treated with 5 μM acriflavine, an FDA-approved small molecule HIF1 inhibitor (49), for 8 h and then with poly(I:C) for 48 and 72 h and apoptosis was evaluated using PI staining. As shown in Figure 4A, poly(I:C)-induced apoptosis was increased in acriflavine/poly(I:C)-treated cells compared with those treated with poly(I:C) alone only slightly after 48 h but more markedly after 72 h. Figure 4B shows the inhibitory effect of acriflavine on HIF1α protein expression.
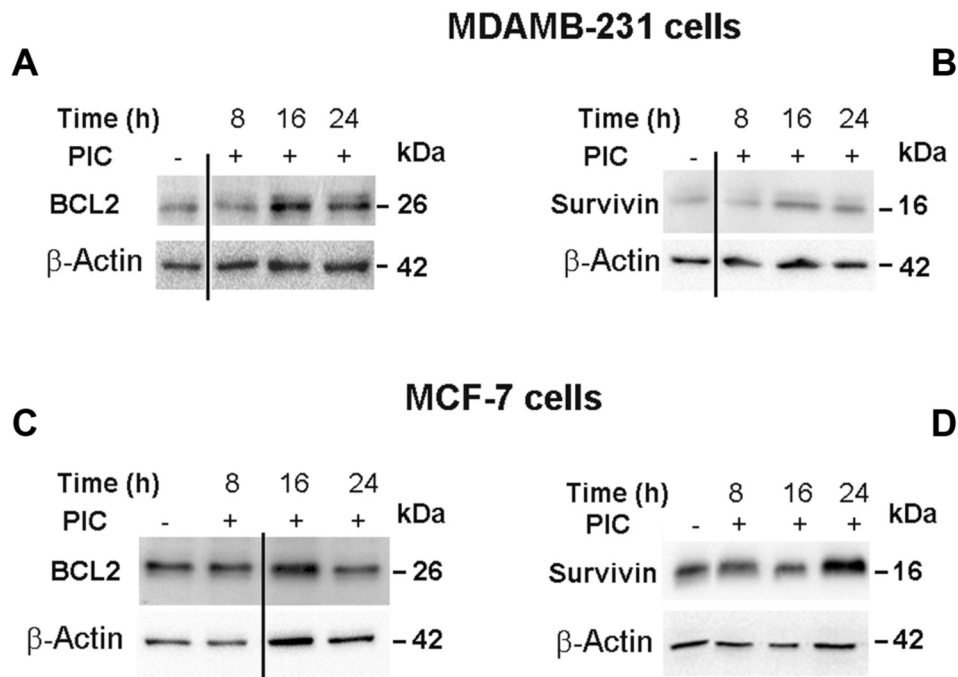
MDAMB-231 cells

Figure 3. *Effect of polyinosinic:polycytidylic acid (PIC) on BCL2 apoptosis regulator (BCL2) and survivin expression in MDA-MB-231 (A, B) and MCF-7 cells (C, D). Both cell lines were stimulated with PIC for the indicated times and whole-cell extracts were analyzed by western blot for BCL2 (left panel) and survivin (right panel). β-Actin was used to control for equal loading of proteins. Each blot is representative of three.*
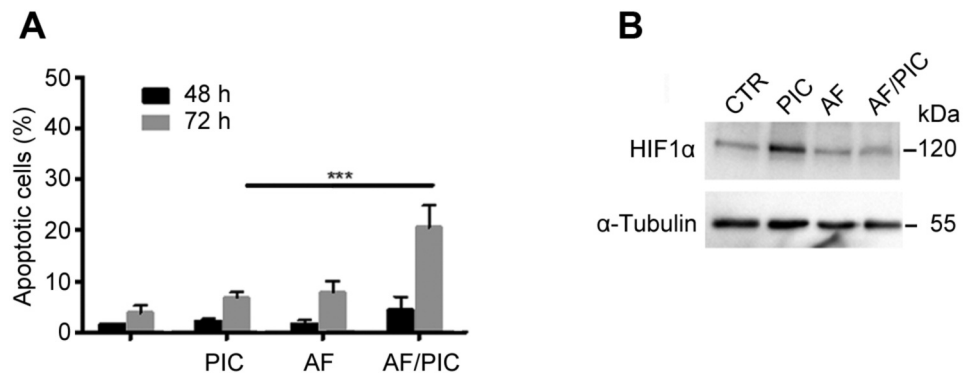

Figure 4. *Effects of hypoxia-inducible factor 1 (HIF1) inhibitor acriflavine (AF) on polyinosinic:polycytidylic acid (PIC)-mediated apoptosis of MDA-MB-231. A: Apoptosis as shown by propidium iodide staining of MDA-MB-231 cells treated with 5 μM AF for the indicated times. By flow cytometry, when the cell DNA content was a peak lower than 2N (sub-G_1 cell population), cells were considered apoptotic. Data represent the mean of three independent experiments. B: Cells were treated for 16 h with PIC with/without AF and the whole-cell lysates were analyzes by western blot to evaluate c-MYC expression. β-Actin was used to control for equal loading of proteins of whole-cell extracts. The blot is representative of three. ***Significantly different from the control (CTR) at p<0.001.*

To investigate whether the up-regulation of HIF1α is crucial for overexpression of c-MYC and TLR3 proteins after poly(I:C) treatment, MDA-MB-231 cells were transiently transfected with siRNA targeting HIF1α or with control siRNA and then treated with poly(I:C). After confirming by western blot that poly(I:C)-induced HIF1α protein expression was significantly reduced in cells with specific HIF1α siRNA (Figure 5A), the levels of c-MYC and TLR3 proteins were evaluated. Western blot showed that HIF1α siRNA significantly inhibited the increase in poly(I:C)-induced c-MYC and TLR3 expression (Figure 5B), demonstrating that c-MYC and TLR3 are targets of the HIF1 complex.
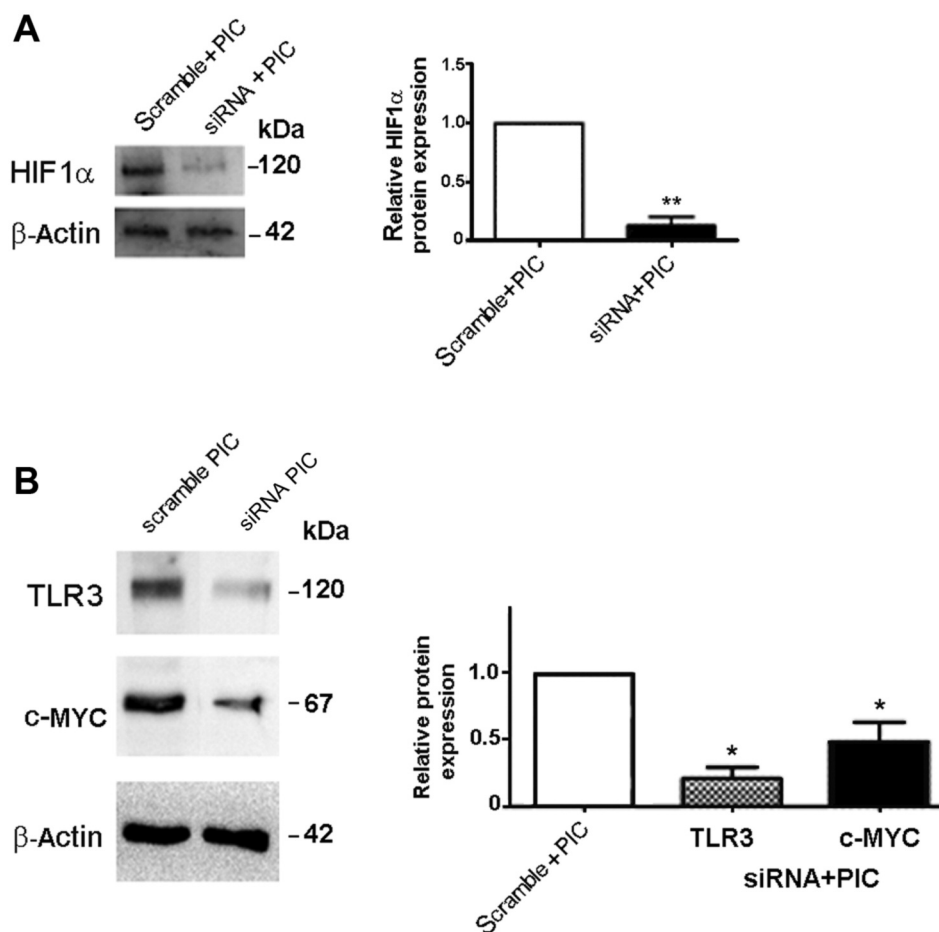
Figure 5. *Specific down-regulation of hypoxia-inducible factor 1α (HIF1α) inhibits polyinosinic:polycytidylic acid (PIC)-induced c-MYC and toll-like receptor-3 (TLR3) protein expression in MDAMB-231 cells. A: MDA-MB-231 cells were transfected with scramble RNA or siRNA against HIF1α, treated with 25 μg/ml PIC for 16 h, then analyzed for HIF1α protein by western blot (left panel). B: c-MYC and TLR3 proteins in transfected MDA-MB-231 cells treated with 25 μg/ml PIC for 16 h were analyzed by western blot (left panel). Densitometric analysis of three separate experiments is shown (right panels). β-Actin was used to control for equal loading of proteins of whole-cell extracts. Significantly different from scramble+PIC at *p<0.05, and **p<0.01.*

TLR3 stimulation induces HIF1-dependent VM in MDA-MB-231 cells. It has been reported that HIF1α protein can promote vasculogenic mimicry (VM) in some types of tumors (50). Here we investigated whether poly(I:C)-induced HIF1α protein promoted the formation of vessel-like channels in MDA-MB-231 cells. As shown in Figure 6A, these cells tend to adopt a chord-like phenotype and formed an arborizing pattern on Matrigel but, interestingly, formed more interlacing VM channels when treated with poly(I:C). To investigate the role of HIF1α in poly(I:C)-induced VM, we analyzed the effect of acriflavine alone or in combination with poly(I:C) on VM channel formation. HIF1α inhibitor acriflavine, as single treatment, slightly reduced the basal formation of vessel-like channels in MDA-MB-231 (data not shown), as expected, given the basal expression of HIF1α in this cell line

(Figure 1). As shown in Figure 6B, HIF1α inhibitor in combination with poly(I:C) abolished VM channel formation compared to poly(I:C) alone, demonstrating the involvement of HIF1α in promoting VM following TLR3 stimulation.

To confirm a key role of HIF1α protein in VM channel formation in breast cancer cells, we investigated the MDA-MB-231 VM levels after treatment with $CoCl_2$, a chemical hypoxia-mimetic, that induces stabilization of HIF1α protein. As shown in Figure 6B, $CoCl_2$, as well as poly(I:C), promoted the formation of vessel-like channels, whereas in combination with HIF1α inhibitor acriflavine, this biological feature was markedly reduced. Data obtained after genetic inhibition of HIF1α confirmed a pivotal role of HIF1α in VM channel formation in MDA-MB-231 cells after poly(I:C) treatment (Figure 6C).
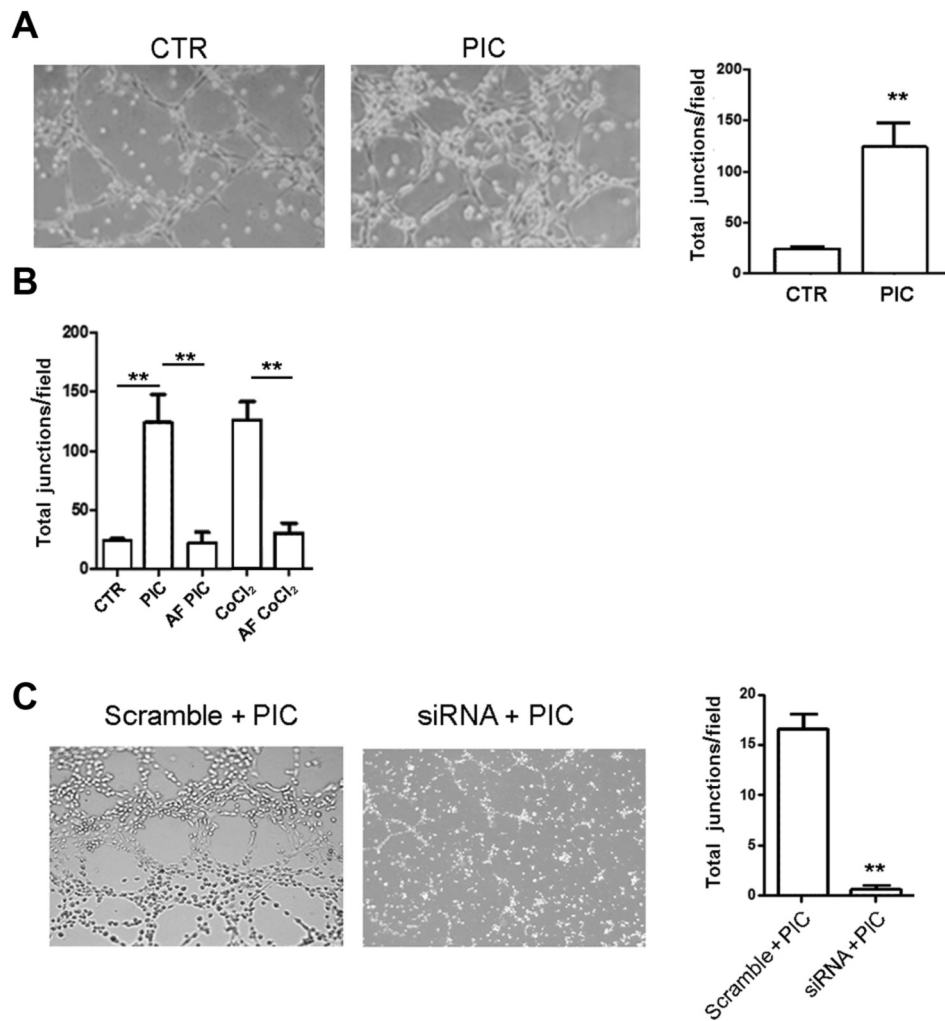
Figure 6. *Toll-like receptor-3 (TLR3) stimulation induces hypoxia-inducible factor 1α (HIF1α)-dependent vasculogenic mimicry (VM) in MDA-MB-231 cells. A: After treatment with 25 μg/ml polyinosinic:polycytidylic acid (PIC), MDA-MB-231 cells exhibited typical pipe-like VM networks on Matrigel compared with untreated cells. Representative images of one of three independent experiments are shown (left panel). The vessel-like channels were counted as the total number of closed polygons in random microscopy fields (right panel). In each well, eight fields/well were counted and three experiments in triplicate were performed. B: MDA-MB-231 cells were pretreated with 100 μM CoCl2 or 25 μg/mL PIC for 3 h with or without 5μM acriflavine (AF), HIF1 inhibitor. After 16 h, VM formation was analyzed and vessel-like channels were counted. C: Representative images from one of three independent experiments of MDA-MB-231 cells transfected with scramble or HIF1α siRNA and treated with 25 μg/ml PIC for 16 h (left panel). The vessel-like channels were counted as total number of closed polygons in random microscope fields (right panel). Significantly different from the control (CTR) or scramble+PIC at **p<0.01.*

TLR3 and HIF1α up-regulation induced by poly(I:C) stimulation in MDAMB-231 cells is downstream of NF-κB and phospho-ERK activation. Since poly(I:C) is known to activate NF-κB and mitogen-activated protein kinases in other cancer cell types (23), in order to evaluate signal transduction pathways upstream of HIF1α activated by poly(I:C), we analyzed the levels of phospho-p65 NF-κB subunit and phospho-ERK in MDAMB-231 cells after poly(I:C) treatment. Western blot analysis showed an increase of phospho-p65 and phospho-ERK starting from 10 min up to 20 min after

poly(I:C) (Figure 7A). To investigate the role of NF-κB and ERK signaling in poly(I:C)-induced expression of HIF1α, cells were pre-treated with BAY 11-7082 and U0126, selective inhibitors of NF-κB and ERK, respectively, before adding poly(I:C). Both inhibitors reduced the level of HIF1α examined after 24 h of poly(I:C) treatment (Figure 7B and C). These data demonstrate that the increase of HIF1α induced by poly(I:C) occurs downstream of NF-κB and ERK activation. Since we showed that HIF1α down-regulation by specific siRNA inhibited poly(I:C)-induced TLR3 up-regulation (Figure 5B),
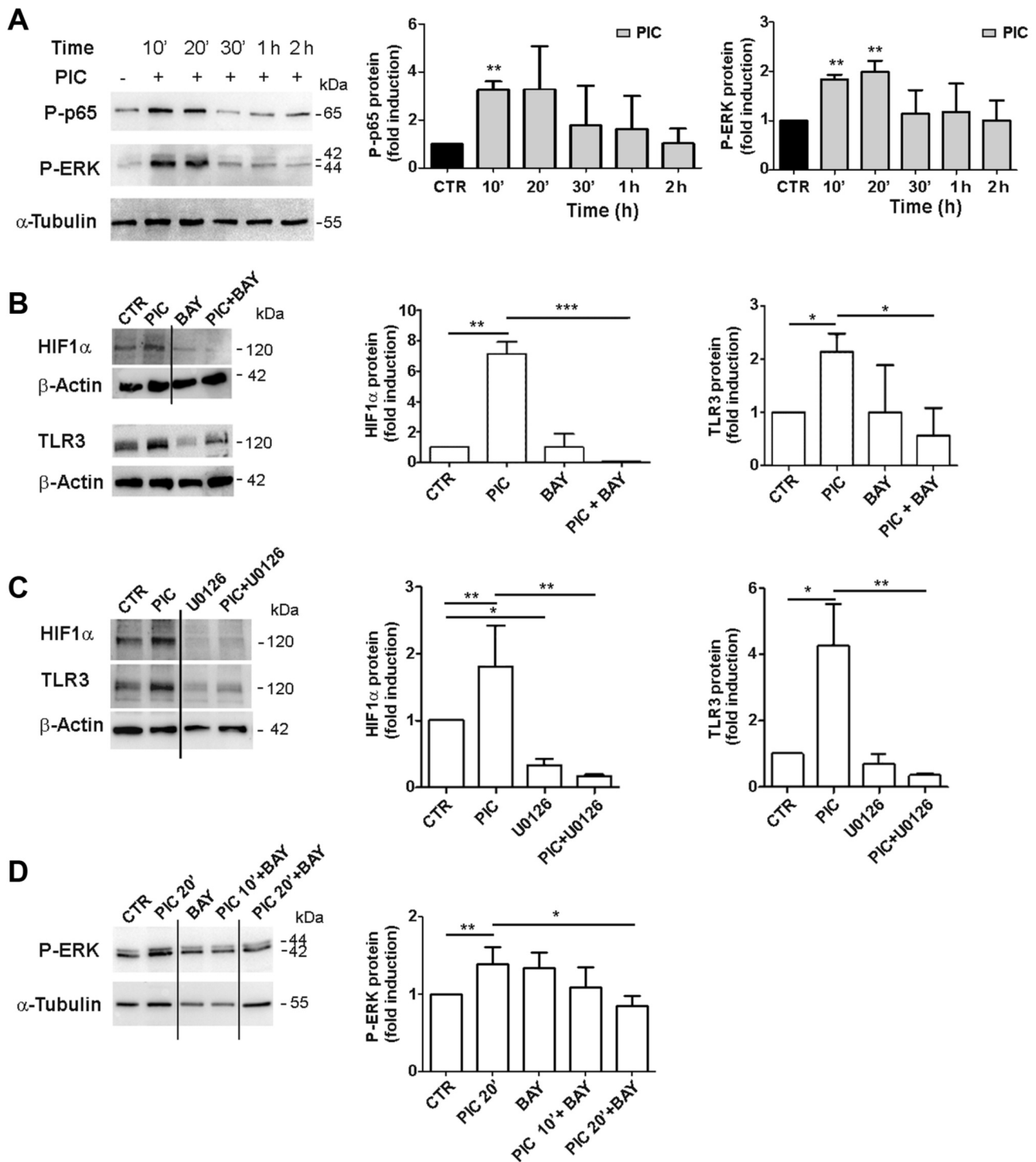
Figure 7. *Toll-like receptor-3 (TLR3) and hypoxia-inducible factor 1α (HIF1α) up-regulation induced by polyinosinic:polycytidylic acid (PIC) in MDA-MB-231 cells is downstream of nuclear factor κB (NF-κB) and extracellular-regulated kinase (ERK) activation. A: MDA-MB-231 cells were treated with 25 μg/ml PIC for different times and whole-cell extracts were analyzed by western blot for phospho-(p)-p65 and p-ERK proteins. B, C: Cells were treated with 10 μM NF-kB inhibitor BAY 11-7082 (B) or 10 μM ERK inhibitor U0126 (C) in addition to 25 μg/ml PIC for 24 h and whole-cell extracts were analyzed by western blot for HIF1α and TLR3 proteins. D: Cells were treated with 10 μM BAY 11-7082 in addition to 25 μg/ml PIC for 24 h and whole-cell extracts analyzed by western blot for phospho-ERK. β-Actin and α-tubulin were used as protein loading controls. The histograms represent the densitometric analysis of three separate experiments with the value for untreated cells set being as 1. Significantly different from the control (CTR) at *p<0.05, **p<0.01 and ***p<0.001.*

we analyzed the effect of BAY 11-7082 and U0126 on TLR3 expression. As expected, NF-κB and ERK inhibition significantly reduced TLR3 expression (Figure 7B and C).

To investigate whether phospho-ERK is downstream of NF-κB in this signaling pathway, MDA-MB-231 cells were pre-treated for 1 h with BAY 11-7082, before adding poly(I:C). We detected reduced levels of phospho-ERK after treatment with BAY+poly(I:C), compared with poly(I:C) alone (Figure 7D). Conversely, treatment with phospho-ERK inhibitor U0126 failed to reduce phospho-p65 induction (data not shown). This demonstrates that the increase of phospho-ERK after poly(I:C) occurs downstream of NF-κB activation.

Our findings are shown in a schematic model of downstream targets after poly(I:C)-induced HIF1α up-regulation by NF-κB signaling pathway in human breast cancer cells (Figure 8).

Discussion

Stimulation of TLRs with microbial components may represent a double-edged sword in cancer: a signal that promotes a pro-inflammatory environment to enhance tumor growth and chemoresistance or a signal that directly induces tumor cell death and activation of antitumor immune response. Data previously obtained in our laboratory on human prostate cancer cell lines showed that poly(I:C) induces a strong TLR3-mediated apoptotic response in LNCaP cells, whereas PC3 cells are less sensitive to this stimulus because of the up-regulation of HIF1α protein in this cancer cell line (25). More recently, an article reported that in the human breast cancer cell lines MDA-MB-231 and MCF-7, poly(I:C) treatment failed to induce apoptosis (51), but the mechanism of poly(I:C) resistance of these breast cancer cells has not been clarified yet. In the present work we show that apoptosis resistance of breast cancer cells is paralleled by increased expression and activity of HIF1α protein. Considering the multifaceted roles of HIF1α in tumor progression and metastasis, this specific pathway is a crucial target for many drugs and a combined therapeutic approach based on HIF1α inhibitors and TLR3 agonist could represent a promising strategy.

Our results suggest that the overexpression of HIF1α protein in human breast cancer cells might play a key role in resistance to poly(I:C) stimulation through both up-regulation of pro-tumoral and anti-apoptotic proteins and, importantly, the induction of VM.

In the present study we observed that poly(I:C) treatment efficiently increased expression of the HIF1α target c-MYC in MDA-MB-231 and MCF-7 cell lines and, interestingly, poly(I:C)-induced nuclear accumulation of c-MYC in MDA-MB-231 cells suggests an increase of c-*MYC* transcriptional activity, enhancing pro-tumor effects, which is in accordance with poly(I:C)-induced features in pharyngeal cancer cells (52). Accordingly, we detected a significant reduction of
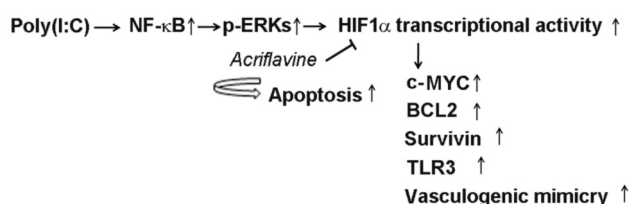

Figure 8. *The scheme summarizes the effect of toll-like receptor-3 (TLR3) stimulation by its ligand polyinosinic:polycytidylic acid [poly(I:C)] on the human triple-negative breast cancer cell line MDA-MB-231. PIC treatment activates the signal transduction pathway involving nuclear factor k B (NF-κB) and extracellular signal-regulated kinases (ERKs) resulting in increased levels and nuclear translocation of hypoxia-inducible factor 1α (HIF1α), with consequent induction of HIF1 transcriptional activity. HIF1 activation mediates the increase of PIC receptor TLR3, and of the protumour and anti-apoptotic proteins c-MYC, BCL2 apoptosis regulator (BCL2) and survivin, and promotes a phenomenon involved in tumor progression called vasculogenic mimicry. Moreover, the inhibition of HIF1 by acriflavine induces increased apoptosis of PIC-treated cells.*

poly(I:C)-induced c-MYC protein after HIF1α down-regulation by siRNA. Unlike c-MYC, we observed an induction of different anti-apoptotic proteins after poly(I:C) treatment in two breast cancer cell lines according to HIF1α expression increase, such as BCL2 up-regulation in MDA-MB-231 cells and survivin increase in MCF-7 cells. This is in accordance with a report that HIF1α is an important transcriptional regulator of survivin expression in non-small lung cell cancer (45) and in multiple myeloma (53).

Moreover, HIF1α-specific siRNA surprisingly led to a reduced level of TLR3. In our hypothesis, the TLR3 activator poly(I:C) induces HIF1α accumulation, indicating that TLR3 is upstream of HIF1α protein. However, it has been demonstrated in oral squamous cell carcinoma that activation of TLR3 stimulated the expression of *HIF1* through NF-κB. In addition, HIF1α increased the expression of TLR3 through direct promoter binding, thus, the TLR3–NF-κB pathway forms a positive feedback loop with HIF1 in oral squamous cell carcinoma (54). Our data highlight the existence of this regulatory loop also in our system.

VM plays an important role in cancer progression by providing an extra blood supply for a malignant tumor. Many studies reported that VM is related to hypoxia-activated signaling pathways and metastatic phenotype (50, 55). It has been demonstrated that the overexpression of HIF1α protein is associated with VM in human gallbladder cell lines (56) as well as in breast cancer (57). TNBC is a subtype which is aggressive, metastasizes and has a poor prognosis. A previous study showed greater VM formation in patients with TNBC than in those with non-TNBC, which supports the hypothesis that VM formation is positively associated with life- threatening TNBC (58). Our present findings indicate that pharmacological and genetic HIF1α inhibition significantly reduced channel formation,

showing that HIF1α is the crucial mediator of VM in human TNBC cell line MDA-MB-231. Moreover, to further verify HIF1α involvement in VM, we mimicked hypoxia using cobalt chloride and observed a strong increase in vessel-like structure formation which was totally blocked by HIF1α siRNA as well as in the presence of the pharmacological HIF1 inhibitor acriflavine. Altogether our data on TNBC agree with other studies in different tumor types (57, 59) and interestingly provide the first evidence that TLR3 stimulation induces HIF1α-mediated VM in a very aggressive breast cancer cell line.

Regarding signaling pathways upstream of poly(I:C)-induced HIF1α in MDA-MB-231 cells, it is known that in normoxic conditions, several stimuli, such as cytokines and growth factors, up-regulate HIF1α at the transcriptional level. Accordingly, we reported that stimulation of TLR3 with poly(I:C) increased HIF1α mRNA expression in prostate cancer cells (25). Han *et al.*'s work demonstrated that up-regulation of HIF1α is directly correlated with activation of TLR3 through NF-κB signaling (54). Similarly, in our work we investigated transcriptional factors and signaling upstream of poly(I:C)-induced HIF1α up-regulation and our data show that the increase of HIF1α is dependent on NF-κB signaling, indicating that NF-κB may regulate HIF1α gene transcription through binding with its promoter, as previously reported (60). Moreover, ERK-associated up-regulation of HIF1α in pancreatic adenocarcinoma was shown (61). In accordance with these previous works, using specific inhibitors, we showed NF-κB-dependent ERK phosphorylation that is crucial in increasing HIF1α and HIF1α-dependent TLR3 up-regulation.

Altogether our results indicate that up-regulation of HIF1α protein in human breast cancer cells might play a key role in resistance to poly(I:C) stimulation through both induction of pro-tumoral and anti-apoptotic proteins and the increase of VM. Studies *in vivo* with combined treatment with poly(I:C) and HIF1 inhibitors are necessary to provide conclusive evidence for TLR3-mediated effects in breast cancer, contributing to the refinement of diagnostic tools and possibly the development of novel targeted therapeutic strategies.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

Conceptualization, E.Z., A.R. and A.F.; methodology, F.S.; A.DA.; R.F.; software, R.F. and A.D.A; formal analysis, F.P.; investigation, F.S.; writing-original draft preparation, A.R. and P.DC; writing-review and editing, F.M. and A.F.; supervision, A.F.; A.R. and P.D.C; funding acquisition, A.F. and A.R.

Acknowledgements

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