Hypoxia-induced down-modulation of PKCε promotes TRAIL-mediated apoptosis of tumor cells

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Abstract. Tumor oxygen status is considered as a prognostic marker that impacts on malignant progression and outcome of tumor therapy. TNF-related apoptosis inducing ligand (TRAIL) plays a key role in cancer immunity, with potential applications in cancer therapy. Protein kinase C (PKC)ε, a transforming oncogene, has a role in the protection of cardiomyocytes and neurons from hypoxia-induced damage while, it can also modulate the susceptibility of tumor cells to TRAIL-induced cell death. Here we demonstrate that hypoxia induces a tumor cell phenotype highly sensitive to the cytotoxic effects of TRAIL. Based on the observation that: i) PKCε expression levels are impaired during hypoxia, ii) the overexpression of PKCε, but not of a kinase-inactive PKCε mutant, is able to revert the hypoxia-induced sensitivity to TRAIL, iii) the down-modulation of PKCε levels by RNA interference, on the contrary, induces the highly TRAIL-sensitive phenotype, iv) the inhibition of hypoxia-inducible transcription factor-1α (HIF-1α) by specific siRNA blocks both the hypoxia-induced down-modulation of PKCε and the induction of the highly TRAIL-sensitive phenotype; we conclude that the HIF-1α upregulation during hypoxia is associated to PKCε down-modulation that likely represents the key molecular event promoting the apoptogenic effects of TRAIL in hypoxic tumor cells.

Introduction

Hypoxic extracellular environments have been described in normal tissues (1) and during chronic inflammatory and malignant diseases (2). Compared with the regular, ordered vasculature of normal tissues, blood vessels in cancer are often highly abnormal, with distended capillaries, leaky walls, and sluggish flow (3). Hypoxic regions are thus typical of virtually all solid tumors. Nevertheless, hypoxia exists also in normal, non-pathological tissues; low oxygen tensions (between 4 and 34 torr, or 0.5-4.5%) have been measured, for instance, in lymphoid organs (bone marrow, thymus, lymph nodes and spleen) and in non-lymphoid organs (bone, liver), as compared with 150 torr or 20% oxygen concentration in the atmosphere (4).

Hypoxia reduces the possibility for a cell to maintain its energy levels and, in severe or prolonged hypoxia, cells initiate the process of programmed cell death. Some cells, however, can adapt to the environmental stress, escape necrosis and apoptosis, and survive. Lymphocytes in general, and cytotoxic cells in particular, have been shown to be able to adapt and work under hypoxia, as reflected by the observation of lymphocyte infiltration and cytotoxicity in hypoxic solid tumors (1). Unfortunately however, in solid tumors *in vivo*, a number of factors associated either directly or indirectly with hypoxia contribute to the resistance to anticancer drugs and to ionizing radiation (5). It is generally believed that hypoxia accelerates malignant progression and metastasis. Most genes induced by hypoxia are regulated by the hypoxia-inducible transcription factor HIF-1α, a protein that therefore plays a crucial role in tumor development (6). At atmospheric oxygen levels, HIF-1α protein is rapidly degraded due to oxygen-dependent hydroxylation by prolyl-hydroxylase domain-containing proteins and subsequent turnover by a von Hippel-Lindau tumor suppressor protein (pVHL)-dependent degradation pathway (6).

The epsilon isoform of protein kinase C (PKCε) has received considerable attention in multiple forms of cell protection from hypoxia (7), the most notable of which is the preconditioning against myocardial ischemia/reperfusion injury, where anti-apoptotic mitochondrial proteins are targeted by PKCε. A similar role of PKCε has been described in the

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protection of neurons from ischemic injury (7). Like other anti-apoptotic molecules, PKCε has been shown to induce transformation in fibroblasts and epithelial cells, generating a highly tumorigenic phenotype *in vivo* (8). Furthermore, PKCε levels have been found to be a predictive biomarker of aggressiveness in breast cancer, and specific disruption of PKCε resulted in a significant inhibition of tumorigenesis and metastasis (9). Recently, we have demonstrated that PKCε is able to protect normal erythroblasts from cell death induced by extracellular apoptogenic agents (10) and emerging evidence shows that PKCε can modulate the susceptibility to cell death induced by molecules belonging to the tumor necrosis factor (TNF) family in gliomas (11) and breast carcinoma cells (12). Thus the expression levels of PKCε, that can be modified by physical (UV, oxygen) and biological stimuli (cytokines), can address cell destiny toward apoptosis, proliferation or malignant transformation (10,13,14).

TNF-apoptosis inducing ligand (TRAIL), a member of the TNF family of proteins, is highly toxic to tumor cells while, with some exceptions, it spares normal cells. This unique feature of selectivity for cancer cells has drawn considerable attention to TRAIL as a potential therapeutic agent against human cancers. Both in its soluble and membrane bound form, TRAIL is used by natural killer cells and cytotoxic T cells to kill tumors (15,16). Hypoxia has been shown either to protect lung and colon carcinoma cell lines from TRAIL-induced apoptosis (17,18), or to increase TRAIL-induced cell death of prostate adenocarcinoma and of hepatoma cell lines (19,20). Moreover, the cytotoxic activity of anti-tumor NK and CD8 T cells is largely based on death ligands/receptor interaction.

There is, however, a lack of data available on the activity of TRAIL in a hypoxic environment. Because virtually all tumors stably or transiently develop in hypoxic/anoxic conditions, we decided to study the effects of hypoxia on tumor cell sensitivity to TRAIL-induced apoptosis. As cellular models, we have chosen different cell lines that: i) have a different spontaneous sensitivities to TRAIL (sensitive: SupT1 and A375; moderately sensitive: Jurkat, DLD2 and K562; resistant: U2OS and HeL), ii) belong to different lineages [hematopoietic (HeL, K562, Jurkat, SupT1), colon carcinoma (DLD2), osteosarcoma (U2OS) and melanoma (A375)] whose growth, differentiation invasiveness are strongly affected by local oxygen tension (21-24).

Materials and methods

Cell cultures and treatment. Erythroleukemia human cell lines K562 and HeL, thymic human cell line SupT1 (CD7+), T lymphoid cell line Jurkat were all cultured in RPMI and 10% of fetal bovine serum (FBS) at the optimal concentration of $5x10^5$ cells/ml. Melanoma human cell line A375 was expanded in DMEM and 10% of FBS, colon carcinoma human cell line DLD2 was cultured in RPMI and 10% of FBS, osteosarcoma human cell line U2OS was expanded in MEM and 10% of FBS. All adherent cell lines (A375, DLD2 and U2OS) were subcultured 1:10 twice at week before cell confluence. Culture media were supplemented with antibiotics and L-glutamine (Euroclone, Milan, Italy). As reversible and cell-permeable proteasome inhibitor we used carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) derivative (Merck KGaA, Darmstadt, Germany). MG132 (25 μM) was added to the cell cultures 1 h before hypoxia exposure. The expression of HIF-1α in normoxia was promoted by treating cells with 200 μM of $CoCl_2$ (Sigma, St Louis, MO) (25,26).

Hypoxia. Hypoxia was generated depleting oxygen from the cell culture atmosphere (1-2 Torr of residual oxygen) by incubating cell culture plates in the GasPak Pouch System (Becton-Dickinson, San Josè, CA) (27). GasPak Pouch System generated 1% oxygen level in 3 h of incubation.

Flow cytometric analysis. Aliquots of $5x10^5$ cells were labeled by a panel of anti-TRAIL-receptor MoAbs (Alexis Biochemical, San Diego, CA). Expression of TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 was analyzed by indirect staining using 1 μg HS101 anti-human TRAIL-R1, HS201 anti-human TRAIL-R2, HS301 anti-human TRAIL-R3, and HS401 anti-human TRAIL-R4 monoclonal antibodies, followed by PE-labeled goat anti-mouse IgG (Beckman Coulter, Fullerton, CA) as a second reagent. Control samples were stained with isotype matched irrelevant MoAbs. Analysis was performed by an Epics XL flow cytometer (Beckman Coulter) and Expo ADC software (Beckman Coulter).

MESF quantification. The absolute number of surface antigens expressed/cell was calculated. To this purpose, the flow cytometer was calibrated with a set of standardized beads (Dako) each with a known amount of fluorochrome (either FITC, RPE or Cy5) expressed in units of MESF (Molecules of Equivalent Soluble Fluorescein). Thus, a standard curve was constructed by plotting MESF values for the beads against the median channel in which the peak was displayed (28).

siRNA design and transfection. Double-strand siRNAs (dsRNA) were designed to target sequences corresponding to nt's 223 to 244, 429 to 450, 942 to 963, and 1158 to 1179 on human PKCε mRNA (NM005400). The target sequences were as follows: 5'-AAGATCAAAATCTGCGAGGCC-3', 5'-AAGATCGAGCTGGCTGTCTTT-3', 5'-AACTACAAGG TCCCTACCTTC-3', and 5'-AAAAAGCTCATTGCTGGT GCC-3'.

The respective sense and antisense RNA sequences were synthesized by Silencer siRNA Construction kit (Ambion, Austin, TX) (29). Non-specific siRNA duplexes containing the same nucleotides, but in irregular sequence (i.e. scrambled PKCε siRNA), were prepared according to the manufacturer's protocol and used as controls. HIF-1α and control siRNA were designed and synthesized as described previously (27), targeting the sequences nt 1378 to 1398 of the human HIF-1α mRNA (AF304431.1). The selectivity of PKCε and HIF-1α siRNA has been widely demonstrated previously (10,27).

The GFP-PKCε expression and control plasmids were kindly provided by Professor Peter Parker (Cancer Research UK, London Research Institute). Kinase inactive control plasmid was generated introducing the K552M mutation to the GFP-tagged PKCε construct (30).

Adherent cells were transfected with 200 nM siRNA by liposomes (Superfect transfection Reagent®, Qiagen, Hilden, Germany) previously described (27,29). The efficiency of siRNA transfection was evaluated by FITC-labeled siRNA

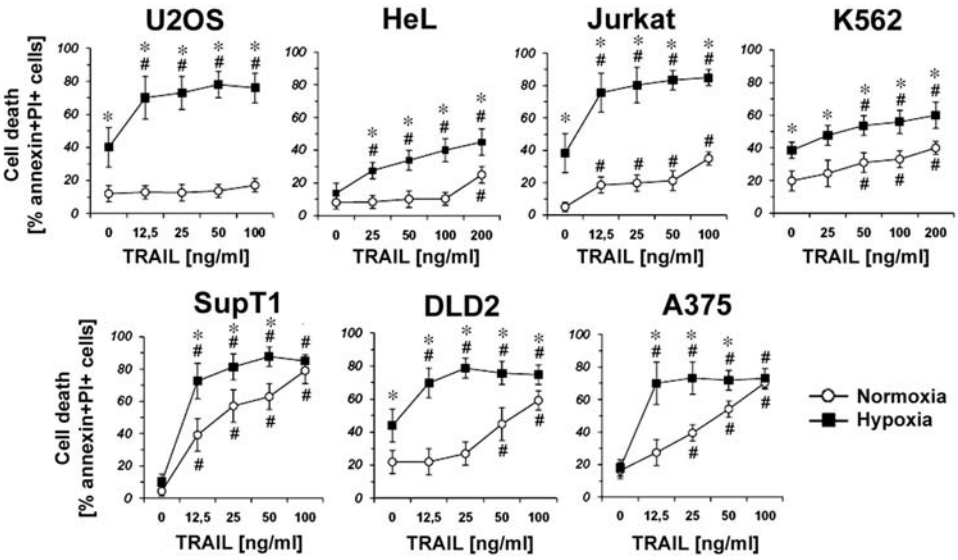

Figure 1. Hypoxia enhances the sensitivity to TRAIL. Apoptosis was analyzed by flow cytometry, staining cells with annexin V-FITC and propidium iodide (PI). Cell lines were treated with TRAIL in normoxia (empty symbols) or hypoxia (filled symbols) for 48 h. TRAIL was used at different concentrations, that are reported in the horizontal axis as ng/ml, while the percentage of killed cells is reported in the vertical axis. Data are means ± standard deviation of 4 independent experiments. ANOVA and Dunnet's test analysis: $^{\#}p<0.05$ comparison vs. TRAIL-untreated cells. t-test analysis: $^{*}p<0.05$ hypoxia vs. normoxia.

(Qiagen) and analyzing the fluorescence emission 24 h post-transfection by flow cytometry.

Semi-quantitative reverse-transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated using the RNeasy mini kit (Qiagen). Total RNA (1 μg) was reverse transcribed with Malone murine leukemia virus (MMV) reverse transcriptase, and progressive dilutions (1/10, 1/50, 1/250) were subjected to PCR amplification to detect ß-actin and PKCε cDNA.

PCR was performed under the following reaction conditions: 95˚C for 30 sec, 56˚C for 30 sec, 72˚C for 30 sec. Thirty-five cycles of amplification and a final extension at 72˚C for 5 min were used. The sequence of primers used for PCR was as follows: ß-actin, 5'-TGACGGGGTCACCCA CACTGTGCCCATCTA-3' (sense) and 5'-CTAGAAGCAT TTGCGGTGGACGATGGAGGG-3' (antisense); PKCε, 5'-C AATGGCCTTCTTAAGATCAAAA-3' (sense) and 5'-CCT GAGAGATCGATGATCACATAC-3' (antisense) (10).

Western blotting. Cultured cells were counted and $2x10^6$ cells were collected at specific time points, washed in PBS, and centrifuged at 200 g for 10 min. Pellets were resuspended in a cell-lysis buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mM Na_3VO_4; 1 mM NaF) supplemented with fresh protease inhibitors, and protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL). Proteins from each sample (30 μg) were then migrated in 5% SDS-acrylamide gels and blotted onto nitrocellulose filters.

Blotted filters were blocked and incubated with specific primary antibodies diluted as described in the manufacturers' protocols. Specifically, rabbit polyclonal anti-PKCε antibodies (Upstate, Lake Placid, NY) were used at the concentration of 1 μg/ml, anti-ß-tubulin (Sigma) was diluted 1:5000 (10), while anti-Bcl-xL polyclonal antibody (Cell Signaling Technology, Beverly, MA) was diluted 1:1000 before use (14).

Filters were washed and further incubated for 1.5 h at room temperature with 1:5000 peroxidase-conjugated anti-rabbit or with 1:2000 peroxidase-conjugated anti-mouse IgG (Pierce) in the primary antibody working solution at room temperature. Specific reactions were revealed with the ECL Supersignal West Pico Chemiluminescent Substrate detection system (Pierce).

Assessment of apoptosis. Apoptotic cells were identified by flow cytometry by annexin V/propidium iodide (PI) staining. Briefly, cells were stained by FITC conjugated annexin V (Actiplate; Valter Occhiena, Torino, Italy) in Ca^{2+} and PI staining buffer, following the manufacturer's protocol. Apoptosis is reported in the histograms as % of annexin V^+ PI^+ cells.

Statistical analysis. The paired t-test was used to compare the mean values from couples of related samples; for multiple comparisons, ANOVA followed by Dunnet's test (Primer of Biostatistic 4.02) was used.

Results

Hypoxia induces a phenotype highly sensitive to TRAIL. When cells were treated with increasing concentrations of TRAIL for 48 h in hypoxia, they showed a significant increase ($^{*}p<0.05$) of sensitivity to the apoptogenic effect of TRAIL as compared to cells grown in normoxia (Fig. 1). Similar results were obtained when TRAIL was administered for 24 h. This effect was evident in TRAIL-sensitive cell lines (SupT1, A375, Jurkat and DLD2) as well as in TRAIL-resistant cell lines (HeL and U2OS). In particular: i) the TRAIL-resistant U2OS and HeL cell lines (that, in normoxia, are not killed by 100 ng/ml of TRAIL) become highly (U2OS) or moderately

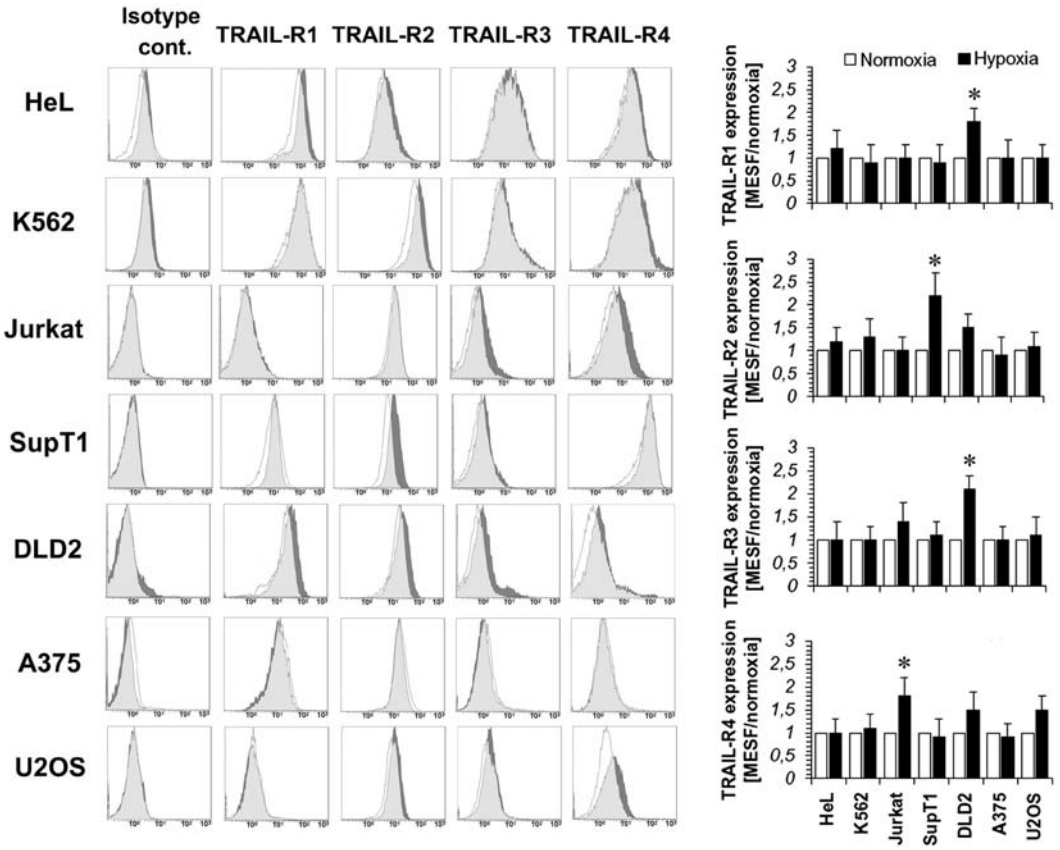

Figure 2. Hypoxia and cell surface expression of TRAIL receptors. Flow cytometry analysis of TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 expression on the reported tumor cell lines cultured 48 h in normoxia or hypoxia. Control samples were stained with isotype matched irrelevant MoAbs (Isotype cont.). Histograms obtained staining normoxic cells (white) are superimposed to those of hypoxic cells (dark grey). Light grey areas correspond to histogram overlappings. Expression of TRAIL receptors is also reported as MESF of hypoxic cell culture/MESF of control cell cultures (cell lines grown in normoxia). Data are reported as means ± standard deviation of 3 independent experiments. t-test analysis: $^{*}p<0.05$ hypoxia vs. normoxia.

(HeL) sensitive to TRAIL in hypoxia ($^{*}p<0.05$), ii) the Jurkat and K562 cell lines (that, in normoxia, are killed at a rate <30% by 100 ng/ml of TRAIL) became highly (Jurkat) or moderately (K562) sensitive to TRAIL in hypoxia ($^{*}p<0.05$), as well, finally, iii) the TRAIL-sensitive cell lines SupT1, A375 and DLD2 (that, in normoxia, are killed at a rate >50% by TRAIL 100 ng/ml of TRAIL) become significantly ($^{*}p<0.05$) more sensitive to TRAIL when grown in hypoxia.

Hypoxia does not affect TRAIL receptor expression. TRAIL promotes apoptosis by binding and inducing the trimerization of TRAIL-R1 and TRAIL-R2. TRAIL-R3 and TRAIL-R4 are unable to induce apoptotic signaling, acting as decoy receptors (15,16). One possible explanation for our observations was the modulation of surface receptors for TRAIL under hypoxic conditions. Fig. 2 shows that hypoxia (48 h) improved TRAIL-R1 expression in DLD2 cells, TRAIL-R2 in SupT1, TRAIL-R3 in DLD2 cells, TRAIL-R4 in Jurkat. The absolute number of surface antigens expressed/cell was also calculated (Fig. 2 histograms). Because i) the magnitude of TRAIL modulation is modest (up to 2-fold increases), and ii) there is no common pattern of receptor modulation across the cell lines analyzed, we did not consider these changes sufficient to offer a mechanistic explanation of the functional data observed above. We therefore started looking for receptor-distal events that, modulated by hypoxia, could be responsible for the generation of the cell phenotype highly sensitive to TRAIL.

Hypoxia impairs PKCε expression. Based on what is known about the role of PKCε in the modulation of the apoptotic signaling emanating from TRAIL-Rs and on its role in the protection from low oxygen atmosphere, we decided to study if its levels were modified by hypoxia. We found that hypoxia constantly reduced PKCε protein levels ($^{*}p<0.05$ vs. normoxic cells) in all the cell lines studied (Fig. 3A).

To understanding if this reduction was due to an increased protein degradation or to a reduced gene transcription, we first treated our cell cultures with the proteasome inhibitor MG132 (25 μM), before lowering the cells to low oxygen tension. The inhibition of the proteasome activity, however, did not prevent at all the down-modulation of PKCε protein levels in hypoxic cells (data not shown). On the contrary, the semi-quantitative RT-PCR analysis of PKCε mRNA showed that hypoxia severely reduced (>5-fold in DLD2 and K562; >25-fold in Jurkat and U2OS) the PKCε mRNA levels in the cell lines tested (Fig. 3B).

PKCε down-modulation induces the phenotype highly sensitive to TRAIL. To formally prove that the down-modulation of PKCε might be responsible for the generation of the highly

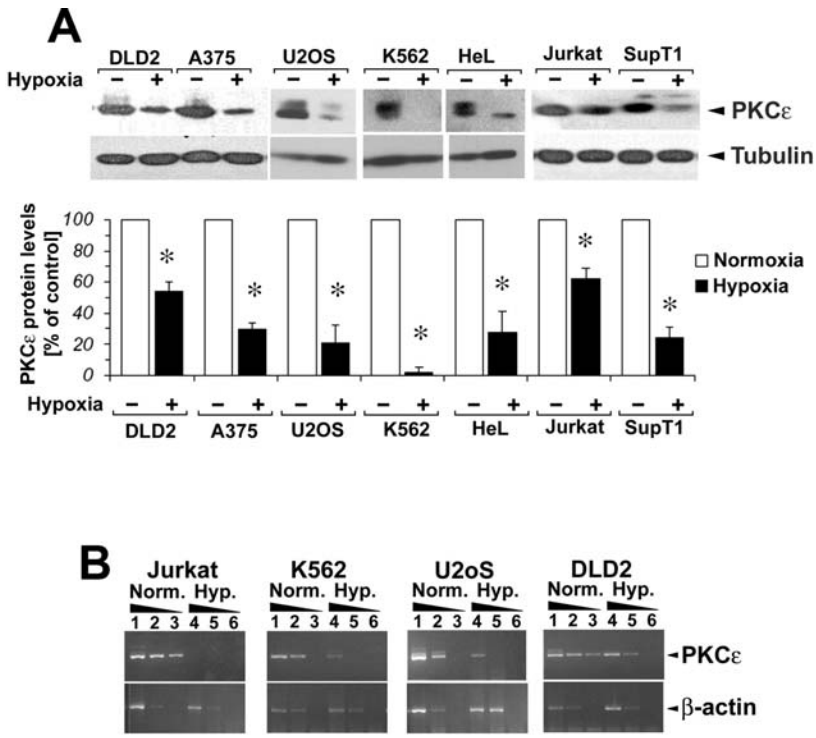

Figure 3. Hypoxia down-modulates PKCε expression. (A) Western blot analysis of PKCε and tubulin protein levels in tumor cells cultured for 48 h under hypoxia (+) and in normoxia (-). Lower lane: densitometric histograms of PKCε expression levels corrected by tubulin expression. Values are expressed as means ± standard deviation of 3 independent experiments and are reported as 100 x hypoxia/normoxia. Results of paired t-test analysis of row data are shown, *p<0.05 hypoxia vs. normoxia. (B) RT-PCR analysis of PKCε and ß-actin mRNA levels of Jurkat, K562, U2OS and DLD2 cell lines grown 24 h in normoxia (Norm.) and in hypoxia (Hyp.). Total RNA was extracted and 1 μg was reverse-transcribed. Different amounts of cDNA were analyzed by PCR: lanes 1 and 4, 1/10 of total cDNA (equivalent to 100 ng of total RNA); lanes 2 and 5 1/50 of total cDNA (equivalent to 20 ng of total RNA); lanes 3 and 6 1/250 of total cDNA (equivalent to 4 ng of total RNA).

sensitive phenotype to TRAIL, we then decided to restore PKCε levels by the transfection of a CMV-mediated PKCε expression vector in hypoxic cells. Cells were transfected and initially cultured in normoxia for 24 h, to ensure PKCε-GFP expression. Then the cell cultures were expanded in hypoxia for additional 24 h. As appropriate negative control, we used a mutated (kinase inactive) PKCε construct (K552M) as well as non-transfected cells (30). Fig. 4A shows the Western blot detection of endogenous PKCε (PKCε) and of recombinant PKCε-GFP in normoxic (lane 1) and hypoxic control, non-transfected, cells (lane 2), in hypoxic cells transfected with the wild-type PKCε and the mutated PKCε (PKCε mut.) (lane 3 and 4, respectively). As shown by densitometric analysis of immunoblots the significant (*p<0.05 vs. normoxic control cells) reduction of PKCε levels induced by hypoxia was counter-balanced by the expression levels of PKCε-GFP protein.

Therefore, given that PKCε transfection completely restored the PKCε expression levels impaired by hypoxia, in the next experiment we evaluated the sensitivity to TRIAL of PKCε-transfected cell lines grown in hypoxic conditions. Fig. 4B shows that the transfection of mutated PKCε (grey filled bars) did not modify the hypoxic cell sensitivity to TRAIL (white bars), while wild-type PKCε (black filled bars) reduced it significantly (*p<0.05 vs. non-transfected controls). In particular, the hypoxia-induced sensitivity to TRAIL was completely abrogated in K562, HeL and DLD2 PKCε-transfected cells and significantly reduced in U2OS and A375 cells.

Furthermore, to reinforce the data on the role of PKCε in the hypoxia-induced cell sensitivity to TRAIL we did the opposite experiment, down-modulating PKCε expression in cells under normoxic conditions. Fig. 5A shows the residual PKCε protein levels 24 h after PKCε siRNA transfection. After further 24 h, cells were treated with or without TRAIL. Apoptosis is reported in Fig. 5B: the reduction of PKCε expression (black bars) is sufficient to significantly increase the sensitivity to TRAIL of all the analyzed cell lines (*p<0.05 vs. non-transfected cells), as expected.

HIF-1α down-modulates PKCε expression. Given the key role of HIF-1α in mediating most of the cellular responses to hypoxia, we then asked if hypoxia-induced down-modulation of PKCε, with the consequent acquisition of the highly sensitive phenotype to TRAIL, might be located downstream hypoxia-induced HIF-1α. We therefore transfected HIF-1α specific siRNA 24 h before the induction of hypoxia, as previously described (27). Fig. 6 shows that under strong reduction of HIF-1α protein levels (lane 4) the expression of PKCε is restored in hypoxic cells (#p<0.05 vs. normoxic untrasfected cells). In other words, the knockdown of HIF-1α abrogated the hypoxia-induced down-modulation of PKCε. Moreover, from the functional point of view, the inhibition of HIF-1α also prevents the development of the hypoxia-induced phenotype highly sensitive to TRAIL; Fig. 6B shows that sensitivity to TRAIL induced apoptosis of cells transfected with HIF-1α siRNAs is significantly reduced as compared to

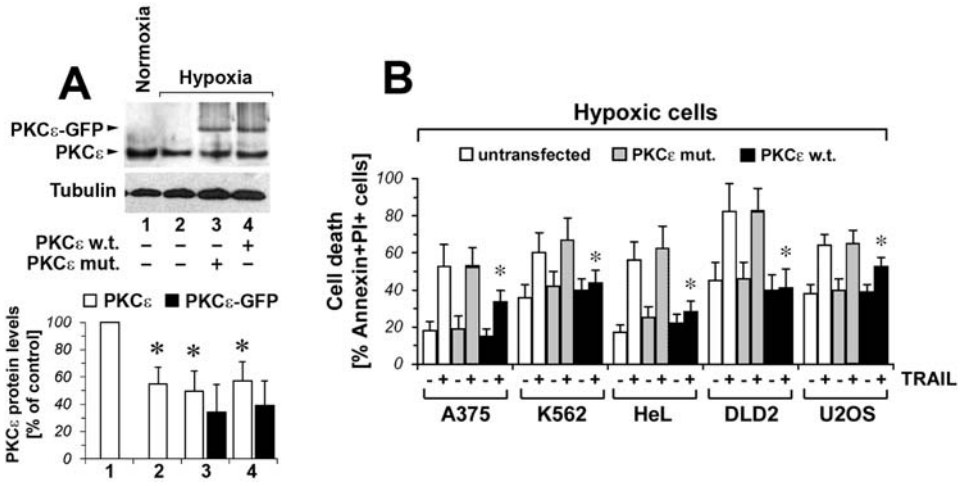

Figure 4. Restoration of PKCε expression protects hypoxic cells from TRAIL-induced apoptosis. (A) Cells were transfected with wild-type PKCε (PKCε w.t., lane 4) or with mutated PKCε (PKCε mut., lane 3). Total PKCε protein was revealed by Western blotting 24 h post transfection. Endogenous PKCε (PKCε) and exogenous GFP-recombinant transfected PKCε (PKCε-GFP) were detected in normoxic (lane 1) and hypoxic cells (lane 2, 3 and 4). Densitometric analysis of PKCε immunoblots was reported as means ± standard deviation of 3 independent experiments: PKCε levels, corrected for tubulin expression levels, are reported as percentage of normoxic control (non-transfected) cells (bar 1). Results of ANOVA followed by Dunnet's test analysis of row data (3 independent experiments) are shown. *p<0.05, comparison vs. hypoxic untransfected cells (lane 1). (B) Cells were transfected with wild-type PKCε (black bars) and with mutated PKCε (grey bars). Twenty-four hours post transfection, TRAIL sensitivity was compared to that of non-transfected controls. TRAIL was administered for 24 h at the following concentrations: A375, 12.5 ng/ml; DLD2 and U2OS, 25 ng/ml; K562 and HeL, 200 ng/ml. Apoptosis is reported as means ± standard deviation of 4 independent experiments. Results of ANOVA followed by Dunnet's test analysis (4 independent experiments) are shown (*p<0.05 vs. white bars).

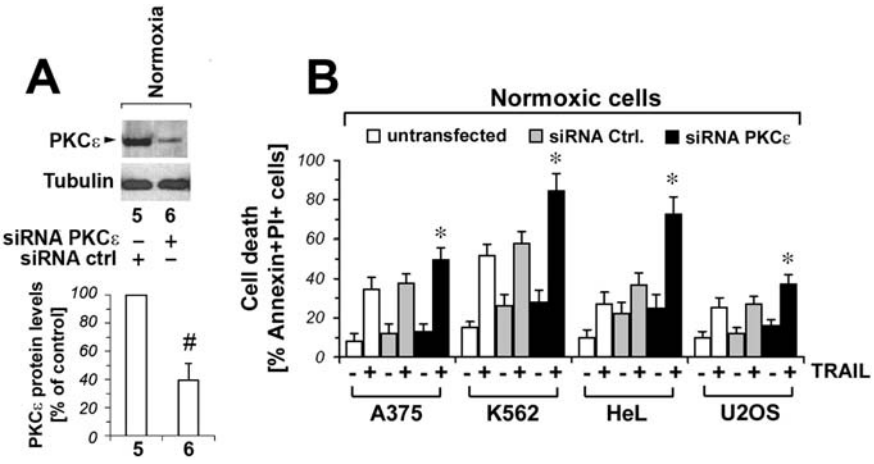

Figure 5. Silencing of PKCε modulates TRAIL-induced apoptosis. (A) Cells were transfected with siRNA for PKCε (lane 6) or control (scrambled) siRNA (lane 5). PKCε expression levels were detected 24 h post transfection, by Western blotting. Densitometric analysis of PKCε is reported as mean ± standard deviation of 3 independent experiments: PKCε levels, corrected for tubulin expression levels, are reported as percentage of normoxic control cells (bar 5). Results of paired t-test analysis of row data (3 independent experiments) are shown (#p<0.05). (B) Cells were transfected with PKCε-specific siRNA (black filled symbol) or with control siRNA (grey filled symbol). Twenty-four hours post transfection cell were grown in normoxia for additional 24 h with or without TRAIL. A375 cells were treated with 25 ng/ml TRAIL whereas HeL, K562 and U2OS cells were treated with 200 ng/ml of TRAIL. Cell death is reported as mean ± standard deviation of 4 independent experiments. Result of ANOVA followed by Dunnet's test analysis (4 independent experiments) are shown. *p<0.05 comparison vs. non-transfected (empty column) TRAIL-treated (+) cells.

that of non-transfected cells or that of cells transfected with control siRNAs (*p<0.05).

To independently support these observations, we promoted HIF-1α expression in normoxia. Cobalt has been used to induce ischemic pre-conditioning *in vivo* (31). Some of the characteristic effects of cobalt are thought to be mediated by interaction with the cellular oxygen-sensing machinery. Like low oxygen tension, cobalt at normoxic conditions is able to stabilize the α-subunit of HIF-1 by blocking its ubiquitination and proteasomal degradation (25-27).

Thus, cell lines were exposed for 24 h to 200 μM $CoCl_2$ at atmospheric oxygen tension. As shown in Fig. 7A, when exposed to $CoCl_2$ all the cell lines studied shown a significant (*p<0.05) reduction of PKCε protein expression levels. As expected, all the cell lines pre-treated for 24 h with $CoCl_2$ acquired the phenotype highly sensitive to TRAIL (Fig. 7B).

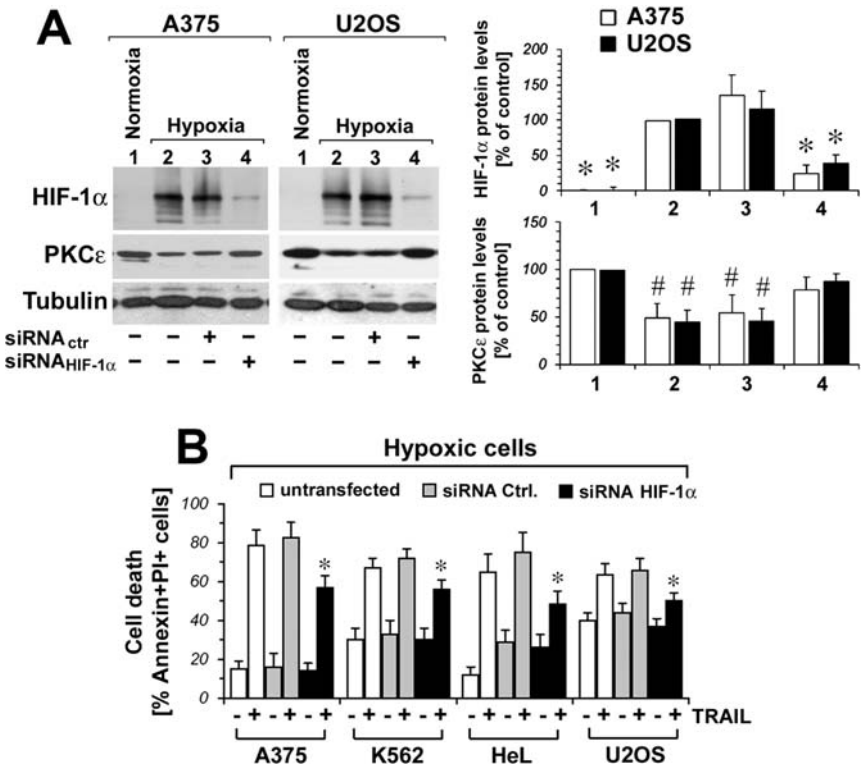

Figure 6. HIF-1α down-modulates PKCε expression and increase TRAIL sensitivity of hypoxic cells. (A) Normoxic (lane 1) and hypoxic (lanes 2, 3 and 4) A375 and U2OS cell lines were transfected with siRNA for HIF-1α (lane 4) or with control (scrambled) siRNA (lane 3) and the expression levels of HIF-1α and PKCε were detected by Western blotting. Twenty-four hours post transfection cells were exposed to hypoxia or normoxia for additional 24 h. Densitometric measurements of HIF-1α and PKCε expression levels, corrected for tubulin expression, are reported as means ± standard deviation of 3 independent experiments (histograms, right panels). Results of ANOVA followed by Dunnet's test analysis of row data (3 independent experiments) are shown. *p<0.05 vs. hypoxic non-transfected cells (lane 2); #p<0.05 vs. normoxic non-transfected cells (lane 1). (B) Cells were transfected with HIF-1α-specific siRNA (black filled symbol) or with control siRNA (grey filled symbol). Twenty-four hours post transfection, A375 and U2OS cells were treated with 25 ng/ml whereas HeL and K562 cells were treated with 200 ng/ml of TRAIL for additional 24 h in hypoxia. Cell death is reported as mean ± standard deviation of 4 independent experiments. Result of ANOVA followed by Dunnet's test analysis (4 independent experiments) are shown. *p<0.05 comparison vs. untransfected (empty column) TRAIL-treated (+) cells.

These results reinforce the evidence for the role of HIF-1α in the hypoxia-induced sensitization to TRAIL via the control of PKCε expression in tumor cells.

Hypoxia induces the down-regulation of Bcl-xL expression. Having shown the hypoxia-generated cascade of events located upstream PKCε down-modulation, we finally investigated the possible pro-apoptotic target(s) located downstream PKCε. By using primary cultures of human normal myeloid progenitors, we have recently demonstrated that PKCε can interfere with the effects of TRAIL by regulating the expression of antiapoptotic mitochondrial factors Bcl-2 and Bcl-xL (10,14). On this basis, we decided to analyze their expression in our model system. While Bcl-2 protein levels did not change under hypoxic cell culture conditions (data not shown), Bcl-xL expression was impaired in the analyzed cell lines (Fig. 8A). To demonstrate that Bcl-xL levels were in fact regulated by PKCε, we overexpressed PKCε (and control mutant PKCε) in K562 and HeL cell lines. Fig. 8B shows that the overexpression of PKCε, but not that of mutated PKCε, promotes the accumulation of the Bcl-xL protein in HeL and K562 cells. On the contrary, the downregulation of the expression of PKCε by specific siRNA impaired the expression of Bcl-xL (Fig. 8C).

Discussion

Tumor development has been linked to hypoxic responses in tumor cells as well as altered apoptotic pathways. Nevertheless, there is a lack of information available on the apoptogenic activity of TRAIL in the hypoxic environment. We have studied a number of tumor cell lines characterized by different tissue origins (bone marrow, thymus, neural crests, bone, colon), growth modality (adhesion or suspension), physical organization (solid or hematological) and different levels of spontaneous sensitivity to TRAIL. Moreover, they belong to a spectrum of lineages for which tumor growth and invasiveness are affected by oxygen tensions. Hypoxia activates different intracellular pathways that involve pro-apoptotic as well as pro-survival molecules such as cytochrome C, p53, caspase-8, members of the Bcl-2 family, and signaling cascades as PI3K/Akt, MEK/ERK, p38, JNK/SAP and PTEN (6).

As a consequence of the differential activation of both apoptotic and anti-apoptotic signaling pathways, oxygen deprivation can induce cell death of some cell types, while other cell lines appear more resistant or even survive hypoxia. Hypoxia has been shown to enhance TRAIL-induced cell death of prostate adenocarcinoma and of hepatoma cell lines by the down-modulation of Akt-mediated FLIP expression

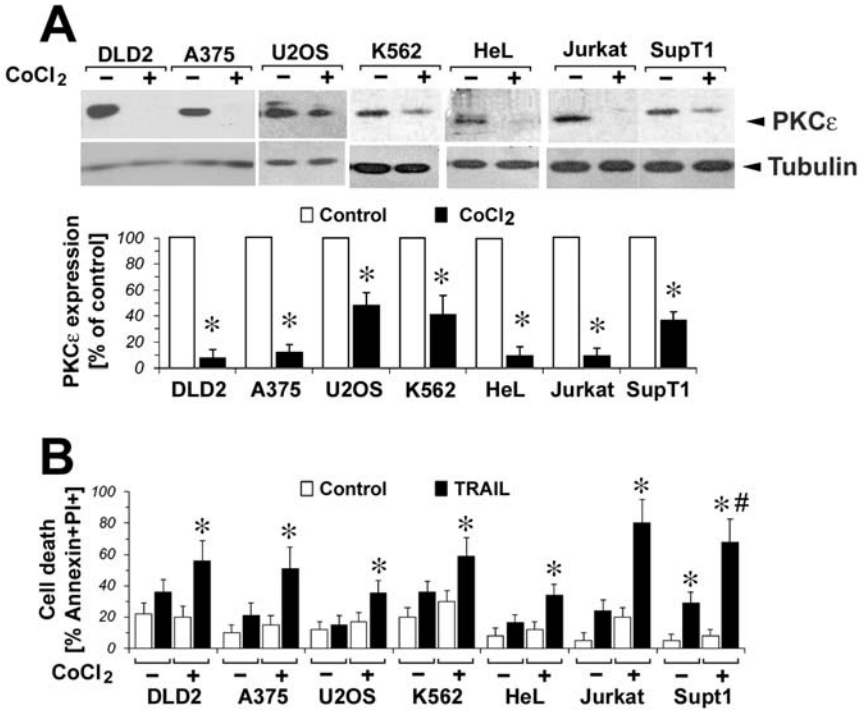

Figure 7. $CoCl_2$ down-regulates PKCε expression and increases TRAIL-induced apoptosis. (A) Cell cultures were treated for 24 h with (+) or without (-) 200 μM $CoCl_2$. PKCε protein levels were detected by Western blotting. Densitometric values, corrected for tubulin expression levels, are shown as percentages of control cells. Mean ± standard deviation of 3 independent experiments are reported. Results of paired t-test analysis of row data (3 independent experiments) are shown. *p<0.05 vs. controls. (B) Cell cultures were treated for 24 h with (+) or without (-) 200 μM of $CoCl_2$, then TRAIL sensitivity was quantified by flow cytometry. A375, Jurkat and SupT1 cells were treated with 12.5 ng/ml, DLD2 with 25 ng/ml, K562 and HeL with 100 ng/ml of TRAIL for additional 48 h. Cell death is reported as means ± standard deviation of 3 independent experiments. Results of ANOVA followed by Dunnet's test analysis (3 independent experiments) are shown. *p<0.05 comparison vs. $CoCl_2$ and TRAIL untreated cells (white bars).

(19) or by the increased expression of TRAIL-R2 (20). However, it has also been reported that hypoxia can up-regulate Bcl-2 expression in the lung carcinoma A431 cell line, while it inhibits the translocation of Bax to mitochondria in the colon carcinoma HCT116 cell line (17,18). Finally, it has been suggested that the hypoxia-induced TRAIL-sensitivity of human tumor cell lines is correlated to the expression levels of p53 and HIF-1α (32).

Hypoxia likely interferes with PKCε expression at the transcriptional level. Even if we cannot formally exclude that hypoxia might promote proteasomic degradation of PKCε, as observed for other proteins (33), nevertheless the MG132 proteasome inhibitor was not able to prevent hypoxia-induced PKCε down-modulation in low oxygen atmosphere. On the contrary, hypoxia interferes with PKCε gene transcription, as we show by RT-PCR experiments.

Our data show that the surface expression of TRAIL receptors remained stable during hypoxia in HeL, K562, A375 and U2OS cell lines, while DLD2 and Jurkat cells showed an increased expression of decoy receptors TRAIL-R3 and TRAIL-R4, respectively. Differently, in agreement with Cao *et al* (20), DLD2 and SupT1 cell lines showed a reproducible upregulation of the expression of the death receptor TRAIL-R1 and TRAIL-R2, respectively. However, the absence of a common pattern in the change of receptor expression across the cell lines examined, suggests that mechanisms other than receptor modulation are involved in hypoxia-induced sensitization to TRAIL.

We show that all the tumor cell lines we used are constantly more sensitive to the apoptogenic effects of TRAIL under hypoxic conditions due to a hypoxia-induced down-modulation of PKCε expression. The down-modulation of PKCε is essential for the generation of a tumor cell phenotype highly sensitive to the cytotoxic effects of TRAIL as the restoration of PKCε protein levels abrogates the hypoxia-induced sensitization to TRAIL. These observations are in agreement with those reported by other groups showing that PKCε has an antiapoptotic activity, preventing breast cancer and glioma cell lines from death receptors-induced apoptosis (11,12).

Among the several molecules potentially able to interfere with the apoptogenic signalling of TRAIL, some Bcl-family members have been shown to be located downstream PKCε in different model systems (12,14). We show that the hypoxia-induced down-modulation of PKCε decreases the cellular levels of Bcl-xL, without affecting Bcl-2 levels. This finding is in line with that known on the antiapoptotic effects of members of the Bcl family in hypoxic cells, as well as with the specific effects of Bcl-xL, that prevents the activation of both the intrinsic and extrinsic pathways of apoptosis in hypoxic cells (34).

Most genes induced by hypoxia are regulated by AP-1, p53, NF-κB and the hypoxia-inducible transcription factor HIF-1α, a protein that therefore plays a crucial role in tumor development (6). A large cohort of HIF-1α-downstream genes has been identified, but few of them are down-modulated by HIF-1α among which genes involved in the cytoskeleton

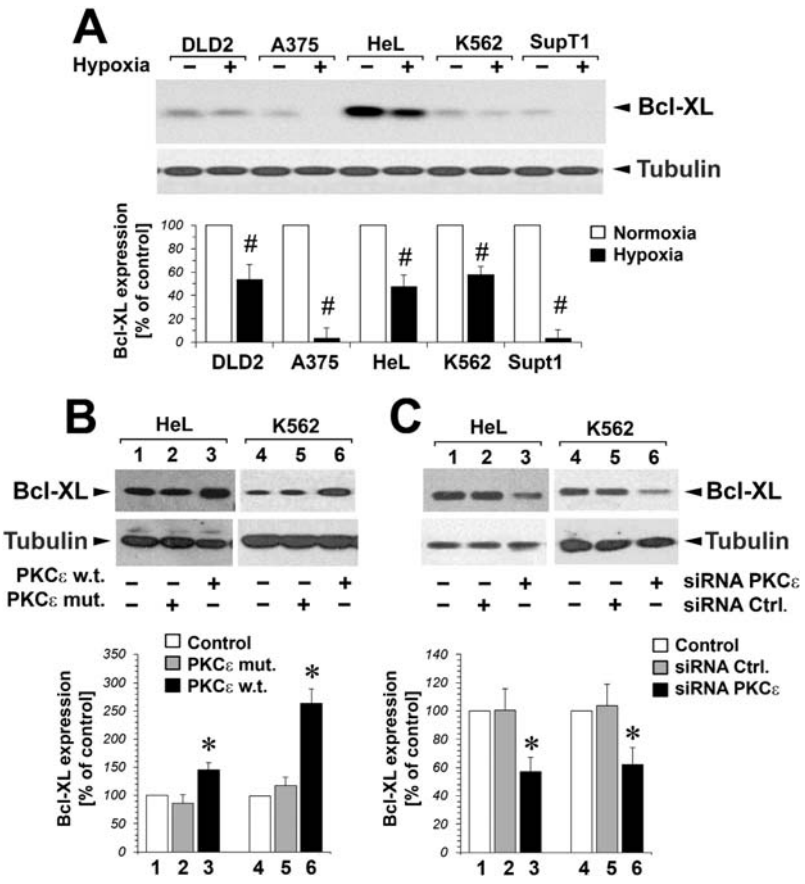

Figure 8. Hypoxia and PKCε modulates Bcl-xL expression. (A) Western blot analysis of Bcl-xL protein level in tumor cells cultured 48 h under hypoxia (+). Reported values, corrected for tubulin expression levels, are showed as percent of control untreated cells. Mean ± standard deviation of 3 independent experiments are reported. Results of paired t-test analysis of row data (3 independent experiments) are shown. *p<0.05 vs. control normoxic cells. (B) Normoxic HeL and K562 cell lines were transfected with CMV-based vector expressing wild-type PKCε (lane 3 and 6) and mutated PKCε (2 and 5) to modulate PKCε protein levels in normoxic cells. Forty-eight hours post transfection (normoxia) the expression of Bcl-xL was analyzed by Western blot analysis and compared to Bcl-xL levels in untransfected cells (lane 1 and 4). Reported values, corrected for tubulin expression levels, are showed as mean ± standard deviation of 4 independent experiments and percent of control untransfected cells. Result of ANOVA followed by Dunnet's test analysis of row data (4 independent experiments) are shown. *p<0.05 comparison vs. control untransfected cells (lane 1 and 4). (C) Normoxic HeL and K562 cell lines were transfected with PKCε siRNA (lane 3 and 6) and with control siRNA (lane 2 and 5). Residual Bcl-xL expression levels were detected by Western blot analysis 48 h post-transfection (normoxia) and compared to untrasfected cells (lane 1 and 4). Obtained values, corrected for tubulin expression levels, are showed as percent of control untransfected cells. Mean ± standard deviation of 4 independent experiments are reported. Result of ANOVA followed by Dunnet's test analysis of row data (4 independent experiments) are shown. *p<0.05 comparison vs. control untransfected cells (lane 1 and 4).

maintenance, mRNA processing and DNA repair genes (35). Furthermore, HIF-1α has been demonstrated to induce estrogen receptor-α down-modulation at post-transcriptional level, promoting its proteasome degradation (33). Recently, HIF-1α expression has been linked to hypoxic tumor cell sensitivity to TRAIL-induced apoptosis (32). Our data suggest that HIF-1α is located upstream PKCε down-modulation in hypoxic cells.

Studies on the promoter elements in the regulation of PKCε gene expression are extremely limited: Bao and coworkers demonstrated the presence of a functional response element-binding protein (CREB) site (36) while, more recently, Zhan and coworkers have sequenced and analyzed a 2,000 bp DNA fragment upstream the transcription starting site of the rat PKCε gene, identifying 4 putative transcription binding sites: AP-1, CREB, CREB/c-Jun-1 and CREB/c-Jun-2 (37).

In parallel, considerable evidence has been accumulated demonstrating that low oxygen tension induces AP-1 in many different cell types both *in vitro* and *in vivo* (38). In this model of hypoxia/anoxia regulated gene expression, AP-1 and HIF-1 functionally associate within a multi-protein transcriptional assembly the enhanceosome. The PKCε promoter contains a functional AP-1 binding site. To the best of our knowledge, no data on the role of AP-1 transcription factor on PKCε expression in hypoxia are currently available.

Based on the series of observations reported in this study showing that: i) the overexpression of PKCε, but not of mutated PKCε - is able to revert the hypoxia-induced highly sensitive phenotype to TRAIL, ii) the inhibition of PKCε levels by siRNA transcription, on the contrary, induces the highly sensitive phenotype to TRAIL in normoxic cells, iii) the inhibition of hypoxia-induced HIF-1α up-regulation by HIF-1α-specific siRNA blocks both the down-modulation of PKCε and the induction of the highly sensitive phenotype to TRAIL, iv) the up-regulation of HIF-1α by $CoCl_2$ in normoxic cells impairs PKCε expression and increases cell sensitivity to TRAIL-induced apopotsis; we conclude that PKCε down-modulation plays a pivotal role in the acquisition of a tumor cell phenotype that positively responds to the apoptogenic

effect of TRAIL. PKCε thus emerges as a key signaling intermediate in hypoxia-induced sensitization of tumor cells to TRAIL. Activation, translocation and phosphorylation levels of PKCε have received considerable attention in multiple forms of cell protection from hypoxia (7), however, transcription and/or protein expression levels in hypoxic tumors cells have not been extensively studied before.

The oxygenation status of a tumor is now thought to have a potential prognostic value in cancer treatment. Tumor masses are often hypoxic, and metastatic tumor cells in lymph nodes grow under hypoxic conditions. The hypoxia induced elevation of HIF-1α promotes a relatively stable PKCε down-modulation that exposes tumor cells to TRAIL-induced cell death.

Given that the use of PKCε inhibitors in cancer therapy is not foreseeable now, the ability of hypoxia to induce PKCε down-modulation promoting the acquisition of the highly sensitive phenotype to TRAIL, should be of great relevance for the use of TRAIL (39) in the anticancer therapy of PKCε$^+$ malignancies.

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