Hypoxia is a key factor in tumor development, contributing to angiogenesis and radiotherapy resistance. Hypoxia-inducible factor-1 (HIF-1) is a major transcription factor regulating the response of cancer cells to hypoxia. However, tumors also contain areas of more severe oxygen depletion, or anoxia. Mechanisms for survival under anoxia are HIF-1α independent in Caenorhabditis elegans and, thus, differ from the hypoxic response. Here we report a differential response of cancer cells to hypoxia and anoxia by demonstrating the induction of activating transcription factor-4 (ATF-4) and growth arrest DNA damage 153 (GADD153) protein specifically in anoxia and the lack of induction in hypoxia. By applying RNAI, ATF-4 induction in anoxia was shown to be independent of HIF-1α, and desferrioxamine mesylate (DFO) and cobalt chloride induced HIF-1α but not ATF-4 or GADD153. Furthermore, the inductive response of ATF-4 and GADD153 was not related to alterations in or arrest of mitochondrial respiration and was independent of von Hippel-Lindau (VHL) disease mutations. In reoxygenated anoxic cells, ATF-4 had a half-life of less than 5 minutes; adding the proteasome inhibitor to normoxic cells up-regulated ATF-4 protein. Extracts from primary human tumors demonstrated more ATF-4 expression in tumors near necrotic areas. Thus, this study demonstrates a novel HIF-1α–independent anoxic mechanism that regulates ATF-4 induction at the protein stability level in tumor cells. (Blood. 2004;103:1876-1882)
Hypoxic incubation
C for 1 hour.°

To investigate the differential response of human cancer cells to hypoxia and anoxia and to determine different regulatory pathways to the hypoxia/HIF-1 system, the expression of ATF-4 and its target gene GADD153 in normoxia (21% O₂), severe hypoxia (0.1% O₂), and anoxia (less than 0.1% O₂) was studied. This report describes the findings that ATF-4 is induced by anoxia rather than hypoxia and that this induction does not involve HIF-1 or electron transport but occurs mainly by stabilization of the protein, which has a half-life after anoxia reoxygenation of less than 5 minutes.

Materials and methods

Cells
The human breast cancer cell lines MCF-7, T47D, MDA-MB 231, 435, 468, the human melanoma cell line LB-4, and the VHL mutant renal cancer cell line 786-0 were provided by Cancer Research UK (London, United Kingdom). Murine neuroblastoma 2a cell line was a gift from Professor Ernst Wagner (Ludwig-Maximilian University, Munich, Germany). Cells were maintained in Dulbecco modified Eagle medium (DMEM, with 4.5 mg/mL glucose) supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL), and 4 mM L-glutamine (Gibco, Paisley, United Kingdom). Previously, 786-0 renal cells had been described, and they were supplemented with 500 μg/mL G418 for selection.

Reagents and plasmids
Rabbit polyclonal antibody to ATF-4 (sc-200) and mouse monoclonal antibody to GADD153 (sc-7351) were from Santa Cruz Biotechnology (Santa Cruz, United States). The Hif-1α antibody used in this study, MCF-7 cells were transfected with 1 μg pcG-ATF4 by using Fugene-6 according to the manufacturer’s protocol (Roche Diagnostics, Sussex, United Kingdom). After 24 hours, cells were lysed by using a urea-denaturing buffer (described below) and analyzed for ATF-4 expression.

RNAi treatment of cells and transfection procedures

Cells were plated onto 100-mm Petri dishes and were grown to approximately 50% confluence before transfection. HIF-1α or inverted control duplex was diluted to give a final concentration of 20 nM in Opti-Mem I (Invitrogen Life Technologies). Twenty-five microliters Oligofectamine transfection reagent (Invitrogen Life Technologies) was added, and the mixture was incubated at room temperature for 25 minutes. Cells were rinsed with Opti-Mem I to remove any residual serum and were incubated with the oligonucleotide duplexes in serum-free conditions for 4 hours at 37°C. Serum was then replenished in the cells, which were further incubated for 24 hours before anoxic pressure was applied, as described above.

To test the anti–ATF-4 antibody used in this study, MCF-7 cells were transfected with 1 μg pcG-ATF4 by using Fugene-6 according to the manufacturer’s protocol (Roche Diagnostics, Sussex, United Kingdom). After 24 hours, cells were lysed by using a urea-denaturing buffer (described below) and analyzed for ATF-4 expression.

Immunoblot analysis

All cell extracts were made in a cold room (4°C). The cell lysis buffer used for preparing total cell extracts was a 1% SDS solution (1 M Tris-HCl, pH 6.8, 5 mM dithiothreitol [DTT], 0.4 M NaCl) supplemented with Complete mini-protease inhibitor cocktail tablets (Roche Diagnostics). Cultured cells were washed rapidly once with ice-cold phosphate-buffered saline (PBS), scraped off, and centrifuged at 13 000 rpm for 25 seconds, and 250 μL urea buffer was added directly to the cell pellet. This was then homogenized rapidly on ice for 15 seconds by using the Ultra-Turrax homogenizer at full speed (IKA, Düsseldorf, Germany). Nuclear and cytoplasmic extracts were based on the method described by Schreiber et al but slightly modified. Cells were washed rapidly once with ice-cold phosphate-buffered saline (PBS), scraped off, and centrifuged for 20 seconds at 13 000 rpm. Cells were resuspended in 4 packed cell volumes of ice-cold buffer A (10 mM HEPES, pH 7.5, 0.1 mM EDTA [ethylenediaminetetraacetic acid], pH 8, 0.1 mM EGTA [ethylene glycol tetracetic acid], pH 8, 10 mM KCl) (Sigma) supplemented with Complete mini-protease inhibitor cocktail tablets (Roche Diagnostics) for cytoplasmic extracts. For nuclear extracts, the cell pellet was resuspended in buffer C (20 mM HEPES, pH 7.5, 1 mM EDTA, pH 8, 1 mM EGTA, pH 8, 0.4 M NaCl) supplemented in Complete mini-protease inhibitor cocktail tablets (Roche Diagnostics). The volume of buffer C used was the same as buffer A.

To make tumor and normal tissue extracts, tumor tissue (or paired tumor/normal tissue) from patients with invasive breast cancer was frozen in liquid nitrogen immediately after surgery and stored at –80°C until extraction. Then 100 to 1000 mg frozen tissue was pulverized on dry ice to a fine powder, and ice-cold extraction buffer (20 mM HEPES, pH 7.4, 1.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM benzamidine, and 10 μg/mL ovomucoid trypsin inhibitor) was added at a
power for 10 minutes [800-W microwave]). After washing in PBS and incubation with 5% normal horse serum (Vector Laboratories, Peterbourough, United Kingdom) for 20 minutes, sections were incubated for 2 hours at room temperature in a negative control antibody (purified rabbit immunoglobulin fraction) at a concentration of 2 μg/mL (DAKO) or in a polyclonal antihuman ATP-4 antibody (Santa Cruz) at the same concentration (2 μg/mL). The reaction was then detected using a standard 3-stage immunoperoxidase technique (Vector Laboratories Elite ABC kit) with the brown chromogen, diamino benzidine (DAB). Sections were counterstained in hematoxylin (British Drug Houses [BDH]), dehydrated, cleared in xylene, and mounted in DPX (BDH).

**Results**

**Induction of ATF-4 by anoxia**

Transfection of MCF-7 cells with the pCG-ATF-4 expression plasmid resulted in a markedly increased expression of a 50-kDa protein (Figure 1A). The same 50-kDa ATP-4 protein was up-regulated in other cancer cells incubated for 16 hours in anoxia (Figure 1A-B). Anoxically induced ATP-4 was nuclear, and no differences in ATF-4 levels between normoxic and anoxic cytoplasmic extracts were detected (Figure 1C). The high expression detected in normoxia in the transfectant likely represents effective transcription and translation from the promoter and the saturation of proteasomal degradation.

**ATF-4 mRNA level in normoxia and anoxia**

RPA demonstrated that ATF-4 mRNA was present at similar levels in normoxia and anoxia in MDA-MB 435 cells (Figure 2A). In a synchronous control, immunoblot analysis demonstrated that ATF-4 protein was induced in anoxia (Figure 2B), suggesting that ATF-4 induction in anoxia is not at the level of transcription or RNA stability.

**Effect of reoxygenation and proteasome inhibition on ATF-4 protein level**

After 16 hours of exposure to anoxia, MDA-MB435 and LB-4 cells were placed for 40 minutes in an incubator with normal 5% CO2 (normoxia, 21% O2). This reoxygenation resulted in a decline in ATF-4 protein levels to those similar for normoxic cells (Figure 3A), suggesting that anoxic ATF-4 protein is

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**Figure 1.** ATF-4 protein is induced in cancer cells under anoxia. (A) ATF-4 overexpression in normoxia (designated as + ATF-4) in MCF-7 cells confirmed the up-regulated band in anoxia to be ATF-4 (50 kDa). (B) Various cancer cell lines, including T47D, MDA-MB 231, 435, 468, and LB-4, were incubated for 16 hours under the corresponding oxygen levels and were analyzed by immunoblot for ATF-4 protein level. N indicates normoxia (21% O2); A, anoxia. (C) MDA-MB 435 and murine N2a cells were incubated for 16 hours in either normoxia or anoxia and were analyzed by immunoblot for ATF-4 protein level in cytoplasmic and nuclear extracts. As indicated, ATF-4 induction in anoxia was only observed in nuclear extracts but not in cytoplasmic extracts. Nuc indicates nuclear; Cyt, cytoplasmic.

**Figure 2.** Levels of ATF-4 mRNA between normoxia and anoxia do not vary. (A) MDA-MB 435 cells were incubated for 16 hours in either normoxia or anoxia, and ATF-4 mRNA levels were analyzed using RPA. As indicated, abundant ATF-4 mRNA was present in similar levels under normoxia and anoxia. (B) Immunoblot control for the RPA demonstrated strong anoxic induction of the ATF-4 protein. n indicates normoxia; A, anoxia; LC, loading control (small nuclear U6 RNA).
normoxia, normoxic and hypoxic cells were treated with 200 μM of the proteasome inhibitor MG132. In normoxia, normoxic and hypoxic cells had already degraded (Figure 3B). To determine whether ATF-4 induction by anoxia was rapidly degraded in normoxia, normoxic and hypoxic cells were treated with 200 and 500 μM of the proteasome inhibitor MG132. In normoxia and hypoxia, proteasome inhibition resulted in up-regulation of the ATF-4 protein (Figure 3C). Thus, collectively these results indicated that ATF-4 induction in anoxia was caused by posttranscriptional, probably proteolytic, mechanisms.

**Induction of ATF-4 by anoxia and relation to GADD153/CHOP10**

**ATF-4 expression in hypoxia and anoxia and correlation with GADD153 and HIF-1α.** Hypoxia (0.1% O2) did not up-regulate ATF-4 or GADD153, whereas anoxia resulted in the strong induction of both proteins in breast cancer cells (Figure 4A). In contrast, HIF-1α was up-regulated by hypoxia and anoxia (Figure 4A). This shows that ATF-4 and GADD153 induction is a response that differentiates between hypoxia and anoxia.

**Figure 3. Reoxygenation of anoxic cells results in a decrease in ATF-4 protein level, and proteasome inhibition results in an increase in ATF-4 protein level.** (A) MDA-MB 435 and LB-4 cells were incubated for 16 hours in normoxia, hypoxia, or anoxia. After 16 hours, anoxic cells (or hypoxic cells, as in the case of MDA-MB 435 cells) were then immediately placed in a normoxic incubator for 40 minutes. This reoxygenation (reox.) of anoxic MDA-MB 435 and LB-4 cells resulted in the down-regulation of ATF-4 protein levels. Reoxygenation of hypoxic MDA-MB 435 cells did not affect the level of ATF-4 protein compared with hypoxic cells. (B) MDA-MB 435 cells were incubated for 16 hours in anoxia, after which the anoxic media of the cells were replaced with normal fresh media (oxygenated), and the cells were incubated in a normoxic incubator for 5 minutes. Cells were then analyzed by immunoblot for ATF-4 protein level. (C) MDA-MB 435 cells were incubated in normoxia for 16 hours, after which 200 or 500 μM (end concentration) of the proteasome inhibitor MG132 was added directly to the cells. Cells were incubated another 16 hours in normoxia or hypoxia and then analyzed using immunoblot for ATF-4 protein levels. N indicates normoxia; H, hypoxia; A, anoxia.

**Figure 4. Induction of ATF-4 by anoxia correlates with the induction of GADD153/CHOP10 but not with that of HIF-1α.** (A) Indicated MDA-MB breast cancer cells were incubated under normoxic, hypoxic, or anoxic conditions for 16 hours and were analyzed by immunoblot for the steady state levels of the indicated proteins: ATF-4, GADD153, HIF-1α, and β-tubulin (as an internal control). (B) MDA-MB435 cells were incubated under anoxia for various times, as indicated, and were analyzed by immunoblot for the indicated proteins. Cells from normoxic conditions were included as a control. N indicates normoxia; H, hypoxia; A, anoxia.

**Time course of anoxic induction of ATF-4.** Figure 4B shows that HIF-1α was already induced within 4 hours of anoxic incubation, whereas both ATF-4 and GADD153 were induced after 16 hours of anoxic incubation. Thus, as reported previously, HIF-1α induction was an early event; in contrast, ATF-4 and GADD153 induction were late responses. Furthermore, though ATF-4 and GADD153 protein levels were maintained after 24 hours of incubation, a decrease was observed in the HIF-1α protein level. These results further demonstrate that the presence of HIF-1α is not required for the maintenance of ATF-4 and GADD153 induction.

**ATF-4 induction in primary tumor extracts, paired primary tumor and normal tissue extracts, and expression near necrotic areas.** Extracts made from patient primary tumors demonstrated ATF-4 expression similarly to the observations made with the in vitro cell culture extracts (Figure 5A). Variations were observed among patient extracts. Some demonstrated greater levels of the 50-kDa ATF-4 protein than others, suggesting that anoxic stress may be more pronounced in some tumors than in others. Interestingly, GADD153 expression was also observed in the tumors, with the greatest levels evident in patients 1 and 4, who also showed the greatest level of ATF-4 expression (Figure 5A). In addition, paired tumor (T)/normal (n) tissue extracts demonstrated ATF-4 expression to be greater in tumors than in normal tissues (Figure 5B). ATF-4 expression was analyzed further through immunostaining of 10 ductal invasive carcinomas of the breast. Enhanced ATF-4 expression (brown) was shown by tumor cells around areas of necrosis (Nec) known to be anoxic (Figure 5C), though it should be noted that ATF-4 staining was not restricted to these areas alone because it was also seen in other tumor areas in some tumors. No such staining was seen when the primary (ATF-4) antibody was replaced with the same concentration of a purified immunoglobulin fraction from a nonspecific rabbit serum (not shown). Nuclei were highlighted in sections using hematoxylin (blue).

**Induction of ATF-4 does not involve HIF-1α, mitochondrial electron transport, or energy depletion.**

HIFs are another group of oxygen-sensitive transcription factors, regulated by proteolysis through the VHL protein. To investigate the relation of the ATF-4 anoxic response to the HIF proteolytic pathway, renal cell lines with mutant VHL were used. The 50-kDa
ATF-4 protein was not induced constitutively in parental 786-0 cells (ie, VA, lacking pVHL) or 786-0 cells with pVHL expression vector, but it was up-regulated in both cells under anoxia only (Figure 6A). These cell lines express HIF-2α rather than HIF-1α and showed the expected constitutive up-regulation in the mutant cells and inducible expression in the cells transfected with wild-type VHL. Thus, anoxic induction of the 50-kDa ATF-4 protein is not dependent on the VHL status of cells.

Treating the cells with RNAi blocked HIF-1α induction in anoxia, whereas cells treated with sense RNA induced HIF-1α in anoxia (Figure 6B). However, RNAi- and sense RNA-treated cells responded to anoxia by inducing ATF-4. Thus, the anoxic response leading to ATF-4 induction is not dependent on the presence of HIF-1α expression in anoxia.

Desferrioxamine or cobalt chloride (200 μM) did not up-regulate ATF-4 or GADD153 but did induce HIF-1α, whereas anoxia up-regulated all 3 proteins. This also shows that DFO and CoCl₂ (at 200 μM) do not imitate the anoxic conditions necessary for ATF-4 and GADD153 induction (Figure 7A).

Tunicamycin (1 mg/mL) induced GADD153 and the 50-kDa ATF-4 protein but failed to induce HIF-1α in MDA-MB 435 cells. Arsenite (2.5 μM) up-regulated ATF-4, GADD153, and HIF-1α; however, ATF-4 induction did not appear as a 50-kDa protein but as a slightly heavier form, suggesting that arsenite induction of ATF-4 involves signaling pathways different from those of anoxia or tunicamycin, which lead to the induction of ATF-4 (Figure 7B). Cyanide (5 mM), in the presence of glucose (4.5 mg/mL), did not induce ATF-4 or GADD153, which shows that blocking the respiratory chain is not the mechanism for the anoxic response. Similar results were also obtained with MDA-MB 468 cells and with cyanide alone (data not shown).

Finally, glucose deprivation using glucose-free DMEM for 16 hours, which deprives cells of glycolytic and oxidative phosphorylation substrates, did not induce HIF1-α or ATF-4 (Figure 7C). Thus, these results indicate that the anoxia signaling pathway does not involve HIF-1α, VHL, adenosine triphosphate (ATP) depletion, or mitochondrial electron transport in inducing the 50-kDa ATF-4 protein.

**Discussion**

The data presented in this paper suggest that cells can sense and discriminate between severe hypoxia (0.1% O₂) and anoxia in an HIF-1α-independent manner. The hypoxia mimetics DFO and CoCl₂ failed to induce ATF-4. The observations that hypoxia did not induce ATF-4 but did induce HIF-1α and that blocking HIF-1α induction in anoxia did not prevent the anoxic ATF-4 response suggest that cells can respond to anoxia independently of the hypoxic HIF-1 signaling pathway. Our data indicate the anoxic ATF-4 protein to be unstable in normoxia and the protein degradation mechanism to be a major pathway controlling ATF-4 induction in cancer cell lines under anoxia. ATF-4 has been shown to contain a nuclear targeting signal in the C-terminus, which may account for the nuclear translocation of increased levels of ATF-4 protein during anoxic stress. ATF-4 mRNA was expressed at similar levels under normoxia and anoxia in MDA-MB-435 cells. This finding is concordant with the observation that ATF-4 mRNA is abundant in human tumor cell lines in normoxia. It also suggests...
primarily that ATF-4 up-regulation is not caused by an increase in mRNA transcription but rather by posttranscriptional events. However, this may be cell type dependent because previously it had been shown that anoxia results in increased levels of ATF-4 mRNA in primary rat fibroblasts.23

One pathway that may contribute to ATF4 regulation is that induced by endoplasmic reticulum (ER) stress, which can be initiated by several mechanisms, including unfolded proteins, tunicamycin, and calcium ionophores. Whether ER stress through tunicamycin mimics anoxic signaling pathways that up-regulate ATF-4 is uncertain, but several proteins induced by ER stress, such as GRP78, are also induced in anoxic conditions.36,37 Protein disulfide isomerase (PDI), which is necessary for disulfide bond formation and, hence, protein folding, is also induced maximally in anoxia.18 In addition, the endoplasmic reticulum resident kinase PERK has been shown recently to become hyper-phosphorylated under hypoxia and anoxia, independent of HIF-1α, leading to the phosphorylation of eIF2α, which then results in hypoxia-induced translational attenuation39 of most mRNAs. Moreover, the PERK-dependent translational attenuation pathway was shown to selectively increase the translation of ATF-4 mRNA during ER stress,40 and this ER-generated signal may mediate an adaptive cellular response to hypoxia.41 Thus, anoxia may also increase the translation efficiency of ATF-4 mRNA, contributing with protein stability pathways to the increase in ATF-4 protein levels. Based on these findings, anoxia but not hypoxia may result in pronounced ER stress, leading to the induction of ATF-4.

Our observations are similar in some respects to those of Dong et al.,22 who showed recently the up-regulation of another factor, the antiapoptotic protein IAP-2, in anoxia through an HIF-1α independent pathway. Milder hypoxic conditions (2% O2) than those used in our experiments (0.1% O2) were shown to induce HIF-1α but not IAP-2, whereas anoxia resulted in the induction of IAP-2, DFO, CoCl2, or arrest of the mitochondrial respiratory chain also could not mimic such anoxic conditions. Dong et al.22 used 1 mg/mL glucose to prevent ATP depletion, and we used 4.5 mg/mL. Therefore, in neither case was ATP depletion a likely explanation. In contrast to our results, IAP-2 induction in anoxia was shown at the transcriptional level to be mainly attributed to activation of the IAP-2 gene promoter.41

We demonstrated ATF-4 expression to be greater in tumors than in normal tissue, but the function of ATF-4 in human tumors remains unknown. It has been shown in ATF-4 knock-out transgenic mice that ATF-4 is critical for processes that require high-level proliferation, such as during fetal-liver hematopoiesis.42 Other knock-out transgenics have suggested ATF-4 to be critical for preventing p53-dependent apoptosis in anterior lens epithelial cells.43,44 Recently, transgenic mice that specifically overexpressed ATF-4 in mammary epithelial cells demonstrated ATF-4 to function as an antiproliferation and a proapoptotic factor during mammary gland development,45 roles that may be relevant to cancer when expressed in anoxic areas. ATF-4 was also shown to block differentiation, another pathway important in cancer development. Thus, the role of ATF-4 in cancer development and progression must be investigated, and the use of ATF-4 as a potential marker to predict the severity of hypoxia in perinecrotic tumor areas must be considered.

 ATF-4 is a substrate for β-TrCP, an F-box protein involved with degradation within the proteasome and showing some similarity to HIF regulation. Although other β-TrCP substrates, such as IκBα and CREB, are degraded in hypoxia (ie, the opposite effect to ATF4),46,47 at the completion of this paper, it was reported that in conditions of severe hypoxic stress, CREB and IκBα are modified by sumo—a small, ubiquitin-like protein—and, hence, stabilized.48 A similar mechanism may be involved in ATF-4 induction under anoxia. The critical factors responsible for ATF-4 stabilization in anoxia remain to be identified, particularly posttranslational modifications, which are being investigated in our laboratory.

References

11. Talks KL, Turley H, Gatter KC, et al. The expression and distribution of the hypoxia-inducible factors HIF-1α and HIF-2α in normal human tissues,


Anoxic induction of ATF-4 through HIF-1–independent pathways of protein stabilization in human cancer cells

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