Ibuprofen-mediated Reduction of Hypoxia-inducible Factors HIF-1α and HIF-2α in Prostate Cancer Cells

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INTRODUCTION

There is increasing evidence suggesting that NSAIDs may have potential use as anticancer agents, either alone or in combination with other cancer therapies (1–8). Whereas NSAID treatment can result in tumor cell death (3, 9, 10), a potentially critical effect of these agents is the inhibition of angiogenesis (1, 4, 5, 11–17). The specific mechanism responsible for the antiangiogenic actions of NSAIDs has not been defined. A number of studies have suggested that the antiangiogenic effect of NSAIDs can be attributed to the specific inhibition of the COX-2 enzyme (11–12, 14–16). However, even the tumor cells that lack COX-1 and COX-2 produce proangiogenic factors and stimulate angiogenesis (12), suggesting that in addition to COX-2, NSAIDs may target other angiogenic pathways as well (12, 13, 17). A major initiator of angiogenesis is hypoxia, which induces a wide variety of genes through the activation of transcription factor HIF-1α, a member of the basic helix-loop-helix PER ARNT SIM gene family (18–20). Under normoxic conditions, HIF-1α interacts with tumor suppressor von Hippel-Lindau protein and is rapidly degraded via ubiquitin-dependent proteasome pathway (21–24). Hypoxia induces a rapid redox-sensitive increase in HIF-1α protein stability and transcriptional activity (25–27), resulting in the activation of target genes involved in erythropoiesis, glycolysis, and angiogenesis (20, 28–31). In addition to the genes required for metabolic adaptation to hypoxia, HIF-1α also enhances the expression of genes coding for growth factors, growth factor receptors, components of the apoptotic pathway, and cell cycle regulators (20, 31, 32). HIF-2α, a recently cloned member of basic helix-loop-helix family, shows close sequence homology and similar pharmacological and regulatory properties as HIF-1α (33–35). Because HIF-1α and HIF-2α can regulate the expression of genes not only involved in angiogenesis but also those that contribute to tumor cell survival and aggressiveness, these HIFs have been suggested as potential targets for antineoplastic therapy (17, 20). Solid tumors contain regions of hypoxia and anoxia (36), and the majority of common human tumors express HIF-1α and HIF-2α (30, 34, 35, 37). Because hypoxia promotes angiogenesis and NSAIDs inhibit angiogenesis, we hypothesized that NSAIDs inhibit the HIF transcription factors. In the

ABSTRACT

Purpose: Hypoxia-inducible factors HIF-1α and HIF-2α are considered to be potential targets for antineoplastic therapy because they regulate the expression of genes that contribute to tumor cell survival, aggressiveness, and angiogenesis. Nonsteroidal anti-inflammatory drugs (NSAIDs) have gained considerable interest as anticancer agents because of their cytotoxic and antiangiogenic properties. The aim of this study was to investigate whether NSAIDs inhibit HIFs and HIF-regulated gene expression in prostate cancer cells.

Experimental Design: PC3 and DU-145 cells were treated with ibuprofen (Ibu) and other NSAIDs under normoxic and hypoxic (95% N2, 5% CO2; <10 ppm O2) conditions. The effect of NSAIDs on HIF proteins was analyzed by Western blot analysis. HIF-regulated proteins, vascular endothelial growth factor (VEGF) and glucose transporter-1 (Glut-1), were analyzed by ELISA and Western blot analysis, respectively.

Results: Exposure of PC3 and DU-145 cells to hypoxic condition up-regulated HIF-1α and HIF-2α proteins. Treatment with Ibu under normoxic and hypoxic conditions reduced the level of HIF-1α and HIF-2α. Ibuprofen-mediated downregulation of HIFs was associated with down-regulation of HIF-regulated proteins VEGF and Glut-1 in cells exposed to hypoxia. Other nonspecific NSAIDs, diclofenac and ketoralac, also inhibited HIF-1α and HIF-2α. The reduction in HIFs was observed in PC3 cells that expressed cyclooxygenase-2 (COX-2) protein as well as in DU-145 cells, which did not express COX-2 protein. COX-2-specific inhibitor NS-398 did not inhibit HIF-1α or VEGF and GLUT-1.

Conclusions: These data indicate that one of the effects of NSAIDs is to reduce HIF protein levels. The inhibition of HIFs by NSAIDs was COX-2 independent.
study presented here, we show that treatment of PC3 and DU-145 prostate carcinoma cell lines with the NSAID Ibu reduced HIF levels under normoxic and hypoxic conditions with a subsequent reduction in the HIF-regulated gene products VEGF and Glut-1. PC3 cells expressed COX-2 protein, whereas DU-145 cells did not. These data suggest that the antiangiogenic actions of NSAIDs may be mediated at least in part via a decrease in these HIFs.

MATERIALS AND METHODS

Materials. PC3 and DU-145 human prostate carcinoma cells from American Type Culture Collection were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and antibiotics. Anti-HIF-1α monoclonal antibody was purchased from Transduction Labs, anti-HIF-2α polyclonal antibody from Novus Biologicals, anti-Glut-1 antibody from Santa Cruz Biotechnology, and anti-COX-2 and antitopoisomerase-1 polyclonal antibodies were from Santa Cruz Biotechnology. Antiactin antibody was purchased from Chemicon. Ibu, diclofenac, and ketorolac were purchased from Sigma Chemicals. Ibu (1 1892; Sigma Chemicals) was dissolved in water (100 mM) and filter sterilized before addition to culture media. NS398 was purchased from Cayman Chemical and prepared as a 100 mM stock in DMSO.

Hypoxia Treatment. Cells were plated in 70-cm² glass flasks and used when ~70% confluent. Before gas equilibration, the media was removed and replaced with fresh media without or with Ibu. After 30 min, flasks were tightly sealed with rubber stoppers, and hypoxia was induced by gassing with a mixture of 95% N₂ and 5% CO₂ for 1 h. After 1 h of gassing, the cells were returned to the incubator in the rubber-stopper-sealed flasks. Previous measurements of the effluent gas flow monitored with a Thermox probe (Ametek, Inc., Pittsburgh, PA) indicated that the oxygen tension in flask was <10 ppm, producing radiobiological hypoxia (38).

Western Blot Analysis. Whole-cell extracts were prepared in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Igepal, 0.1% SDS, 1 mM EDTA, 600 μM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT and complete protease inhibitor cocktail tablet (Roche Diagnostics Corp., Cat. no. 1836153). After incubating on ice for 20 min, cell extracts were centrifuged at 20,000 × g for 10 min, and supernatants were collected. Protein concentrations were determined by Bio-Rad DC protein assay. Sixty μg protein from normoxic samples and 30 μg protein from hypoxic samples were separated on 6% gels for HIF-1α and HIF-2α analyses or 10% gels for Glut-1 and COX-2 analyses. Topo-1 and actin were used as loading controls.

VEGF Levels. Cells were plated in glass flasks (15 cm²) in 2 ml of media, treated with 2 mM Ibu, and subjected to hypoxia as described earlier. Media was collected, centrifuged at 750 × g for 5 min, and stored at -70°C. Concurrently, cells were trypsinized and counted. The concentration of the secreted VEGF₁₆₅ isofrom in the media was determined with an ELISA kit (R&D Systems) according to the manufacturer’s instructions and was expressed as ng VEGF/10⁶ cells. Data were analyzed by two-tailed t test analysis.

RESULTS

In the PC3 and DU-145 cell lines, the levels of the HIF-1α protein detected under normoxic conditions were dependent on the length of time in culture media (Fig. 1A). After 24–48 h in media containing 10% fetal bovine serum, HIF-1α protein was readily detectable in both cell lines (Fig. 1A, left panels). However, HIF-1α levels were rapidly reduced when the conditioned media were replaced with fresh media (Fig. 1A, right panels); over the next 24 h, HIF-1α levels then returned to those observed before replacement of the conditioned media. Exposure of cell lines to 2 mM Ibu in conditioned media resulted in a rapid reduction in HIF-1α levels by 60 min (Fig. 1A, left panels), which remained at an undetectable level for 24 h. When Ibu was added in fresh media, the increase in HIF-1α levels that oc-
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Ibuprofen reduced constitutive HIF-1α/HIF-2α levels, and the accumulation of HIF-1α/HIF-2α after addition of fresh media. To determine the effects of Ibuprofen on hypoxia-induced HIF-1α protein, PC3 and DU-145 cells were grown in glass flasks and gassed with 95%/5% N₂/CO₂ for 1 h. As shown in Fig. 2A, as compared with normoxic conditions, HIF-1α levels were significantly increased in both cell lines after the 1-h gassing procedure. HIF-1α remained elevated at 4 h and decreased by 24 h (Fig. 2B). Addition of 2 mM Ibuprofen significantly reduced HIF-1α levels as determined after the 1-h gassing procedure (Fig. 2A). At 4 h, the inhibitory effect of Ibuprofen, although present, was only ~50% as compared with HIF-1α in cells exposed to hypoxia without the drug. At 24 h, Ibuprofen had essentially no effect in PC3 cells and actually increased HIF-1α levels in the hypoxic DU-145 cells. These data indicate that Ibuprofen reduces the initial hypoxia-induced increase in HIF-1α.

In addition to HIF-1α, a critical transcription factor mediating hypoxia-induced changes in gene expression is HIF-2α. To determine the effects of Ibuprofen on HIF-2α levels, PCs and DU-145 cells were exposed to this NSAID under normoxic and hypoxic conditions. The antiangiogenic effect of NSAIDs such as Ibuprofen has been suggested to involve COX-2 (11, 12, 14–16). To determine whether the Ibuprofen-mediated reduction in HIFs is dependent on COX-2, the levels of this protein were determined in the two prostate carcinoma cell lines (Fig. 2D). PCs expressed similar levels of COX-2 under normoxic and hypoxic conditions. Ibuprofen treatment resulted in an increase in COX-2 levels under both conditions, which is consistent with previous results with a number of NSAIDs (9, 39). However, with respect to a role for COX-2 in the Ibuprofen-mediated reduction in HIF-1α and HIF-2α levels, the most significant data were obtained from DU-145 cells. In this cell line, COX-2 protein was not detected under normoxic conditions, and no increase was detected after hypoxia or Ibuprofen treatment. These data suggest that COX-2 does not mediate the effects of Ibuprofen on HIF protein levels.
The data presented above indicate that Ibu reduces the levels of the HIF-1α and HIF-2α transcription factors. To gain insight into the potential physiological significance of this effect, the expression of two gene products that are induced by hypoxia and regulated by HIF were determined. VEGF is a critical angiogenic factor and is well established to be increased under hypoxia and at least, in part, regulated by HIF (20, 31). Therefore, the effects of Ibu on secreted VEGF levels were determined under normoxic and hypoxic conditions. VEGF was detectable in the media from normoxic PC3 cells and was dramatically increased by 24 h of hypoxia (Fig. 3A). Whereas Ibu had little or no effect on VEGF levels under normoxic conditions after 24 h, the VEGF levels after 24 h of hypoxia in the presence of 2 mM Ibu were reduced by 50% (P < 0.01). Similar effects were observed for DU-145 cells. The VEGF concentrations (mean ± SD, n = 3) in media from untreated and Ibu-treated normoxic DU-145 cells at 24 h were 4.81 ± 0.82 ng/10^6 cells and 4.09 ± 0.81 ng/10^6 cells, respectively. Hypoxia increased the VEGF concentration to 7.72 ± 0.60 ng/10^6 cells; Ibu treatment of hypoxic DU-145 cells resulted in a reduction in VEGF levels to 3.97 ± 1.43 ng/10^6 cells.

An additional protein induced by hypoxia and regulated by the HIFs is Glut-1. In Western blots of cell extracts, Glut-1 protein migrated as a broad multiband complex between M_r 45,000 and M_r 60,000 (Fig. 3, B and C), which is consistent with previous studies (40). Glut-1 protein levels were increased in PC3 and DU-145 cells after 24 h of hypoxia (Fig. 3, B and C). Whereas Ibu had essentially no effect on Glut-1 levels under normoxic conditions, 2 mM Ibu treatment under hypoxic conditions resulted in a significant reduction in Glut-1 in both cell lines. These data indicate that Ibu also decreases the levels of proteins corresponding to HIF-regulated genes under hypoxic condition.

To determine the concentration of Ibu required to inhibit HIF-1α and HIF-regulated proteins VEGF and Glut-1 under hypoxia, PC3 and DU-145 cells were treated with different concentrations of Ibu and exposed to hypoxia. HIF-1α levels were analyzed at 1 h, and VEGF and Glut-1 were analyzed at 24 h. Inhibition of HIF-1α was not seen in cells that were treated with lower concentrations of Ibu (0.5–1 mM; Fig. 4A). At these concentrations, Ibu did not inhibit VEGF (Fig. 4B) and Glut-1...
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with 100 µM NS398 (data not shown), so we treated PC3 cells in 0.1% serum media under hypoxia. The data presented in this study show that the up-regulation of HIF-1α and HIF-2α in prostate cancer cells exposed to severe hypoxia was followed by the up-regulation of VEGF and Glut-1 and that Ibu inhibited this up-regulation.

The presence of HIF-1α in prostate cancer cells under normoxic condition was first demonstrated by Zhong et al. (43), suggesting a decoupling of HIF-1α protein expression from O2 tension. More recently, HIF-1α expression has been observed in a variety of cancer cell lines under nonhypoxic conditions (30, 33, 34, 45). The data presented here showed that after growth in unchanged media under normoxic conditions for 24 h, HIF-1α and HIF-2α were readily detectable in the prostate carcinoma cell lines PC3 and DU-145. However, when the conditioned media was removed and fresh media was added, there was a rapid and almost complete loss of HIF-1α protein, which returned to initial levels by 24 h. It appears that changing the media removes an essential growth factor(s) necessary to maintain HIF-1α levels under nonhypoxic condition. The increase in HIF-1α within 24 h of adding fresh media suggests that the factor(s) originates from the cells. Indeed, recent studies have shown that various growth factors induce HIF-1α (20, 31, 44, 46), which, in turn, induces expression of genes coding for growth factor receptors and binding proteins (32, 46). The expression of HIF-1α in tumor cells may thus generate an autocrine loop (32) maintaining its own expression in cancer cells.

Although Ibu reduced HIF-1α levels in cells grown under normoxic and hypoxic conditions, the reduction was more complete in normoxic cells. Previous studies have reported that the inhibition of HIF-1α expression by inhibition of the phosphatidylinositol 3′-kinase pathway is also more effective under normoxic than under hypoxic conditions (30, 44). HIF is known to be regulated by changes in phosphorylation (20, 47). Moreover, the regulation of HIF-1α may operate through different pathways/processes under normoxic and hypoxic conditions (44). Thus, to completely limit HIF-1α expression, it may be necessary to target more than one regulatory process. HIF-1α levels decreased rapidly after the addition of Ibu. The specific mechanism by which Ibu reduces HIF is currently under investigation; its delineation should provide insight into the fundamental processes regulating the expression of this protein.

In this study, the increase in HIF-1α was evident at the end of 1-h gassing period. Thereafter, the protein levels remained high for up to 4 h of hypoxia and returned to basal levels by 24 h. This is in agreement with an earlier finding in HeLaS3 cells where maximum accumulation of HIF-1α protein was

**DISCUSSION**

Several recent studies indicate that a variety of common human tumors express HIF-1α and HIF-2α (30, 34, 35, 37). The expression of HIF-regulated proteins VEGF and Glut-1 is up-regulated in the hypoxic regions of tumors (41, 42). The data presented in this study show that the up-regulation of HIF-1α and HIF-2α in prostate cancer cells exposed to severe hypoxia was followed by the up-regulation of VEGF and Glut-1 and that Ibu inhibited this up-regulation.

(Fig. 4C) in PC3 and DU-145 cells. Reduction in VEGF and Glut-1 was seen only at a concentration at which HIF-1α was inhibited.

To determine whether the inhibition of HIF-1α was specific to Ibu or it was a feature shared by other NSAIDs as well, PC3 and DU-145 (data not shown) cells were treated with other nonspecific NSAIDs diclofenac and ketorolac. These NSAIDs inhibited HIF-1α (Fig. 5A, left panel) as well as HIF-2α (data not shown) under normoxic condition and also under hypoxic condition (Fig. 5A, right panel) at varying degrees at the concentrations studied. Treatment of PC3 and DU-145 cells with COX-2-specific NSAID NS398 at 100 µM concentration under the same experimental conditions (10% serum media) failed to inhibit HIF-1α (Fig. 5B). When cell were treated with NS398 in 0.1% serum media, slight inhibition of HIF-1α was observed (data not shown), so we treated PC3 cells in 0.1% serum media with 100 µM NS398 for 1 h and then exposed to hypoxia. There was essentially no effect on hypoxic accumulation of HIF-1α with NS398 at 1 h (Fig. 5B, right panel) as compared with the significant inhibition seen with 2 mM Ibu (Figs. 2A and 5A) at this time point. Furthermore, treatment with 100 µM NS398 failed to inhibit the HIF-regulated proteins VEGF (Fig. 5C) and Glut-1 (Fig. 5D) at 24 h.
seen at 1 h after anoxic/hypoxic exposure, and the level was maintained for 4 h (48). We observed an increase in HIF-regulated gene products VEGF and Glut-1 at 24 h subsequent to the up-regulation of HIF-1α at 1–4 h. Although HIF-1α was partially inhibited at 4 h in Ibu-treated cells, Ibu had essentially no effect at 24 h in PC3 cells, and actually HIF-1α level was higher compared with the hypoxic control in DU-145 cells. Despite lack of inhibition of HIF-1α by Ibu at 24 h under hypoxic condition, both VEGF and Glut-1 were inhibited. This apparent discrepancy between the time courses of HIF-1 and VEGF/Glut-1 inhibition after Ibu treatment may reflect the different time requirements for the sequential activation of a transcription factor, the subsequent expression of the secondary response gene and the ultimate changes in level of the corresponding protein. Alternatively, the HIF-1α protein observed at 24 h of continuous hypoxia may not be functionally active. It has been suggested that the predominant HIF-1α protein species accumulating at later time points probably undergo post-translational modification as they migrate at a higher position in the gel (18). Our data suggests that the reduction in the initial hypoxia-induced increase in HIF-1α by Ibu is sufficient to reduce the increase in HIF-1-regulated gene products at 24 h. Although under normoxic condition Ibu completely inhibited HIF-1α, no inhibition of VEGF and Glut-1 was observed. It appears that the basal expression of these proteins under normoxic condition may be regulated by multiple pathways.

Whereas a number of agents that inhibit signaling pathways have been reported to reduce HIF-1α, the effects of these agents on HIF-2α have not been reported. However, it would appear that if the therapeutic goal were to reduce the expression of genes regulated by HIFs, it would be necessary to target both HIF-1α and HIF-2α. The data presented here clearly illustrate that Ibu targets both proteins. The reduction in HIF-1α and HIF-2α may account for the reduction in hypoxia-induced VEGF and Glut-1 proteins in Ibu-treated cells. Thus, decreasing both forms of HIF may contribute to the anticancer effects, both in prevention and treatment, of NSAIDs.

Several studies have demonstrated that NSAIDs inhibit tumor growth by inhibiting angiogenesis (1, 4, 5, 49–51). NSAID-mediated inhibition of angiogenesis is generally attributed to the inhibition of COX-2 because NSAIDs are primarily associated with the inhibition of COXs (11–16). It is well documented that in addition to COX-2, angiogenesis is also regulated by HIFs under hypoxia by increasing angiogenic factors, including VEGF and VEGF receptor, Flt-1 (17, 20, 28, 31, 52). The inhibition of VEGF by Ibu under hypoxic condition in this study appears to be regulated by HIFs. This is supported by the following observations. PC3 and DU-145 cell lines used in this study have different COX-2 profiles. PC3 cells expressed COX-2 protein constitutively, whereas DU-145 cells did not contain detectable COX-2 protein. Hypoxia had no effect on COX-2 protein level in both cell lines. On the contrary, hypoxia up-regulated HIFs in both cell types with a subsequent increase in VEGF secretion. The inhibition of HIFs by Ibu resulted in the inhibition of VEGF in both cell types. In the absence of inhibition of HIF-1α, there was no inhibition of VEGF and Glut-1 in cells treated with lower concentrations of Ibu or 100 μM NS398. Finally, the concentration of Ibu required to inhibit HIF-1α and HIF-regulated gene products, VEGF and GLUT-1, was higher than the concentration that is required to inhibit prostaglandin synthesis. Our data support the recent observations of Jones et al. (17) who showed that in gastric microvascular endothelial cells NSAIDs indomethacin and NS398 inhibited hypoxia-induced accumulation of HIF-1α, resulting in inhibition of hypoxia-induced VEGF/Flt-1 expression. However, in this study, NS398 did not inhibit hypoxic accumulation of HIF-1α and HIF-regulated proteins in prostate cancer cells at the concentration at which NS398 was effective in endothelial cells. This discrepancy could be because of the differences in experimental conditions or normal versus cancer cell types.

Inhibition of endothelial cell proliferation, tube formation, and VEGF expression is seen at relatively high concentrations of indomethacin (0.25–0.5 mM) or NS398 (100 μM; Refs. 13, 17, 53). Although NSAIDs inhibit prostaglandin synthesis at micromolar concentrations, several other effects of NSAIDs, including induction of apoptosis, cytotoxicity, inhibition of various transcription factors, and kinases, require concentrations that are well above those that inhibit of prostaglandin synthesis (3, 8–10, 13, 39, 53). In vitro, even the COX-2-specific inhibitors appear to have a prostaglandin-dependent and prostaglandin-independent component (13). The in vivo inhibition of angiogenesis and tumor growth by some of the COX-2-specific NSAIDs is recently attributed to the NSAID-mediated inhibition of host stromal and angiogenic vasculature COX-2 (15, 16). The significance of inhibition of HIFs and HIF-regulated gene products by NSAIDs needs to be additionally investigated in animal tumor models.

HIF-1α has clearly been shown to aid in a cell’s ability to adapt to the hypoxic milieu. Moreover, it has also been implicated in tumor cell survival and proliferation (54). Studies have shown that growth of tumors derived from HIF-1α-inactivated cells was significantly slower than matched cells containing the functional protein (19, 55). Furthermore, tumors derived from HIF-1α-deficient cells were also deficient in expression of VEGF, a critical tumor angiogenic factor (19, 55, 56). Finally, constitutive expression of HIF-1α was reported to render pancreatic cancer cells resistant to apoptosis induced by hypoxia or glucose deprivation (45). It has been shown that severe hypoxic conditions up-regulate apoptosis inhibitory protein IAP-2 (57). Compared with adjacent normal tissues, HIF-1α is overexpressed in the majority of the common human cancers evaluated (37). Thus, in recent years, HIF-1α has emerged as a potentially important therapeutic target for cancer therapy (20, 58). Strategies suggested for HIF-1α targeting include disruption of the normal coactivational response to hypoxia (59), the use of decoy oligonucleotides (58), and gene therapy approach based on hypoxia response element-regulated gene expression that exploits the presence of hypoxia/anoxia in tumors for the induction of therapeutic genes (60). In addition to these genetic approaches, pharmacological intervention of HIF is also actively pursued in many laboratories (61–63). Our study along with the earlier study by Jones et al. (17) suggests that a therapeutically feasible approach of targeting HIFs is through the use of NSAIDs.

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REFERENCES


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