Hypoxia activates the cyclooxygenase-2–prostaglandin E synthase axis

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Hypoxia-inducible factors (HIFs), in particular HIF-1α, have been implicated in tumor biology. However, HIF target genes in the esophageal tumor microenvironment remain elusive. Gene expression profiling was performed upon hypoxia-exposed non-transformed immortalized human esophageal epithelial cells, EPC2-hTERT, and comparing with a gene signature of esophageal squamous cell carcinoma (ESCC). In addition to known HIF-1α target genes such as carbonic anhydrase 9, insulin-like growth factor binding protein-3 (IGFBP3) and cyclooxygenase (COX)-2, prostaglandin E synthase (PTGES) was identified as a novel target gene among the commonly upregulated genes in ESCC as well as the cells exposed to hypoxia. The PTGES induction was augmented upon stabilization of HIF-1α by hypoxia or cobalt chloride under normoxic conditions and suppressed by dominant-negative HIF-1α. Whereas PTGES messenger RNA (mRNA) was negatively regulated by normoxia, PTGES protein remained stable upon reoxygenation. Prostaglandin E2 (PGE2) biosynthesis was documented in transformed human esophageal cells by ectopic expression of PTGES as well as RNA interference directed against PTGES. Moreover, hypoxia stimulated PGE2 production in a HIF-1α-dependent manner. In ESCC, PTGES was overexpressed frequently at the mRNA and protein levels. Finally, COX-2 and PTGES were colocalized in primary tumors along with HIF-1α and IGFBP3. Activation of the COX-2–PTGES axis in primary tumors was further corroborated by concomitant upregulation of interleukin-1β and downregulation of hydroxyprostaglandin dehydrogenase. Thus, PTGES is a novel HIF-1α target gene, involved in prostaglandin E biosynthesis in the esophageal tumor hypoxic microenvironment, and this has implications in diverse tumor types, especially of squamous origin.

Introduction

Oxygen tension is among the key elements in the tumor microenvironment that influences cancer development and progression. Hypoxia-inducible factors (HIFs), comprising an oxygen-sensitive α-subunit and a constitutively expressed β-subunit, facilitate global cellular adaptation to hypoxia and oxygen delivery by transcriptionally activating genes essential in various processes such as glucose transport, glycolysis, angiogenesis and erythropoiesis (1,2).

The normal esophageal epithelium expresses very little HIF-2α protein (3). In contrast, HIF-1α is expressed highly in 30–70% of primary esophageal squamous cell carcinomas (ESCCs) and associated with induction of vascular endothelial growth factors, tumor invasion, lymphatic invasion or lymph node metastasis and a lower post-operative survival rate (4–7). Interestingly, the normal esophageal epithelium adjacent directly to the tumor expresses HIF-1α to a variable extent, implying changes in the esophageal tumor microenvironment (8). Moreover, HIF-1α expression has been detected in >50% of early-stage esophageal cancers (8). HIF-1α protein expression is highly inducible by hypoxia treatment in cultured esophageal cancer cell lines (5), suggesting that overexpression of HIF-1α in primary esophageal tumors is mainly accounted for by the lack of oxygen availability. However, information is limited regarding the expression and function of HIF target genes in the hypoxic esophageal tumor microenvironment.

Prostaglandin E2 (PGE2)-mediated signaling and the enzymes regulating its biosynthesis play a pivotal role in cancer development (9). In particular, cyclooxygenase (COX)-2 has been studied extensively as a key rate-limiting enzyme for prostanooids biosynthesis, implicated in the pathogenesis, disease progression and poor survival rates in various tumor types, including ESCC (10–12). Prostaglandin E synthase (PTGES) has emerged as another essential enzyme not only functioning downstream of COX-2 but also being activated by proinflammatory stimuli such as interleukin-1β (IL-1β) and lipopolysaccharide (13). PTGES is upregulated in gastrointestinal cancers and premalignant lesions such as colonic adenomatous polyps (14–18). Although PTGES has been shown to be expressed in esophageal adenocarcinoma (19), its expression and regulatory mechanisms in ESCC remain to be elucidated.

In this study, we carried out gene array experiments using an immortalized human esophageal epithelial cell line, EPC2-hTERT (20), exposed to hypoxia. Comparison of hypoxic gene signature in EPC2-hTERT with gene expression profiling data in primary esophageal tumors revealed the regulation of prostanooids biosynthesis by the COX-2–PTGES enzyme axis as a novel hypoxia target pathway in esophageal cancer.

Materials and methods

Tissue samples

Esophageal tissues were procured via surgery at the Okayama University Hospital (M.T., Y.S. and Y.N.), Kitano Hospital (MK) and the Hospital of the University of Pennsylvania through the Cooperative Human Tissue Network. All were pathologically diagnosed as ESCC. Forty paired tumors and adjacent normal tissues, including 55 pairs on tissue microarray, were available as paraffin blocks. Frozen tissues were available for RNA (13 cases) and protein (15 cases) analyses. All the clinical materials were obtained from informed consent patients in accordance with Institutional Review Board standards and guidelines.

Cell cultures

Primary normal human esophageal cells EPC1 and EPC2 and non-transformed immortalized diploid human esophageal cell line, EPC2-hTERT, and its derivative transformed by epidermal growth factor receptor, p53R175H and cyclin D1, were grown under normoxic conditions (21% O2, 5% CO2 and 95% humidity) at 37°C in Keratinocyte-SFM serum-free medium (Invitrogen, Carlsbad, CA) as described previously (20–23). Subconfluent cells were exposed to hypoxia (0.2 or 1% O2) in an Invivö2 hypoxia chamber (Ruskinn Technologies, Bridgend, UK). Cobalt chloride (Sigma Aldrich, St. Louis, MO) was used at a concentration of 250 μM.

Abbreviations: cDNA, complementary DNA; COX, cyclooxygenase; ESCC, esophageal squamous cell carcinoma; HIF, hypoxia-inducible factor; HPGD, hydroxprostaglandin dehydrogenase; IGFBP3, insulin-like growth factor binding protein-3; IL, interleukin; mRNA, messenger RNA; PCR, polymerase chain reaction; PGE2, prostaglandin E2; PTGES, prostaglandin E synthase.

These authors contributed equally to this work.
Constructs and retrovirus- and lentivirus-mediated gene transduction

Wild-type and dominant-negative HIF-1α complementary DNAs (cDNAs) in pcDNA3.1 (+) were isolated by restriction digestion with BamHI for wild-type HIF-1α and polymerase chain reaction (PCR) with restriction site-tagged sense 5'-gcagatcaagcaacactgctgggtttgccggtagtagtcatctatcatcatca-tgaggcaatgtactgttcagttggacaacctggccgtagggg-3' and reverse 5'-gtcaggtgatatcaacatcaaaatcaagtctgga-tct-3' primers for dominant-negative HIF-1α, respectively, and subcloned into pBABE-puro-loxP at their BamHI site for the former and BamHI and SalI sites for the latter, resulting in creation of pBABE-puro-loxP-WT-HIF-1α and pBABE-puro-loxP-DN-HIF-1α, respectively. PTGES cDNA derived from TE7 esophageal cancer cell line (25) was subcloned into pBABE-bl (23), resulting in creation of pBABE-bl-PTGES. The constructs were verified by DNA sequencing. Replication-competent retrovirus was produced as described previously (23).

Lentiviral pGIPZ expressing short hairpin RNA directed against human PTGES (clone ID # V2LHS_67663 and V2LHS_67660) or a non-silencing pGIPZ lentiviral shRNAtrans control vector (Open Biosystems, Huntsville, AL) was transfected into HEK-293T cells with Arrest-In Transfection Reagent (Open Biosystems) according to the manufacturer's instructions. The virus-containing medium was harvested 48 and 72 h after transfection, filtered (0.45 µm) (Pall Gelman Laboratory, Ann Arbor, MI) and stored at −80°C until use.

For virus infection, 0.5–1.5 × 10⁶ cells were seeded per well in a six-well tissue culture plate and subjected to the spin infection method in the presence of 4 µg/ml polybrene (Sigma–Aldrich Corp., St Louis, MO) as described previously (25). Cells transfected with wild-type HIF-1α, dominant-negative HIF-1α or an empty vector (pBABE-puro-loxP) was selected with 5 µg/ml of puromycin (Invitrogen) for 5 days following retrovirus infection. To establish PTGES overexpressing cells and empty vector (pBABE-bl)–transduced control cells, 10 µg/ml of G418 (Invitrogen) was added 24 h after infection and selected for 5 days. Cells transduced with short hairpin RNA were analyzed by fluorescence-activated cell sorter and sorted as the top 20% of the most highly green fluorescent protein–expressing cells in the Flow Cytometry and Cell Sorter Facility at the University of Pennsylvania.

Transient transfection and dual-luciferase assays

HIF-mediated transcriptional activity was determined using (eHRE)-Luc, containing three copies of the hypoxia response element from the EPO gene enhancer in pGL3 (Promega, Madison, WI) (26). Transfection was carried out using the FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. Briefly, 1 × 10⁶ cells were seeded well per well in 24-well plates 24 h before transfection. Four hundred nanograms of the (eHRE)-Luc or pGL3-Promoter (Promega) control vector plasmid DNA was transfected along with 5 ng of pHRL-SV40-renilla luciferase vector (Promega) to calculate the variation of transfection efficiency among wells. Cells were exposed to hypoxia or cobalt chloride 24 h after transfection and incubated for 24 h before cell lysis. Luciferase activities were determined using the Dual-Luciferase™ Reporter Assay system (Promega) and the ORION Microplate Luminometer (Berthold Detection Systems USA, Oak Ridge, TN). The mean of firefly luciferase activity was normalized with the cotransfected renilla luciferase activity. Transfection was carried out at least three times, and variation between experiments was not >15%.

RNA isolation, cDNA synthesis and real-time reverse transcription–PCR

Total RNA was isolated from cultured cells and snap-frozen tissues with the RNasy Mini Kit and the RNasy Fibrous Tissue Midi Kit (Qiagen, Valencia, CA, respectively). Integrity of total RNA was determined by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). cDNA was synthesized with the Superscript™ First Strand Synthesis System (Invitrogen) as described previously (25).

Real-time PCR was done using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with TaqMan® Gene Expression Assays (Applied Biosystems) for PTGES (Hs0010420_m1), COX-2 (Hs0015313_m1), hydroxyprostaglandin dehydrogenase (HPGD) (Hs001629), BCL2 and carbonic anhydrase 9 (CA9) (Hs00154208_m1). SYBR green reagent (Applied Biosystems) was used to quantitate messenger RNA (mRNA) for insulin-like growth factor binding protein-3 (IGFBP3) and β-actin as described previously (25). All PCRs were performed in triplicate. The relative expression level of each mRNA was normalized to the β-actin mRNA level as an internal control.

Microarray analysis

Gene array experiments were performed using an Affymetrix gene chip (U133+2.0) (Affymetrix, Santa Clara, CA) upon RNA representing EPC2-hTERT exposed to 0.2% O₂ (severe hypoxia), 1% O₂ (moderate hypoxia) or 21% O₂ (normoxia) for 24 h. Preparation of complementary RNA, hybridization and scanning of the arrays were performed as described previously (25). Similarly, gene array experiments were done on paired ESCC tumor and normal tissues (n = 5). CEL files (probe level data) were exported from Affymetrix® GeneChip® Operating Software that was also used to calculate detection flags, which were exported in .chp files. Probe level data (.CEL files) were imported with Partek Genomics Suite (v. 6.4; Partek, St Louis, MO). The data were normalized and summarized to the probe set level using GeneChop Robust Multiarray Average, yielding log, intensities for all probe sets on the array. These intensity values were then filtered based on the detection flags, retaining only those probe sets flagged as present in at least one of four samples for each of the hypoxia-exposed cell experiments and those present in at least 4 of 10 for the tumor and normal tissue samples. Significance analysis of microarrays (Ver. 3.02) was done to identify differentially expressed transcripts and calculate Q-values, d-scores and fold change on all probe sets passing filter. The hypoxia datasets were treated as a two-class unpaired design, whereas the ESCC tumor dataset was treated as a two-class paired experiment. Further analysis was done to create gene lists and a Venn diagram based on cutoff values: fold change >2, Q <1% for 0.2% O₂, Q <3% for 1% O₂ and Q <10% for 21% O₂. Microarray data submission # GSE1753 for hypoxia experiments and GSE17351 for ESCC tumors were deposited with Gene Expression Omnibus at the NCBI (http://www.ncbi.nlm.nih.gov/geo).

Western blotting

Western blotting was done as described previously (25). Twenty micrograms of cleared cell lysate or tissue homogenate was denatured and fractionated on a NuPAGE Bis–Tris 4–12% gel (Invitrogen) and transferred to Immobilon-P membranes (Millipore, Billerica, MA). The membranes were incubated with anti-HIF-1α (H1alpha7) (Novus Biologicals, Littleton, CO) at 1:500 dilution, anti-PTGES (Oxford Biomedical Research, Oxford, MO) at 1:1000 dilution, anti-IGFBP3 (DSL–Rc00536) (Diagnostic Systems Laboratories, Webster, TX) at 1:1000 dilution or anti-β-actin (AC-74) (Sigma Aldrich) at 1:3000 dilution. Following incubation with horseradish peroxidase-conjugated secondary antibodies at 1:5000 dilution for 1 h at room temperature, the signal was developed by enhanced chemiluminescence solution (ECL Plus; Amersham Biosciences, GE Healthcare, Little Chalfont, UK) and detected on Blue Lite Autorad Film (ISC BioExpress, Kaysville, UT). β-Actin was detected to confirm equal protein loading.

Enzyme-linked immunosorbent assay

PGE₂ levels in culture supernatants were quantified by enzyme-linked immunosorbent assays (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions. Briefly, 1.5 × 10⁵ cells were seeded per 60 mm dish in 5 ml of fresh medium and incubated for 48 h. Absorbance was measured at 410 nm by BioTek Synergy™ HT Multi-Mode Microplate Reader. Since hypoxia reduced cell proliferation by 30–50% compared with normoxic controls (data not shown), the PGE₂ concentrations were normalized by cell number at the time of harvesting culture supernatants.

Immunohistochemistry

Immunohistochemistry was performed with the VECTASTAIN® ABC kit (Vector Laboratories, Burlingame, CA). In brief, sections were incubated with anti-PTGES rabbit polyclonal antibody (Oxford Biomedical Research) at 1:150 dilution, anti-COX-2 mouse monoclonal antibody (Cayman Chemical) at 1:250 dilution, anti-HIF-1α rabbit polyclonal antibody (Novus Biologicals) at 1:5000 dilution or anti-human IGFBP-3 mouse monoclonal antibody (Clone 84728.111) (R&D Systems, Minneapolis, MN) at 1:250 dilution, followed by incubation with biotinylated secondary IgG and signal development using the DAB Peroxidase Substrate Kit (Vector Laboratories). Signal was intensified for PGE₂ using Catalyzed Signal Amplification system (Dako, Carpinteria, CA). The intensity was scored as negative (0), weakly positive (0.5), definitively positive (1), strongly positive (1.5) or very strongly positive (2).

Statistical analysis

Data from triplicate experiments are presented as mean ± standard error and were analyzed by two-tailed Student’s t-test. P <0.05 was considered significant.

Results

Hypoxic gene signature reveals PTGES as a potential HIF-inducible gene essential in esophageal tumor biology

To delineate the role of hypoxia in esophageal epithelial and tumor biology, we first carried out gene array experiments using a non-transformed immortalized diploid esophageal carcinoma cell line, EPC2-hTERT (20). Unlike cancer cell lines, EPC2-hTERT has no genetic alterations at
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PTGES protein, but not mRNA, is stabilized under normoxic conditions

We next explored the kinetics of PTGES mRNA and protein expression as a novel HIF-inducible gene. Under moderate hypoxic conditions (1% \(O_2\), PTGES mRNA was typically induced within 12 h and increased further as a function of time in EPC2-hTERT cells (Figure 3A). PTGES protein was induced by hypoxia exhibiting kinetics similar to other HIF-1\(\alpha\) target genes such as CA9 and IGFBP3 (Figure 3B-C and data not shown). These results indicate that the HIF-dependent transcriptional activity is not only necessary but also sufficient for PTGES induction under hypoxic conditions.

PTGES contributes to biosynthesis of PGE\(_2\)

To elucidate the biological activity of PTGES in esophageal epithelial cells, we ectopically expressed PTGES by retrovirus-mediated stable transduction (Figure 4A) or knocked down PTGES by RNA interference (Figure 4B). When overexpressed, PTGES increased the PGE\(_2\) level in conditioned media (Figure 4A). RNA interference prevented hypoxic induction of PTGES and suppressed basal PGE\(_2\) production under normoxic conditions (Figure 4B). These data indicate that PTGES functionally contributes to production of PGE\(_2\). Moreover, hypoxia stimulated PGE\(_2\) production and that was enhanced by ectopically expressed wild-type HIF-1\(\alpha\) and suppressed by dominant-negative HIF-1\(\alpha\) (Figure 4C), in agreement with HIF-1\(\alpha\)-mediated

early passages that may affect the cellular response to hypoxia. When EPC2-hTERT was exposed to moderate (1% \(O_2\)) or severe (0.2% \(O_2\)) hypoxia, \(\geq\)2-fold of gene expression was detected by 133 and 790 probe sets on the Affymetrix U133 Plus 2.0 chip, respectively (Figure 1A). Both oxygen tensions induced 68 probe sets commonly, although lower oxygen tension induced higher levels of mRNA in most of the genes induced by hypoxia (Figure 1A and supplementary Table 1 is available at Carcinogenesis Online). They included 52 HIF target genes such as carbonic anhydrase 9, glucose transporter SLC2A1 (a.k.a. GLUT1), 6-phosphofructo-2-kinase/fructose-2, 6-bifosphatases PFKFB3 and PFKFB4, phosphoglycerate kinase 1 (PGK1), prolyl hydroxylases Egln1 (PHD2) and Egln3 (PHD3), and IGFBP3. Severe and moderate hypoxia induced 31 (59.6%) and 16 (30.8%) of such HIF inducible genes, respectively (27,28) (supplementary Table 2 is available at Carcinogenesis Online).

To gain insight into the role of HIF in hypoxic tumor microenvironment, the above hypoxic gene array results were compared with a set of gene array data (GSE17351) representing five paired primary esophageal tumors (ESCC) and adjacent normal mucosa. Only five genes, PTGES (also known as mPGES-1), IGFBP3, GLUT1, Lysyl oxidase-like 2 (LOXL2) and proline 4-hydroxylase(P4HA1), appeared to be overexpressed commonly in primary tumors as well as hypoxia at a high filtering stringency (<3% of the false discovery rate). i.e. \(Q\)-values) for hypoxic conditions (Figure 1A), whereas 28 genes passed filtering at a low stringency without setting a \(Q\)-value cut-off (supplementary Table 3 is available at Carcinogenesis Online). Among these were HIF target genes regulating glucose metabolism (e.g. GLUT1 and PDK1) and cancer invasion and metastasis through stromal remodeling (e.g. LOXL2). Our previous studies implied IGFBP3 in the pathogenesis of ESCC (23,25). To explore novel HIF target genes in esophageal tumor biology, we first considered those induced as a function of the extent of hypoxia. Robust induction of PTGES at lower oxygen tension (151.1-fold, \(Q = 0.00%\)) (Figure 1A and supplementary Table 3 is available at Carcinogenesis Online) was appealing given the role of PGE\(_2\) in tumor biology, especially regulation of blood flow and inflammation. Interestingly, PTGS2 (COX-2) was also induced by 0.2% \(O_2\) (supplementary Tables 2 and 3 are available at Carcinogenesis Online). Thus, we hypothesized that tumor microenvironment is permissive for activation of the COX-2–PTGES axis leading to increased biosynthesis of PGE\(_2\) (Figure 1B).

Fig. 1. Hypoxia may activate the COX-2–PTGES axis in ESCC. Gene array experiments imply the hypoxic activation of the central pathway regulating prostaglandin biosynthesis in the tumor microenvironment. (A) Venn diagram representing the overlap of genes that are significantly different in tumors relative to normal tissues (\(n = 5\), severe hypoxia (0.2% \(O_2\)) and moderate hypoxia (1% \(O_2\)) relative to normoxia (21% \(O_2\)). Fold change, \(>2; Q<10\%\) for ESCC, \(Q<1\%\) for 0.2% \(O_2\) and \(Q<3\%\) for 1% \(O_2\). (B) PTGES, also known as mPGES-1, generates PGE\(_2\) from PGH\(_2\). COX-2, also known as PTGS2, produces PGH\(_2\) from arachidonic acids (AA). HPGD mediates PGE\(_2\) degradation. IL-1\(\beta\) is known to activate the COX-2–PTGES axis. Although HIF is known to induce COX-2, hypoxia regulation of PTGES remains to be elucidated.
PTGES regulation. However, relatively modest (<2-fold) induction of PGE\(_2\) by hypoxia (Figure 4C) may imply a negative feedback mechanism limiting prostanoïd biosynthesis. In line with such a notion was induction of HPGD, a chief enzyme catalyzing prostaglandin, under moderate hypoxic conditions (1% \(O_2\)) (supplementary Figure 3B is available at Carcinogenesis Online). Interestingly enough, gene array data on primary tumors showed downregulation of HPGD as validated by quantitative reverse transcription–PCR showing sharp suppression in 10 of 13 (76.9%) primary ESCC samples (supplementary Figure 3B is available at Carcinogenesis Online), suggesting that loss of HPGD expression may lead to stabilization of PGE\(_2\) in tumor tissues.

The COX-2–PTGES enzyme axis is activated in ESCC

Our in vitro data indicate that PTGES is a novel HIF-inducible gene regulating PGE\(_2\) biosynthesis in concert with other factors such as COX-2 and HPGD. In addition, gene array data indicated upregulation of PTGES and COX-2 in primary ESCC tumors (supplementary Table 3 is available at Carcinogenesis Online). In fact, quantitative reverse transcription–PCR documented \( \geq \)2-fold overexpression of mRNA for PTGES and COX-2 in 8 of 13 (61.5%) and 5 of 13 (38.5%), respectively, in tumors compared with adjacent normal mucosa (Figure 5A and B), although there was no correlation at the mRNA levels between PTGES and COX-2 (Pearson correlation coefficient \(= -0.085; P = 0.783\)). Western blotting revealed PTGES upregulation in 7 of 13 (53.8%) tumor tissue samples evaluated (Figure 5C). When immunohistochemistry was done on an tissue microarray representing 35 paired ESCC and adjacent normal mucosa as well as independent seven primary tumor samples, including those from the identical cases (case # 1–5 in Figure 5) used for gene array experiments, PTGES was found negative or weakly positive in the normal esophageal epithelium (Figure 6A). In tumors, PTGES colocalized to the cyttoplasm along with other HIF target genes such as IGFBP3 and COX-2 (Figure 6A and data not shown), although the expression levels were not correlated between PTGES and COX-2 (Figure 6B, Pearson correlation coefficient \(= 0.329; P = 0.054\)). In addition, the expression level of each protein tended to spatially vary within a single tumor (data not shown), suggesting the heterogeneous nature or gradient of the tumor hypoxic microenvironment. Nonetheless, PTGES was upregulated markedly in 5 of 35 (14.3%) and moderately in 11 of 35 (31.4%) in the tumor tissue microarray samples (Figure 6B), consistent with the frequency of PTGES mRNA overexpression in tumor tissues (Figure 5A). Lack of correlation in the protein and mRNA expression level between PTGES and COX-2 may be accounted for by differential regulation by independent factors other than hypoxia.

One such regulatory factor may be the inflammatory cytokine IL-1\(\beta\), known to induce COX-2 (33), which appeared to be frequently overexpressed in 8 of 13 (61.5%) ESCC tissue samples (supplementary Figure 4A is available at Carcinogenesis Online). Interestingly, the expression level of IL-1\(\beta\) was found to be correlated with that of COX-2 (supplementary Figure 4B is available at Carcinogenesis Online) but not that of PTGES (Pearson correlation coefficient \(= -0.276; P = 0.361\)). In culture, IL-1\(\beta\) stimulated PGE\(_2\) production (supplementary Figure 5A is available at Carcinogenesis Online), albeit to a lesser extent compared with ectopically expressed PTGES (Figure 4A). Interestingly, IL-1\(\beta\) failed to enhance PGE\(_2\) production when PTGES was overexpressed (supplementary Figure 5A is available at Carcinogenesis Online). Moreover, IL-1\(\beta\) did not affect the hypoxic induction of HIF-1\(\alpha\) or PTGES in EPC2-hTERT cell derivatives (supplementary Figure 5B is available at Carcinogenesis Online and data not shown), suggesting that IL-1\(\beta\) may not regulate PTGES directly in esophageal epithelial cells, whereas HIF-1\(\alpha\) can regulate both COX2 and PTGES.

Discussion

This is the first demonstration of PTGES as a novel target of hypoxia in esophageal epithelial cells (Figures 1–3), regulating PGE\(_2\) production in culture (Figure 4) in addition to its overexpression in a subset of primary ESCC (Figures 5 and 6). In agreement with its potential
role in carcinogenesis, PTGES has been shown to transform HEK293 cells in concert with COX-2 (13). When COX-2 and PTGES were simultaneously targeted in the gastric glandular epithelium in transgenic mice, development of hyperplastic tumors was observed along with induction of inflammatory cytokines, chemokines and growth factors, increased PGE$_2$ levels and recruitment of macrophages (34). Genetic deletion of PTGES in $ApcMin$ mice suppressed the size and number of preneoplastic aberrant crypt foci as well as tumor growth (35). These mouse models imply a complex cross talk between the COX-2–PTGES axis and tumor microenvironment. Thus, future studies should determine the interplay among PTGES, COX-2 and HPGD as well as the role of influential factors such as hypoxia and inflammation in tumor tissues.

HIF-dependent PTGES induction in this study is a novel finding. Grimmer et al. (36) have recently demonstrated that hypoxia induced PTGES along with HIF-1$\alpha$ in chondrocytes. They implied HIF by antagonizing hypoxic induction of PTGES with 2-methoxyestradiol. However, 2-methoxyestradiol affects HIF-1$\alpha$ (37) as well as other transcription factors such as nuclear factor-kappaB and p53 (38,39), which can be activated by cellular stress under hypoxic conditions. PTGES is expressed in KYSE-170 and KYSE-270 ESCC lines and augmented by exposure to acidified cell culture medium supplemented with chenedoxycyclic acid and trypsin (40), implying a role for PTGES in gastroesophageal reflux disease and Barrett’s esophagus. Using a rat esophagoduodenal anastomosis model, Jang et al. (41) showed activation of the COX-2–PTGES axis in the reflux-induced esophageal squamous dysplasia and Barrett’s metaplasia. These data indicate cellular stress may induce PTGES in esophageal cells. Consistent with this premise, glutathione peroxidase 2, an antioxidant enzyme, appeared to inhibit PGE$_2$ production through downregulation of COX-2 and PTGES in HT-29 colon cancer cells (42). Thus, hypoxic induction of PTGES may involve reactive oxygen species that may serve as signals from a cellular oxygen sensor leading to stabilization of HIF (43).

The primary role of HIF in PTGES mRNA expression under hypoxic conditions was reinforced by our experiments using cobalt chloride under normoxic conditions (Figure 2E), excluding the direct transactivation of PTGES by hypoxia-inducible transcription factors other than HIF. In addition, dominant-negative HIF antagonized PTGES induction (Figure 2E and F), indicating the requirement of HIF. The mechanisms underlying transcriptional regulation of PTGES remain to be explored. Within 5 kb from the predicted transcription start site, there are three potential HIF-responsive elements (A/G)(CGT(G/C)C (−976 to −971, −466 to −461 and −317 to −312). In addition, three other potential sites exist within the first intron of PTGES. However, our data do not exclude the possibility that transcription factors activated by HIF, but not HIF per se may transactivate PTGES. Thus, future studies should determine HIF-responsive elements in the promoter and adjacent regulatory regions of PTGES.

Gene expression under hypoxic conditions is subjected to unique regulation through transcriptional as well as translational mechanisms.
Under hypoxic stress, global translation is reduced to conserve energy (44). However, hypoxia is permissive for selective translation of the mRNAs encoding proteins essential in hypoxic adaptation (45). Corroborating the premise for PTGES as a hypoxia-inducible gene, more pronounced upregulation of PTGES as well as CA9 proteins was observed at 24 h or later upon hypoxic exposure (Figure 3B).

In addition to COX-2 and PTGES, the other upstream and downstream molecules are thought to influence the PGE2 level and its signaling pathway. PGE2 receptor EP2 is overexpressed in a subset of ESCC and correlate with poor prognosis (46), although our quantitative reverse transcription–PCR data showed EP2 upregulation in only 1 of 13 ESCC samples (data not shown). IL-1β, a COX2 inducing cytokine (33), may cooperate with PTGES in PGE2 biosynthesis without affecting PTGES expression (supplementary Figure 5 is available at Carcinogenesis Online). However, lack of IL-1β-mediated PGE2 stimulatory effect in the presence of ectopically expressed PTGES (supplementary Figure 5A is available at Carcinogenesis Online) or relatively modest PGE2 induction under hypoxic conditions

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**Fig. 4.** PTGES and hypoxia regulate PGE2 production in human esophageal cells. Western blotting confirmed ectopic expression of PTGES (A) and the suppression of PTGES expression by RNA interference (B) in EPC2-hTERT-EGFR-p53R175H-cyclin D1 cells, an EPC2-hTERT cell derivative. Ectopically expressed PTGES was detectable under normoxic conditions in the PTGES but not empty control vector (Bla)-transduced cells in (A). Hypoxia (1% O2) induced PTGES in transformed EPC2-hTERT, although two independent short hairpin RNA (shRNA) sequences (PTGES1 and PTGES2) suppressed PTGES expression compared with scrambled short hairpin RNA sequence (control) in (B). Histograms represent the PGE2 levels in conditioned media of the PTGES-manipulated cells in (A and B). *P < 0.01 versus Bla in (A); **P < 0.01 versus control in (B) (n = 3). In (C), PGE2 levels were determined in conditioned media of the indicated EPC2-hTERT cell derivatives described in Figure 2. *P < 0.05 versus puro under normoxia (n = 3). Note that the PGE2 levels in basal conditions appeared to be 10–30 pg/ml per 10^6 cells in EPC2-hTERT and its derivatives.

**Fig. 5.** PTGES is overexpressed in ESCC. The mRNA levels for PTGES (A) and COX-2 (B) in ESCC tissues were determined by real-time reverse transcription–PCR. **P < 0.001 versus normal; *P < 0.05 versus normal; ns, not significant versus normal (n = 3). (C) PTGES protein was detected in ESCC tissues. N, normal; T, tumor.
(Figure 4C) may imply negative feedback mechanisms. Such an idea may be supported by hypoxic induction of HPGD mRNA in EPC2-hTERT cells (supplementary Figure 3A is available at Carcinogenesis Online); HPGD is downregulated in several gastric and colorectal cancers (47–49). HPGD inactivation may serve as a mechanism of resistance to celecoxib chemoprevention of colon tumors (50). Interestingly, HPGD mRNA appeared to be severely downregulated in all our ESCC samples tested (supplementary Figure 3B is available at Carcinogenesis Online), which has not been reported previously. These data reinforce the critical role of the COX-2–PTGES–PGE2 axis in esophageal carcinogenesis.

In summary, our data suggest that PTGES may play an essential role in biosynthesis of PGE2 in esophageal epithelial cells in response to hypoxia and other factors in tissue microenvironment such as inflammatory cytokines. Whereas PTGES mRNA expression is regulated by HIF-dependent transcriptional activation, PTGES protein is stabilized upon reoxygenation. Thus, the regulation of PTGES may be subject to complex levels of regulation involving other intrinsic enzymes such as COX-2 and HPGD.

Supplementary material

Supplementary Tables 1–3 and Figures 1–5 can be found at http://carcin.oxfordjournals.org/

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References


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