Identification of COX-2 as a major actor of the transcriptomic adaptation of endothelial and tumor cells to cyclic hypoxia[1]

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ABSTRACT

Cyclic hypoxia in tumors originates from heterogeneities in red blood cell flux and therefore influences tumor cells but also endothelial cells lining tumor blood vessels. The impact of fluctuations in pO_2 on the gene expression pattern of endothelial cells is largely unknown. Here, we compared the transcriptomic profile of endothelial cells exposed to cyclic hypoxia vs continuous hypoxia or normoxia. Microarray analyses identified the upregulation of a limited number of early genes that were more selectively triggered by cyclic hypoxia. Among them, we selected ADAMTS1, BMP2 and PTGS2 and used qPCR to perform time course studies. A transient increase in mRNA expression was confirmed for each of them in response to cyclic hypoxia. For PTGS2, this was sufficient to increase the expression and the activity of COX2 (the protein product of PTGS2) in both endothelial and tumor cells. The hypoxia-inducible factor 1α (HIF-1α) was exquisitely stabilized by cyclic hypoxia and favored COX-2 induction as validated by the use of echinomycin and HIF-1α targeting siRNA. Finally, using a specific COX-2 inhibitor, we documented that COX-2 accounted for the higher cell survival and angiogenic potential conferred by cyclic hypoxia.

In conclusion, our study documents that PTGS2/COX2 is part of a cyclic hypoxia gene signature and largely accounts for the unique phenotype of endothelial cells exposed to fluctuations in pO_2. Our data indicate that this may be extended to tumor cells, thereby offering new perspectives for the clustering of tumors expressing COX2 together with other cyclic hypoxia-responsive genes.

INTRODUCTION

Tumor hypoxia is nowadays recognized as a biologically unstable phenomenon (1-5). This feature referred to as intermittent or cyclic hypoxia may be described by considering two timescales of fluctuations (see (6) for review). A low frequency wave (hours to days) is attributable to the remodelling of the angiogenic tumor vascular network (7,8) whereas shorter cycles (minutes to hours) are directly caused by variations in red blood cell flux within tumor microvessels (3,9,10). Several mechanisms are described to account for these fluctuations including vasomotion of resistance vessels (11,12) and lack of deformability of hypoxic red blood cells (13,14). The phenotype of tumor cells is therefore directly influenced by the cyclic component of oxygen delivery in the tumor. Increased invasiveness and metastases were previously reported in response to tumor cell exposure to intermittent hypoxia (15,16). Another consequence, although poorly investigated, is that tumor microvessels, in particular endothelial cells at the interface with blood flow, are also influenced by fluctuations in O_2-transporting red blood cells. This may occur directly through, for instance, an unequal distribution of red blood cells at bifurcation points leading to a temporarily non-perfused vessel (or a vessel perfused with RBC-free serum) (6). Cycles of angiogenesis/vascular remodelling may also favor conditions where a given tumor area may undergo fluctuations in pO_2.

Here, we examined the impact of cyclic hypoxia on the transcriptomic profile of endothelial cells to get insights on pathways selectively triggered by fluctuations in pO_2. The use of endothelial cells for this screening instead of tumor cells was justified since (i) as stated above, endothelial cells lining tumor blood vessels are also exposed to periods of hypoxia and this may therefore influence their phenotype, including their angiogenic potential and (ii) the use of

genetically stable endothelial cells should allow to identify adaptation changes to cyclic hypoxia independently of alterations in the tumor cell genome (like mutations or chromosome aberrations). We found that among a short list of differentially regulated genes by O_2 status, prostaglandin synthase-2 (PTGS2) was a major target of cyclic hypoxia-triggered adaptation in endothelial cells and tumor cells. Using a pharmacological inhibitor of COX-2 (the protein encoded by PTGS2), we identified the functional roles of this enzyme in driving both endothelial cell survival and angiogenesis in response to cyclic hypoxia.

MATERIAL AND METHODS.

Cell culture and hypoxia protocols.

Human umbilical vein endothelial cells (HUVEC) were routinely cultured in a specific endothelial cell growth medium (EGM, Lonza, Belgium). Hepatocarcinoma cells (TLT) and cervix cancer cells (SiHa) were cultured in DMEM (Invitrogen, Belgium) with 10% fetal calf serum. Before treatments, cells were serum-starved; endothelial cells were incubated in endothelial basal medium (EBM, Lonza, Belgium) and tumor cells in serum-free DMEM.

To reach and control hypoxic conditions, cells were transferred in a modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA) and flushed for 10 minutes with a gas mixture of 5% CO_2-95% N_2 gas till the chamber atmosphere reaches a pO_2 value of 0.5% (as determined by oximetry). The hypoxic chamber was then sealed and placed in a 37°C incubator for the selected period of time. The cyclic hypoxia protocol consisted in 3 periods of 1 h-hypoxia interrupted by 30 min-reoxygenation whereas 3 hours of uninterrupted exposure to hypoxia were used for the continuous hypoxia protocol. Cells were either collected at the end of hypoxia (ie, the last period of hypoxia in the cycling protocol) or after one hour of reoxygenation (Figure 1). Cells maintained in normoxia were used as controls.

RNA isolation and microarray analysis

Total RNA was extracted with TriPure reagent (Roche, Belgium). Quantification and integrity of RNA were assessed by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Microarray analysis was performed on GeneChip human genome U133A 2.0 Array from Affymetrix (Santa Clara, CA). Target labeling for the expression analysis was performed from 5 µg total RNA and GeneChips were automatically stained and washed in a fluidics station,

as recommended by the manufacturer (Affymetrix). Genes were sorted as follows to determine the cyclic hypoxia signature: genes found to be "present" according to Gene Chip Operating software (GCOS, Affymetrix) and their expression levels changed by a factor >2 (and maintained for at least 1h) when comparing cyclic hypoxia *vs.* normoxia and by a factor <2 when comparing continuous hypoxia *vs.* normoxia, as determined by Gene Spring software (Agilent Technologies).

Real-time quantitative PCR

Reverse transcription was performed from total RNA using the SuperScript II RNase H^- reverse transcriptase and random hexamer primers (Invitrogen). Real-time quantitative PCR analyses were performed in triplicate using iQ SYBR Green Supermix (BioRad). The following specific primers were designed according to GenBank sequence ; HPRT (hypoxanthine phosphoribosyl transferase), ACTB (beta-actine) and RPL19 (ribosomal protein L19) sets were used for the housekeeping genes.

Genes	**Forward primers**	**Reverse primers**
Human sequences:		
PTGS2	CAGCCATACAGCAAATCCTTG	AATCCTGTCCGGGTACAATC
ADAMTS1	GTTCCTGTGGGCTGTCCTAC	ACCGAAGACGAGGACGAAG
BMP2	ACGCTCTTTCAATGGACGTG	GGAAGCAGCAACGCTAGAAG
HPRT	TGGCGTCGTGATTAGTGATG	CACCCTTTCCAAATCCTCAG
ACTB	ACAGAGCCTCGCCTTTGC	ACCCATGCCCACCATCAC
RPL19	CAAGCGGATTCTCATGGAACA	TGGTCAGCCAGGAGCTTCTT
Mouse sequences:		
PTGS2	AGCAGATGACTGCCCAACTC	GGGTCAGGGATGAACTCTCTC
ACTB	GATCATTGCTCCTCCTGAGC	ACTCCTGCTTGCTGATCCAC

PCR fluorescence data were obtained and analysed with the IQ5 instrument (Biorad). Ct (number of cycles needed to generate a fluorescent signal above a predefined threshold) was determined for each sample and the relative mRNA expression, expressed as fold-variation, was calculated using the $2^{-\Delta\Delta Ct}$ formula after normalization to housekeeping genes (ΔCt) and determination of the difference in Ct ($\Delta\Delta$Ct) between the various conditions tested.

Immunoblotting

Cells were lysed in RIPA buffer and immunoblotting was performed on total cell extracts as previously described (17,18). Anti-COX-2 antibody was from Cayman Chemicals (Ann Arbor, MI) and anti-β-actin from Sigma (Sigma-Aldrich, Belgium).

HIF-1α Inhibition

To evaluate HIF implication in COX-2 regulation, cells were incubated at the beginning of the hypoxia protocol with 10 ng/ml echinomycin, a HIF-1 DNA binding-activity inhibitor (Sigma), or its solvant DMSO. In other experiments, cells were transfected with HIF-1α-targeting siRNA (AACTGGACACAGTGTGTTTGA) or with control, non-targeting siRNA (AllStars negative siRNA, Qiagen) using Lipofectin reagent (Invitrogen) 24 hours before initiating the hypoxia protocols.

Prostaglandin production

Evaluation of COX-2 activity was made through immunoassay-based measurements of prostaglandins, i.e. PgE_2 and PgF_1a as PgI_2 stable metabolite and TxB_2 as TxA_2 metabolite, according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI). Prostaglandin

release in the endothelial and tumor cell supernatants for a 18h-period (post-hypoxia) were determined and normalized in regard to the protein concentrations determined from the corresponding cell lysates.

Survival and tube formation assays.

Cell survival was evaluated in a clonogenic assay according to a protocol previously developed (18). This test (generally reserved to tumor cells) integrates a pro-apoptotic stress for endothelial cells which need to recover from an important dilution factor at the time of plating. Briefly, cells were seeded at 1500 cells per 60 mm-dish and submitted to hypoxia protocols 24 hours later. Cells were then incubated for 7 days in full medium containing 10µM NS398 (COX-2 inhibitor) or DMSO (drug solvant) before crystal violet staining and counting of colonies. Endothelial tube formation was determined in serum-free conditions by seeding 4.10^4 endothelial cells (pre-exposed or not to hypoxia protocols) in 24-well dishes containing Matrigel (BD Biosciences) and 10µM NS398 or DMSO. Formation of capillary-like tubular structures was assessed by counting the number of tube intersections in different fields and expressed an angiogenic index.

Statistical analysis

Data are presented for convenience as mean ± SE. Statistical analyses were made using Student's t test or one-way ANOVA where appropriate.

RESULTS.

Cyclic hypoxia is associated with a specific gene expression profile in endothelial cells.

In order to identify the actors that participate in the endothelial cell adaptation to cyclic hypoxia, we performed studies exploiting high-density microarray technology. We compared the transcriptomic patterns of endothelial cells exposed to cyclic hypoxia *vs.* continuous hypoxia and normoxia (see schematic representation in Figure 1). The scheme protocol of cyclic hypoxia (1h-hypoxia/30 min-reoxygenation) was based on previous measurements (by our group) of fluctutations in the tumor vasculature occurring at the frequency of 0.5 to 1 cycle per hour (5,11,19).

We found that the expression of 231 genes was modified by a factor >2 in response to cyclic hypoxia *vs.* normoxia. As stated in the Methods section, to be considered significantly altered, changes in gene expression had to be maintained for one more hour. Also, we further restricted this set of genes to those not similarly regulated by continuous hypoxia (i.e., the expression of which not modified by a factor >2 *vs.* normoxic conditions). This led us to establish a short list of 12 genes up-regulated by cyclic hypoxia (Table 1).

Based on previously documented functions in endothelial cell biology, we picked out 3 upregulated genes (namely, ADAMTS1, BMP2 and PTGS2) and verified the extent of the changes in expression after cyclic hypoxia using qPCR. Slight differences were identified between microarray and qPCR data but the preferential upregulation of the three genes in response to cyclic hypoxia was confirmed (see Table 2). A time course study, however, revealed that the upregulation of ADAMTS1, BMP2 and PTGS2 was very transient since the expression of each transcript was back to basal levels as soon as 2-6 hours after the end of the last period of

hypoxia (Figure 2 A-C). No significant alteration in the expression of the three transcripts was observed in response to continuous hypoxia (Figure 2 A-C).

Cyclic hypoxia induces COX-2 expression in a HIF-1α-dependent manner.

Because of the previously reported role of COX-2 (the PTGS2 gene product) in preventing cell apoptosis in response to a large variety of stress, we then focused our work on this protein. Immunoblotting experiments revealed that the transient increase in COX-2 mRNA observed in response to cyclic hypoxia (see Figure 2A) did translate into an upregulation of COX-2 protein expression picking 6-18 hours later (Figures 3A and 3B). That continuous hypoxia did not impact on the COX-2 expression was also confirmed (Figure 3C). We previously reported that cyclic hypoxia stimulated the expression of HIF-1α despite the reoxygenation phases (18). This finding was extended in this study where the extent of HIF-1α signal was found to be strikingly higher in response to cyclic 3x1h-hypoxia than continuous 3h-hypoxia (7.2-fold *vs*. 2.1-fold, $P<0.01$, $n=3$) (Figure 3D). To determine the role of HIF-1α in the upregulation of COX-2, we used echinomycin, a pharmacological inhibitor of HRE binding as well as a specific siRNA targeting HIF-1α. Both echinomycin (Figure 3E) and HIF-1α siRNA (Figure 3F) prevented the increase in COX-2 mRNA transcript expression observed in response to cyclic hypoxia. Note that both strategies also reduced the expression of COX-2 mRNA in continuous hypoxia conditions (Figures 3E and 3F).

Cyclic hypoxia selectively increases COX-2 expression and activity in tumor cells.

We then determined whether the increase in COX-2 expression in endothelial cells was a phenomenon also observed in tumor cells. Using both human and mouse tumor cell lines (cervix

cancer SiHa and hepatocarcinoma TLT, respectively), we documented that COX-2 mRNA expression was upregulated in response to cyclic hypoxia but not to continuous hypoxia (Figure 4A). Importantly, this translated into an increase in COX-2 protein expression (Figure 4B) and enzymatic activity as authenticated by the measurements of the production of PgE_2, PgI_2 and TxA_2 (Figure 4C). We found that cyclic hypoxia was associated with a strong production of either prostanoid whereas continuous hypoxia did not influence COX-2 activity (Figure 4C). Of note, absolute levels of released prostaglandins were much higher in tumor cells than in endothelial cells (6.2 ± 1.4 *vs.* 0.24 ± 0.02 ng PGE2/μg prot., respectively; n=6). A 50%-higher level of PGE2 production was however reproducibly observed in endothelial cells in response to cyclic hypoxia *vs*. continuous hypoxia (not shown).

Cyclic hypoxia stimulates endothelial cell survival and tubulogenesis in a COX-2-dependent manner.

We then aimed to evaluate the role of COX-2 activity on endothelial cell survival and angiogenesis. Clonogenic assays revealed that cyclic hypoxia significantly increased the number of endothelial cell colonies formed *vs.* normoxia and continuous hypoxia (Figure 5A). The COX-2 inhibitor NS398 prevented this increase in endothelial cell survival whereas it failed to significantly influence survival in cells exposed to normoxia or continuous hypoxia. Similarly, while NS398 prevented the stimulatory effect of cyclic hypoxia on the capacity of endothelial cells to form tubes when cultured on Matrigel, NS398 did not alter angiogenesis under normoxia (Figure 5B). Also, although continuous hypoxia did stimulate angiogenesis above background, this was not prevented by the presence of NS398 (Figure 5B).

DISCUSSION.

A key finding of this study is the identification of a specific transcriptomic signature in response to cyclic hypoxia *vs.* continuous hypoxia. Two major insights about adaptation to cyclic hypoxia may be derived from these data. First, using a model of genetically stable cells (ie, endothelial cells), we brought the proof of principle that fluctuations in the pO2 values fostered the expression of specific sets of early genes that influence the cell phenotype. In particular, we found that cyclic hypoxia could induce the expression of PTGS2 in both non-tumor (ie, endothelial) and tumor cells (mouse hepatocarcinoma, human cervix cancer). Second, our data underscore the role of COX-2 (the PTGS2 product) in mediating the beneficial effects of cyclic hypoxia on endothelial cells. Using a specific COX-2 inhibitor, we documented that the endothelial cell survival and their capacity to form tubular structures, both promoted by exposure to cyclic hypoxia, were largely dependent on COX-2 activation. Our results document that while the endogenous COX-2 activation in endothelial cells is sufficient to exert these effects (see Figs 5A and 5B), major release of prostaglandins by tumor cells exposed to cyclic hypoxia (Fig. 4C) also supports a paracrine role of COX-2 activation in tumor cells. Moreover, recent studies indicate a positive feedback loop wherein the induction of COX-2, through the production of PGE2, promotes HIF-1 transcriptional activity and VEGF induction (20).

COX-2 was previously reported as a HIF-1α target in tumor cells (20) and endothelial cells (21). Our data identify this gene as a yet more exquisite target of HIF-1α in response to cyclic hypoxia. Indeed, after 3h-continuous hypoxia, the induction of COX-2 was barely detectable at the mRNA level and no significant changes were observed at the protein level in

endothelial and tumor cells. By contrast, the cyclic 3x1h-hypoxia protocol was sufficient to elicit a strong increase in PTGS2 mRNA transcript and COX-2 protein and activity. In other reports exploring the effects of continuous hypoxia, longer periods of hypoxia (ie, 6 to 24 hours) were actually needed to detect significant alterations in COX-2 protein levels (20,22). The higher extent of HIF-1α stabilization in response to cyclic hypoxia (Figs 3D) certainly accounts for a large part of the striking increase in COX-2 expression. It should be noted however that both pharmacological and genetic blockade of HIF-1α failed to completely prevent the impact of cyclic hypoxia on PTGS2 expression (Figs 3E and 3F). This suggests the possible synergistic effects of other transcription factors triggered by the alternation of hypoxia-reoxygenation. In particular, ROS-dependent activation of sensitive transcription factors are currently examined in our lab using adamts-1 and bmp2 as a model since, contrary to PTGS2, we could not block their induction by cyclic hypoxia through HIF-1α inhibition (not shown).

The status of COX-2 as a marker of cancer progression is matter of debate: both positive and negative correlations are reported in the literature (23-29). Although this may arise from methodological aspects such as multi- *vs*. univariable analysis or the mode of scoring, the multiple regulatory pathways leading to PTGS2 upregulation could also account for part of these discrepancies (ie, different co-regulated genes would be necessary to determine whether ptgs2 expression is associated with poor patient survival). Our data indicate that in the context of intermittent hypoxia, COX-2 is associated with cell survival and angiogenesis, and could therefore be part of a signature associated with bad prognosis. This may be particularly relevant in the selection of cancer patients prone to receive COX inhibitors as adjunct treatments. Indeed, although the use of non-steroidal anti-inflammatory drugs was associated with a better outcome

for different cancer patients, the enthusiasm was lately tempered by reports of GI toxicity and increased cardiovascular risk (30,31). Targeting COX inhibitors to the right patient population is therefore increasingly recognized as a safer way to exploit the potential of these drugs. The individualization of COX inhibition-based therapy already led to the demonstration of a profitable co-administration with conventional anti-cancer modalities (26,32).

Interestingly, PTGS2/COX-2 was recently identified as a key actor in metastatic progression in pre- and clinical studies (33). Using the same set of patients, we found that 81% of the genes associated with PTGS2/COX-2 upregulation in our experiments could discriminate between patients with or without metastases (Supplementary Figure 1). Interestingly, the same authors recently reported that the COX-2 expression actively participated in the assembly of new tumor blood vessels and favored the breaching of capillaries leading to the intravasation of tumor cells and their seeding as distant metastases (34); the latter processed being blocked by mouse pretreatment with celecoxib, a COX inhibitor. Together with other studies reporting the higher metastatic potential of cells submitted to cyclic hypoxia (15,16) (including also preliminary results from our lab using the exact same 3x1h-hypoxia protocol used in this study (not shown)), these data give credential to a key role of acute modulations of the microenvironment in tumor cell invasiveness.

In conclusion, our study identified the PTGS2 gene as part of a transcriptomic signature selectively induced by cyclic hypoxia and its protein product, COX-2, as a key mediator of the increased endothelial cell survival and angiogenesis observed in response to cyclic hypoxia. Together with the observation that cyclic hypoxia favors COX-2 expression and activity also in tumor cells, these findings provide a new rationale to select cancer patients who may benefit from COX inhibitors.

REFERENCES.

(1) Brown JM. Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism of reoxygenation. Br J Radiol 1979;52:650-6.

(2) Chaplin DJ, Olive PL, Durand RE. Intermittent blood flow in a murine tumor: radiobiological effects. Cancer Res 1987;47:597-601.

(3) Kimura H, Braun RD, Ong ET, et al. Fluctuations in red cell flux in tumor microvessels can lead to transient hypoxia and reoxygenation in tumor parenchyma. Cancer Res 1996;56:5522-8.

(4) Cardenas-Navia LI, Yu D, Braun RD, et al. Tumor-dependent kinetics of partial pressure of oxygen fluctuations during air and oxygen breathing. Cancer Res 2004;64:6010-7.

(5) Baudelet C, Ansiaux R, Jordan BF, et al. Physiological noise in murine solid tumours using T2*-weighted gradient-echo imaging: a marker of tumour acute hypoxia? Phys Med Biol 2004;49:3389-411.

(6) Dewhirst MW, Cao Y, Moeller B. Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. Nat Rev Cancer 2008;8:425-37.

(7) Jain RK. Molecular regulation of vessel maturation. Nat Med 2003;9:685-93.

(8) Carmeliet P. Angiogenesis in life, disease and medicine. Nature 2005;438:932-6.

(9) Lanzen J, Braun RD, Klitzman B, et al. Direct demonstration of instabilities in oxygen concentrations within the extravascular compartment of an experimental tumor. Cancer Res 2006;66:2219-23.

(10) Brurberg KG, Skogmo HK, Graff BA, Olsen DR, Rofstad EK. Fluctuations in pO2 in poorly and well-oxygenated spontaneous canine tumors before and during fractionated radiation therapy. Radiother Oncol 2005;77:220-6.

(11) Baudelet C, Cron GO, Ansiaux R, et al. The role of vessel maturation and vessel functionality in spontaneous fluctuations of T2*-weighted GRE signal within tumors. NMR Biomed 2006;19:69-76.

(12) Brurberg KG, Benjaminsen IC, Dorum LM, Rofstad EK. Fluctuations in tumor blood perfusion assessed by dynamic contrast-enhanced MRI. Magn Reson Med 2007;58:473-81.

(13) Pries AR, Schonfeld D, Gaehtgens P, Kiani MF, Cokelet GR. Diameter variability and microvascular flow resistance. Am J Physiol 1997;272:H2716-H2725.

(14) Kavanagh BD, Coffey BE, Needham D, Hochmuth RM, Dewhirst MW. The effect of flunarizine on erythrocyte suspension viscosity under conditions of extreme hypoxia, low pH, and lactate treatment. Br J Cancer 1993;67:734-41.

(15) Cairns RA, Hill RP. Acute hypoxia enhances spontaneous lymph node metastasis in an orthotopic murine model of human cervical carcinoma. Cancer Res 2004;64:2054-61.

(16) Cairns RA, Kalliomaki T, Hill RP. Acute (cyclic) hypoxia enhances spontaneous metastasis of KHT murine tumors. Cancer Res 2001;61:8903-8.

(17) Sonveaux P, Martinive P, DeWever J, et al. Caveolin-1 expression is critical for vascular endothelial growth factor-induced ischemic hindlimb collateralization and nitric oxide-mediated angiogenesis. Circ Res 2004;95:154-61.

(18) Martinive P, Defresne F, Bouzin C, et al. Preconditioning of the tumor vasculature and tumor cells by intermittent hypoxia: implications for anticancer therapies. Cancer Res 2006;66:11736-44.

(19) Baudelet C, Gallez B. How does blood oxygen level-dependent (BOLD) contrast correlate with oxygen partial pressure (pO2) inside tumors? Magn Reson Med 2002;48:980-6.

(20) Kaidi A, Qualtrough D, Williams AC, Paraskeva C. Direct transcriptional up-regulation of cyclooxygenase-2 by hypoxia-inducible factor (HIF)-1 promotes colorectal tumor cell survival and enhances HIF-1 transcriptional activity during hypoxia. Cancer Res 2006;66:6683-91.

(21) Schmedtje JF, Jr., Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. J Biol Chem 1997;272:601-8.

(22) Schmedtje JF, Jr., Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. J Biol Chem 1997;272:601-8.

(23) Tomozawa S, Tsuno NH, Sunami E, et al. Cyclooxygenase-2 overexpression correlates with tumour recurrence, especially haematogenous metastasis, of colorectal cancer. Br J Cancer 2000;83:324-8.

(24) Zhang H, Sun XF. Overexpression of cyclooxygenase-2 correlates with advanced stages of colorectal cancer. Am J Gastroenterol 2002;97:1037-41.

(25) Fidler MJ, Argiris A, Patel JD, et al. The potential predictive value of cyclooxygenase-2 expression and increased risk of gastrointestinal hemorrhage in advanced non-small cell lung cancer patients treated with erlotinib and celecoxib. Clin Cancer Res 2008;14:2088-94.

(26) Edelman MJ, Watson D, Wang X, et al. Eicosanoid modulation in advanced lung cancer: cyclooxygenase-2 expression is a positive predictive factor for celecoxib + chemotherapy--Cancer and Leukemia Group B Trial 30203. J Clin Oncol 2008;26:848-55.

(27) Sheehan KM, Sheahan K, O'Donoghue DP, et al. The relationship between cyclooxygenase-2 expression and colorectal cancer. JAMA 1999;282:1254-7.

(28) Fux R, Schwab M, Thon KP, Gleiter CH, Fritz P. Cyclooxygenase-2 expression in human colorectal cancer is unrelated to overall patient survival. Clin Cancer Res 2005;11:4754-60.

(29) Papadimitrakopoulou VA, William WN, Jr., Dannenberg AJ, et al. Pilot randomized phase II study of celecoxib in oral premalignant lesions. Clin Cancer Res 2008;14:2095-101.

(30) Hippisley-Cox J, Coupland C. Risk of myocardial infarction in patients taking cyclo-oxygenase-2 inhibitors or conventional non-steroidal anti-inflammatory drugs: population based nested case-control analysis. BMJ 2005;330:1366.

(31) Bertagnolli MM. Chemoprevention of colorectal cancer with cyclooxygenase-2 inhibitors: two steps forward, one step back. Lancet Oncol 2007;8:439-43.

(32) Fabi A, Metro G, Papaldo P, et al. Impact of celecoxib on capecitabine tolerability and activity in pretreated metastatic breast cancer: results of a phase II study with biomarker evaluation. Cancer Chemother Pharmacol 2008;62:717-25.

(33) Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. Nature 2005;436:518-24.

(34) Gupta GP, Nguyen DX, Chiang AC, et al. Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. Nature 2007;446:765-70.

Table 1. List of selectively upregulated genes in endothelial cells exposed to cyclic hypoxia. Data are presented *as fold-induction vs. normoxia* for the following conditions: cyclic hypoxia, cyclic hypoxia + 1 hour reoxygenation, continuous hypoxia and continuous hypoxia + 1 hour reoxygenation. Data were derived from Affymetrix microarrays (GeneChip® Human Genome U133A 2.0) using criteria of gene selection as detailed in the Method section (GEO access: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lrgdfkossqgeqjg&acc=GSE12246)

Gene codes	Affymetrix references	Gene names	Cyclic hypoxia	Cyclic hypoxia +1h reox.	Continuous hypoxia	Continuous hypoxia + 1h reox.
ADAMTS1	222162_s_at	ADAM metallopeptidase with thrombospondin type 1	**2.8**	**6.3**	1.0	1.2
H3F3B	211998_at	H3 histone, family 3B	**2.6**	**2.5**	1.3	0.9
KCNJ2	206765_at	potassium inwardly-rectifying channel J2	**2.4**	**2.5**	1.3	1.3
PLAGL1	207002_s_at	pleiomorphic adenoma gene-like 1	**2.3**	**2.5**	1.2	1.9
UBE2J1	217824_at	ubiquitin-conjugating enzyme E2, J1	**2.2**	**2.5**	1.3	1.4
PCDH17	205656_at	protocadherin 17	**2.1**	**2.1**	1.5	0.9
BMP2	205289_at	Bone morphogenetic protein2	**2.1**	**2.7**	1.2	1.2
AKAP8	203848_at	A kinase (PRKA) anchor protein 8	**2.1**	**2.6**	1.6	1.5
ARIH2	201230_s_at	ariadne homolog 2	**2.1**	**2.4**	1.3	1.4
EFNB2	202668_at	ephrinB2	**2.0**	**2.0**	1.5	1.2
Chr6 orf 62	222309_at	Chr6 orf 62	**2.0**	**2.2**	0.7	1.6
PTGS2	204748_at	prostaglandin-endoperoxide synthase 2	**2.0**	**2.5**	1.1	1.6

Table 2. Relative expression of ADAMTS1, BMP2 and PTGS2 genes in endothelial cells exposed to cyclic and continuous hypoxia. Data are presented *as fold-induction vs. normoxia* for the following conditions: cyclic hypoxia, cyclic hypoxia + 1 hour reoxygenation, continuous hypoxia and continuous hypoxia + 1hour reoxygenation. Data were derived from qPCR amplification curves and changes in expression were calculated, after normalization, with the ΔΔCt method; all the values corresponding to cyclic hypoxia (+1h reox) conditions are significantly different from 1 (***P<0.01, n=3-9).

Gene codes	Cyclic hypoxia	Cyclic hypoxia + 1h reox.	Continuous hypoxia	Continuous hypoxia + 1h reox.
ADAMTS1	**3.0**	**3.0**	1.0	1.1
BMP2	**2.3**	**2.3**	0.9	0.9
PTGS2	**3.5**	**2.9**	0.9	1.8

Figures legends.

Figure 1. Protocols of cyclic and continuous hypoxia. Endothelial and tumor cells were exposed to either three periods of 1h-hypoxia (0.5% O_2) interrupted by 30 min reoxygenation or three continuous hours of hypoxia, respectively. In both conditions, cells were collected and lysed for further mRNA extraction at the end of these protocols as well as after one additional hour reoxygenation, or for protein extraction at selected times after reoxygenation.

Figure 2. Time course of the changes in (A) PTGS2, (B) ADAMTS1 and (C) BMP2 mRNA expression. Bar graphs show the relative expression (calculated from corresponding qPCR data) of either gene in endothelial cells at the indicated periods of time after cyclic (left) and continuous (right) hypoxia protocols, as detailed in Figure 1. The reference value corresponds to the expression level in normoxic conditions (Nx). ** $P < 0.005$, *** $P < 0.001$ *vs*. normoxia, n = 3-9.

Figure 3. Increase in COX-2 protein expression after cyclic hypoxia is HIF-1α dependent. Endothelial cells were cultured under normoxia or exposed to cyclic or continuous hypoxia as detailed in Figure 1. Cells were collected 6 hours after the hypoxia protocols (**A**) or after the indicated period of times (**B, C**). Cell lysates were then processed in Western blotting for COX-2 detection; the reference value corresponds to the COX-2 protein level in normoxic conditions (Nx). Cells were also collected directly at the end of either hypoxia protocols and immunobloted for HIF-1α (**D**). In some experiments, endothelial cells were treated with 10 nM echinomycin (**E**) or transfected with HIF-1α-targeting siRNA (**F**); control cells were exposed to solvant (DMSO) or transfected with control siRNA. Cells were then collected one hour after the end of either hypoxia protocols and the impact of cyclic and continuous hypoxia *vs.* normoxia on COX-2 mRNA expression was determined by qPCR

analyses. In the siRNA transfection experiments, the extent of HIF-1α accumulation in hypoxic conditions was consistently found increased by ~2-fold *vs.* non-transfected cells. *P<0.05, **P<0.005 *vs.* corresponding untreated conditions (n = 3-4).

Figure 4. COX-2 expression and activity is selectively increased in response to cyclic hypoxia in tumor cells. Cervix cancer (SiHa) and hepatocarcinoma (TLT) cells were cultured under normoxia or exposed to cyclic or continuous hypoxia as detailed in Figure 1. Cells were collected 1h (A) or 18h (B) after the hypoxia protocols and further processed in qPCR and immunoblotting experiments, respectively. **A.** Bar graph shows the changes in PTGS2 mRNA expression in response to hypoxia *vs*. normoxia. **B.** Representative Western blot confirms the increase in COX-2 protein expression in TLT hepatocarcinoma cells exposed to cyclic hypoxia. **C.** Bar graphs show the 18h-accumulation of different prostanoids in the TLT hepatocarcinoma cell culture medium, as determined by immunoassay. ***P<0.001 vs. normoxia and continuous hypoxia (n = 6).

Figure 5. Cyclic hypoxia induces endothelial cell proliferation and tube formation in a COX-2-dependent manner. Endothelial cells were cultured under normoxia or exposed to cyclic or continuous hypoxia as detailed in Figure 1. In some experiments, cells were treated with the COX-2 inhibitor NS398 (10μM); control cells were exposed to solvant (DMSO). **A.** Bar graph represents the extent of endothelial cell survival determined in a clonogenic assay by counting the number of colonies of >50 cells. **B.** Bar graph represents the angiogenic index determined by measuring the number of tube intersections formed 12 hours after plating on Matrigel. The inset shows a representative picture of computerized tube network formed in the absence (left) or the presence (right) of NS398 under cyclic hypoxia. *P<0.05, **P<0.005, ***P<0.001, n = 4-6.

Figure 1.

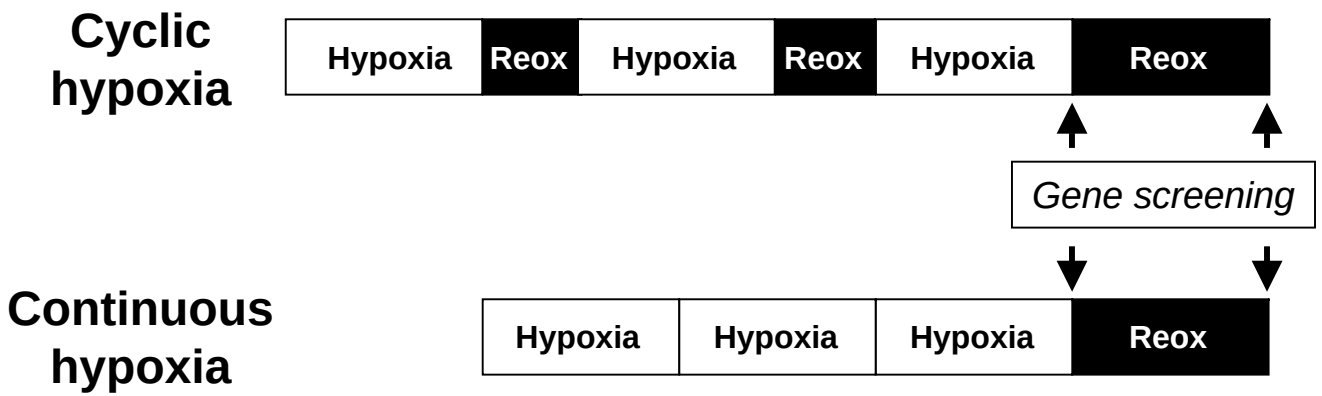

Cyclic hypoxia
Hypoxia
Reox
Hypoxia
Reox
Hypoxia
Reox
Gene screening
Continuous hypoxia
Hypoxia
Hypoxia
Hypoxia
Reox

Figure 2.

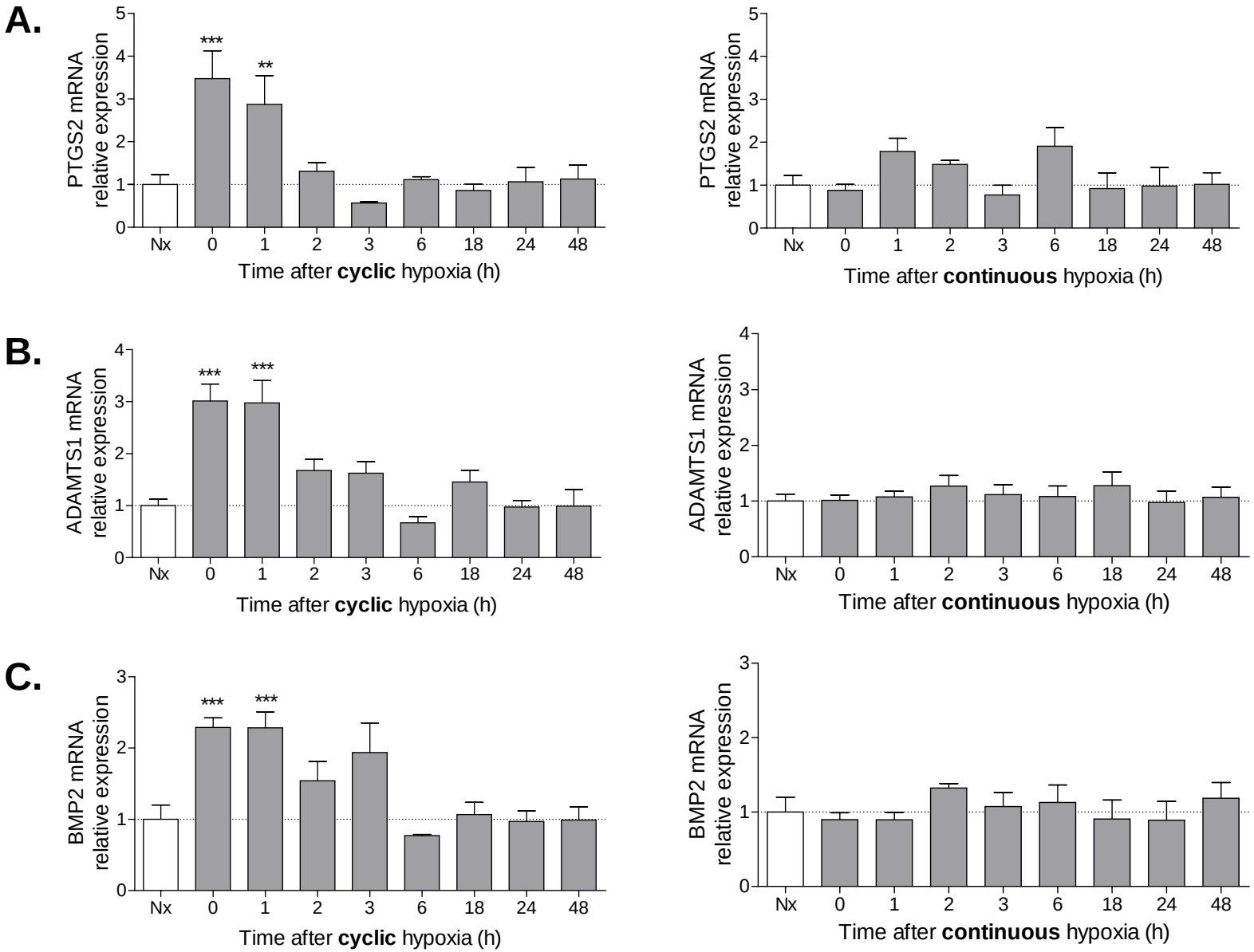

A.
PTGS2 mRNA relative expression
***
**
Nx 0 1 2 3 6 18 24 48
Time after **cyclic** hypoxia (h)
PTGS2 mRNA relative expression
Nx 0 1 2 3 6 18 24 48
Time after **continuous** hypoxia (h)
B.
ADAMTS1 mRNA relative expression
***
***
Nx 0 1 2 3 6 18 24 48
Time after **cyclic** hypoxia (h)
ADAMTS1 mRNA relative expression
Nx 0 1 2 3 6 18 24 48
Time after **continuous** hypoxia (h)
C.
BMP2 mRNA relative expression
***
***
Nx 0 1 2 3 6 18 24 48
Time after **cyclic** hypoxia (h)
BMP2 mRNA relative expression
Nx 0 1 2 3 6 18 24 48
Time after **continuous** hypoxia (h)

Figure 3.

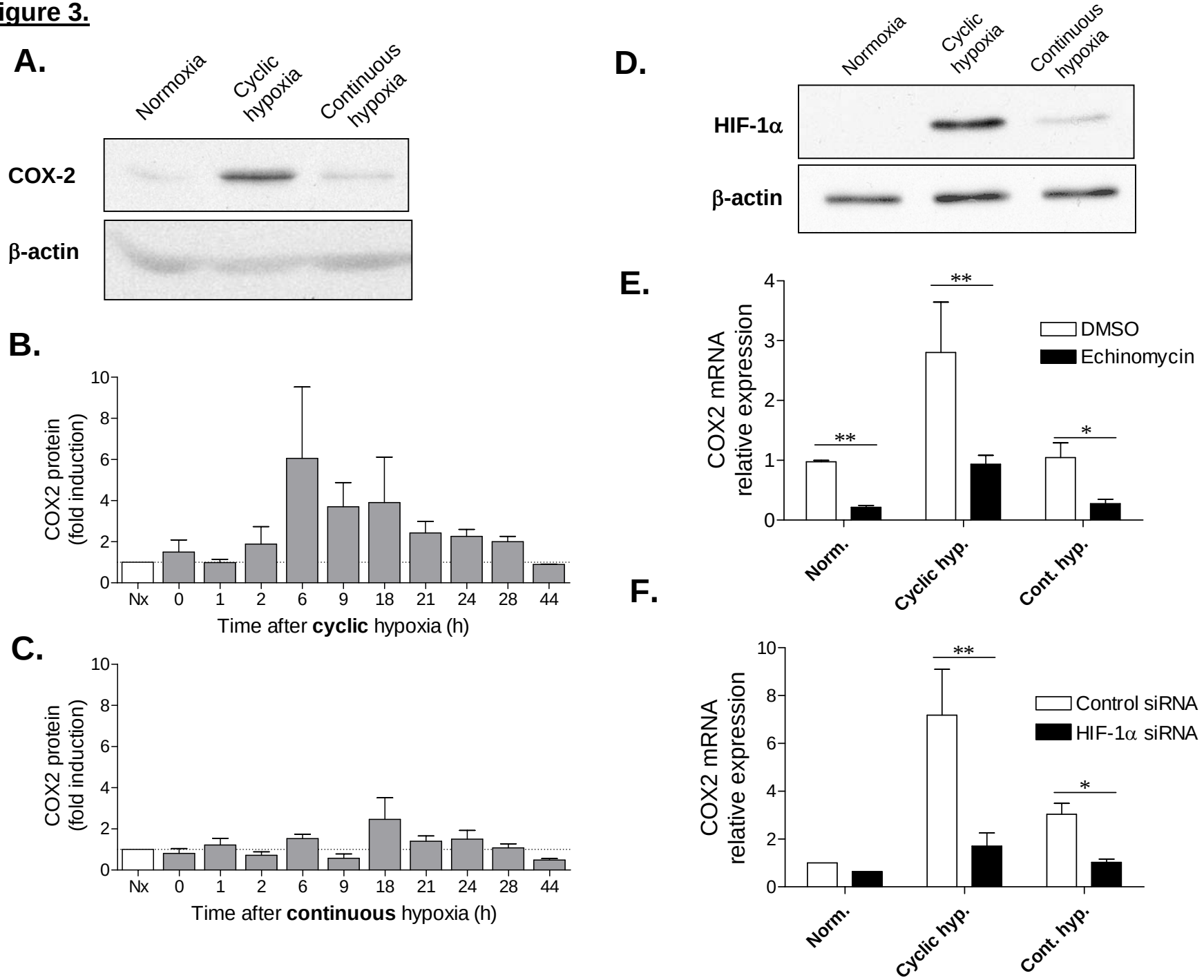

A.
Normoxia
Cyclic hypoxia
Continuous hypoxia
COX-2
β-actin
B.
COX2 protein (fold induction)
Nx 0 1 2 6 9 18 21 24 28 44
Time after cyclic hypoxia (h)
C.
COX2 protein (fold induction)
Nx 0 1 2 6 9 18 21 24 28 44
Time after continuous hypoxia (h)
D.
Normoxia
Cyclic hypoxia
Continuous hypoxia
HIF-1α
β-actin
E.
COX2 mRNA relative expression
DMSO
Echinomycin
Norm.
Cyclic hyp.
Cont. hyp.
F.
COX2 mRNA relative expression
Control siRNA
HIF-1α siRNA
Norm.
Cyclic hyp.
Cont. hyp.

Figure 4.

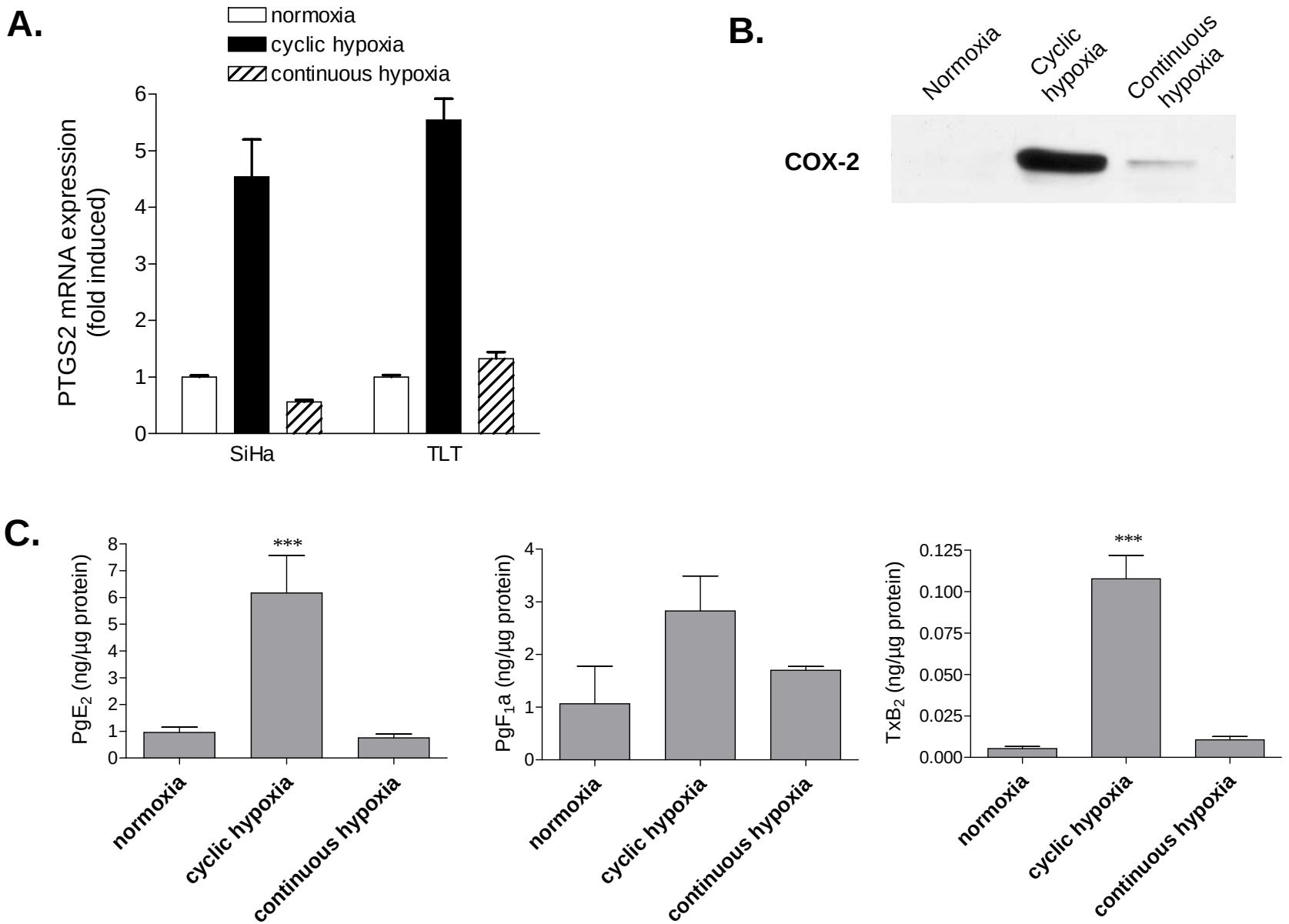
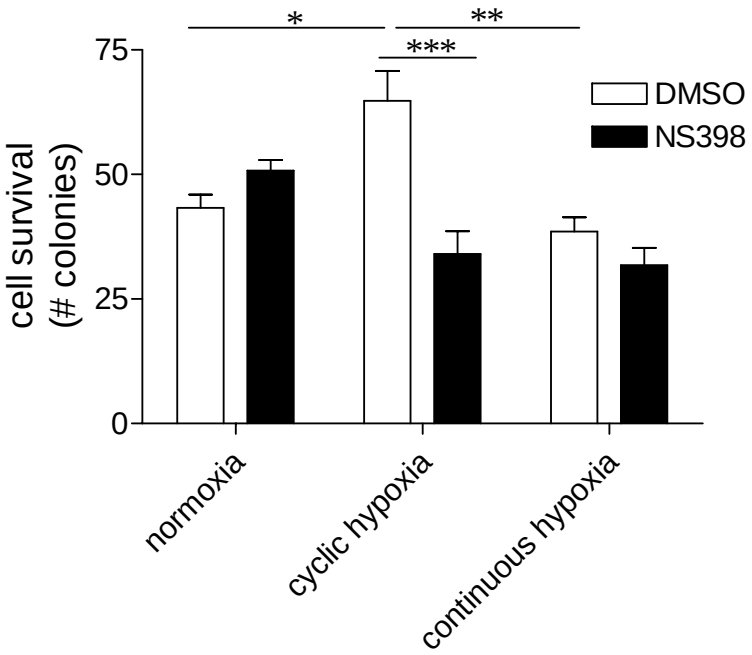
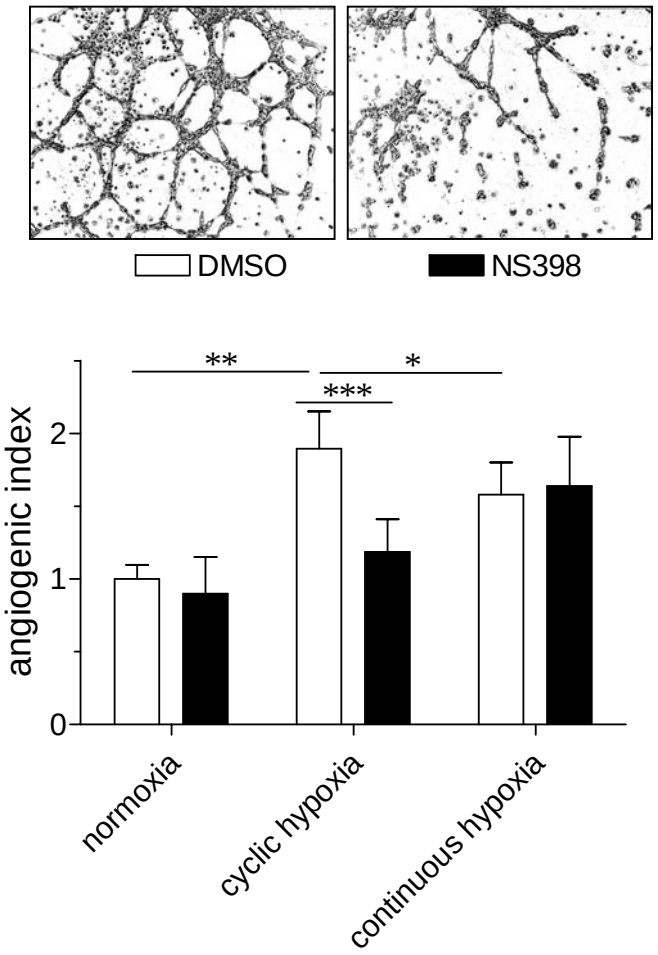

A.
normoxia
cyclic hypoxia
continuous hypoxia
PTGS2 mRNA expression (fold induced)
0
1
2
3
4
5
6
SiHa
TLT
B.
Normoxia
Cyclic hypoxia
Continuous hypoxia
COX-2
C.
PgE2 (ng/µg protein)
***
PgF1a (ng/µg protein)
TxB2 (ng/µg protein)
normoxia
cyclic hypoxia
continuous hypoxia

Figure 5.

A.


*
**
***
75
50
25
0
cell survival
(# colonies)
DMSO
NS398
normoxia
cyclic hypoxia
continuous hypoxia

B.


DMSO
NS398
**
*
***
2
1
0
angiogenic index
normoxia
cyclic hypoxia
continuous hypoxia