Effects of Hypoxia on Monocyte Inflammatory Mediator Production

DISSOCIATION BETWEEN CHANGES IN CYCLOOXYGENASE-2 EXPRESSION AND EICOSANOID SYNTHESIS a

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Blood-derived monocytes are found at sites of inflammation as well as in solid tumors and atherosclerotic arteries. They are an abundant source of inflammatory eicosanoids such as prostaglandin E2 (PGE2) and thromboxane A2, which are formed via arachidonic acid (AA) metabolism by cyclooxygenase-1/2 (COX-1/2). In vitro studies of inflammatory mediator production are conducted invariably in room air, which does not reflect the oxygen tensions found in monocyte-containing lesions, which are frequently hypoxic. In this work we examined the effects of hypoxia at levels reported in these lesions, on monocyte COX-2 expression, the related events that lead to either a tumor necrosis factor (TNF)-α or other proinflammatory cytokine stimulus, and on COX-2 activity. We show that monocytes increase in COX-2 expression and COX-2 activity when exposed to hypoxia. The results suggest the existence of an autocrine loop involving COX-2 and a cPLA2 immunoblot. Additionally, in Fig. 10, lane 3 of the normoxic panel was reused in lane 2 of the hypoxic panel.

The histopathology of inflamed lesions shows infiltration with monocytes, which are an abundant source of clinically important inflammatory mediators. The eicosanoids, prostaglandin E2 (PGE2) and thromboxane A2 (TXA2), are produced via the cyclooxygenase (COX) activity of monocytes. Both have roles in inflammation. PGE2 can cause hyperalgesia and vasodilation, and TXA2 is a facilitator of inflammatory cytokine function (1, 2). COX-1 is constitutively expressed in most tissues and synthesizes prostaglandins for normal physiological function (3). COX-2, not normally expressed by monocytes, may be induced rapidly by inflammatory stimuli such as cytokines or bacterial endotoxin (4). Consequently, COX-2 activity is commonly associated with inflammatory responses because of its augmentation of eicosanoid biosynthesis (5, 6).

Studies of COX-2 expression, cPLA2 activation, and eicosanoid synthesis were performed under normoxic and hypoxic conditions. In Fig. 4, the “no LPS” lanes in the GAPDH Northern blots were duplicated between normoxic and hypoxic conditions. In Fig. 6A, several bands were duplicated in the COX-2 immunoblot. In Fig. 10, the first lanes between normoxic and hypoxic conditions were duplicated in the phosphorylated cPLA2 immunoblot. Additionally, in Fig. 10, lane 3 of the normoxic panel was reused in lane 2 of the hypoxic panel.

This article has been retracted by the publisher. An investigation by the Journal determined the following. In Fig 4, the “no LPS” lanes in the GAPDH Northern blots were duplicated between normoxic and hypoxic conditions. In Fig. 6A, several bands were duplicated in the COX-2 immunoblot. In Fig. 10, the first lanes between normoxic and hypoxic conditions were duplicated in the phosphorylated cPLA2 immunoblot. Additionally, in Fig. 10, lane 3 of the normoxic panel was reused in lane 2 of the hypoxic panel.

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‡ The abbreviations used are: PGE2, prostaglandin E2; AA, arachidonic acid; cPLA2, cytosolic phospholipase A2; COX, cyclooxygenase; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MAPK, MAFK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PEI, polyethyleneimine; RIA, radioimmunoassay; TNF-α, tumor necrosis factor-α; TXA2, thromboxane A2; TXB2, thromboxane B2.

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Eicosanoid Measurement—TXA₂ has a t₁/₂ of ~30 s under physiological conditions and is hydrolyzed to the stable metabolite TxB₂, which was measured. PGE₂ and TxB₂ levels were determined by RIAs. The TxB₂ assay used rabbit antiserum raised against thromboxane-conjugated TxB₂ (18).

**Fig. 1.** Effect of hypoxia on COX-2 mRNA and protein. Monocytes (5 × 10⁶) were stimulated with 2 ng/ml LPS and 1 ng/ml TNF-α or 2 ng/ml interleukin (IL)-1β for 24 h in normoxia or hypoxia. Cells were processed for Western blot analysis as described under “Methods.” Blots are representative of three separate experiments, the mean values of which are shown in the graph. Bars with different letters are significantly different (p < 0.05).

**Fig. 2.** Effect of hypoxia on COX-2 protein induction in monocytes with various stimuli. Monocytes (5 × 10⁶) were stimulated with 20 ng/ml LPS, 1 ng/ml TNF-α, or 2 ng/ml interleukin (IL)-1β for 24 h in normoxia or hypoxia. Cells were processed for Western blot analysis as described under “Methods.” Blots are representative of three separate experiments, the mean values of which are shown in the graph. *p < 0.05 compared with the same stimuli in normoxic monocytes.

20 to 0.312 μg/ml TNF-α, 1:3 were added together with 50 μg/ml leupeptin (matched pair antibody) against 0.05 μg/ml mouse monoclonal (matched pair antibody) and 0.05 μg/ml human TNF-α (10 μg/ml leupeptin, 10 μg/ml aprotinin) and 60 μg of 2× phosphate buffer (0.125 M Tris-maleate, pH 7.4, 5% (w/v) dried milk, 0.15% Tween 20, 0.1% β-mercaptoethanol). Samples were heated at 95 °C for 7 min before storing at −20 °C. Proteins (50 μg) were separated on 9% SDS-PAGE and then transferred onto either a Sequi-Blot™ polyvinylidene difluoride membrane or a nitrocellulose membrane at ~4 °C for 16 h at ~300 mA. The membrane was blocked for 1 h at 25 °C in Tris-buffered saline (25 mM Tris-HCl, 0.2 M NaCl, 0.15% Tween 20, pH 7.6) containing 5% (w/v) dried milk. Subsequently, membranes were treated with the relevant antibodies at the following dilutions: polyclonal COX-2, 1:10,000; anti-TX synthase, 1:10,000; polyclonal phospho-p38 MAPK, 1:1,000; phospho-p44/42 MAPK, 1:1,000; polyclonal phospho-p38 MAPK, 1:1,000; phospho-p44/42 MAPK, 1:1,000; and monoclonal β-actin antibody. These were followed by horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse antibodies. Bound antibodies were revealed with the Supersignal WestPico chemiluminescent system following the manufacturer’s protocol (Pierce).

**Northern Blot—** Total RNA was isolated using TriZol (Invitrogen) according to manufacturer’s protocol. Total RNA (10 μg/lane) was heated at 68 °C for 10 min, electrophoresed on a 1% agarose-formaldehyde gel, transferred to a positively charged nylon membrane (Hybond N+, Amersham Biosciences, Inc.), and UV cross-linked. Membranes were prehybridized for 2 h at 55 °C and subsequently hybridized overnight at 43 °C with random primer [32P]dCTP-labeled human COX-2 cDNA or gyceraldehyde-3-phosphate dehydrogenase probe using a DIG DNA Labeling Kit (Bresatec, Adelaide, Australia). The COX-2 cDNA probe was prepared by reverse transcription-PCR as described (19). Equal RNA loading efficiency was determined by visualization of 28 S and 18 S bands using a densitometer.
**RESULTS**

**Effect of Hypoxia on Monocyte COX-2 Message and Protein**—U937 monocytic cells (2 × 10⁶ cells/2 ml) were transfected with 4 µg of the COX-2–531 construct using Jet PEI and incubated for 5 h. The medium was changed and stimulated with 20 µg/ml serum-treated zymosan for 8 h in normoxia or hypoxia. Cells were then lysed with Passive Lysis Buffer and assayed for luciferase activity (n = 3). *p < 0.05 compared with normoxic monocytes. Results are representative of four separate experiments in triplicate.

**Effect of Hypoxia on COX-2 mRNA Stability.** Monocytes (5 × 10⁶) were transiently stimulated with 200 ng/ml LPS for 3 h (37 °C) in normoxia or hypoxia. Actinomycin D (AD) (5 µg/ml) was then added, and the level of COX-2 mRNA was assessed for a further 3 h by Northern analysis. The blot is representative of three separate experiments, the mean values of which are shown in the graph as percent change from time 0 h. *p < 0.05 compared with the equivalent times in hypoxic monocytes. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Effect of Hypoxia on COX-2 activity.** Monocytes (5 × 10⁶) were transiently stimulated with 200 ng/ml LPS for 3, 6, and 18 h in normoxia or hypoxia. Supernatants were collected and assayed for PGE₂ and TXA₂, compared with normoxic monocytes.

**Transplantation—U937 monocytic cells were plated in 12-well plates (2 × 10⁶ cells/2 ml) in RPMI with 10% FCS and 50 ng/ml phorbol 12-myristate 13-acetate, which promotes differentiation after 3–5 days of treatment (22). After differentiation, cells were transfected using Jet PEI (PolyTransfection), according to the manufacturer's instructions. Briefly, 4 µg of the pGL3-COX-2–531 construct was suspended in 75 µl of 150 mM sterile NaCl solution. Also 4 µl of Jet PEI solution was suspended in 75 µl of 150 mM sterile NaCl solution. The Jet PEINaCl solution and incubated at room temperature for 30 min. The medium in the wells was then changed to fresh medium, and 150 µl of the DNA/Jet PEI was added to each well. The transfection was allowed to proceed for 5 h, and the medium replaced again with either hypoxic or normoxic medium. The cells were then stimulated with 100 ng/ml serum-treated zymosan for specified times. Following the transfection period, the medium was removed and discarded and the cells lysed with Passive Lysis Buffer supplied in the Dual Luciferase™ Reporter Assay Kit. The lysate was then assayed for luciferase activity.

**Statistical Analysis—**Results are expressed as the mean ± S.E. of triplicate incubations. Analysis of variance followed by the Newman-Keuls multiple comparisons test was used to identify the statistically significant differences between treatments using WINKS (Texasoft, Cedar Hill, TX).

**Fig. 3. Effect of hypoxia on COX-2 transcription in monocytes.** U937 monocytic cells (2 × 10⁶ cells/2 ml) were transfected with 4 µg of the COX-2–531 construct using Jet PEI and incubated for 5 h. The medium was changed and stimulated with 20 µg/ml serum-treated zymosan for 8 h in normoxia or hypoxia. Cells were then lysed with Passive Lysis Buffer and assayed for luciferase activity (n = 3). *p < 0.05 compared with normoxic monocytes. Results are representative of four separate experiments in triplicate.

**Fig. 4. Effect of hypoxia on COX-2 mRNA stability.** Monocytes (5 × 10⁶) were transiently stimulated with 200 ng/ml LPS for 3 h (37 °C) in normoxia or hypoxia. Actinomycin D (AD) (5 µg/ml) was then added, and the level of COX-2 mRNA was assessed for a further 3 h by Northern analysis. The blot is representative of three separate experiments, the mean values of which are shown in the graph as percent change from time 0 h. *p < 0.05 compared with the equivalent times in hypoxic monocytes. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
human monocytes in a time-dependent manner over 18 h. The up-regulation of COX-2 mRNA and protein was greatly potentiated by hypoxia (1% O2) (Fig. 1). This augmentation of COX-2 expression by hypoxia was observed with a variety of costimuli (Fig. 2).

It was reported that hypoxia can increase transcription of COX-2 in endothelial cells (21), and therefore this mode of regulation in monocytes was examined. Many attempts to transfect fresh human monocytes transiently with a COX-2 promoter/luciferase reporter construct were unsuccessful. However, the human monocytic cell line U937 was transfectable, and these cells were used. Hypoxia augmented activity of the 531-bp segment of the COX-2 promoter in U937 cells (Fig. 3). Another mode of regulation of COX-2 levels can occur post-transcriptionally with stabilization of mRNA in response to LPS or interleukin-1β (23), although this has not been examined in hypoxia. Therefore, the effect of hypoxia on COX-2 mRNA stability in monocytes was examined. Monocytes were transiently stimulated with LPS for 15 min in normoxia or hypoxia and then washed and incubated in fresh normoxic or hypoxic medium for 3 h to allow synthesis of COX-2 mRNA. Actinomycin D was added to inhibit further transcription, and the level of COX-2 mRNA was measured for a further 3 h. COX-2 mRNA levels decreased in normoxia by more than 90% within 3 h after the addition of actinomycin D (Fig. 4). By comparison, COX-2 mRNA levels decreased in hypoxia by less than 20% within 3 h after the addition (Fig. 4).

Effect of Hypoxia on Monocyte COX-2 Activity—Fresh monocytes were treated with LPS for 18 h at 37 °C in normoxia or hypoxia, and the accumulation of PGE2 and TXA2 in the cell supernatants was measured. There was a marked reduction in the accumulation of PGE2 and TXA2 synthesis in hypoxia (Fig. 5). Similar time courses and reduced eicosanoid synthesis in hypoxia were also observed when LPS was transient, with LPS removal after 15 min (data not shown). The reduced synthesis of these eicosanoids in hypoxia did not correlate with the increased expression of COX-2 protein in hypoxia, described
above. Possible explanations for the disparate hypoxia-induced changes in COX-2 expression and eicosanoid synthesis were sought.

**Effect of Heme on COX-2 Activity in Hypoxia**—COX-2 is a heme-containing enzyme, and cellular heme levels can be reduced by heme oxygenase, including the inducible isoform, heme oxygenase-1, which may be up-regulated during hypoxia (24–26). Therefore, the cellular levels of heme may become limiting for adequate COX-2 constitution in hypoxia and may explain the dissociation of up-regulated COX-2 protein and activity. The addition of heme or a heme oxygenase inhibitor, zinc-protoporphyrin IX, under previously reported conditions (25) did not affect the amount of COX-2 protein (Fig. 6a) or the production of PGE$_2$ and TXA$_2$ (Fig. 6b) protein in normoxia or hypoxia under these experimental conditions, suggesting that heme oxygenase activity or heme levels are not responsible for reduced COX-2 activity in hypoxia.

**Dependence of COX-2 Activity on O$_2$ as a Cosubstrate in Hypoxia**—COX-2 utilizes oxygen as a cosubstrate during the conversion of AA to prostaglandin H$_2$, the common precursor of PGE$_2$ and TXA$_2$. In this study, O$_2$ in the incubation chamber was set at 1%; cf. ~20% for air at sea level. This level of hypoxia reduced dissolved oxygen in the incubation medium to 33 mm Hg. To determine whether these levels of O$_2$ were rate-limiting for eicosanoid synthesis, monocytes were first incubated in hypoxia with LPS to induce COX-2. After 18 h, cells were washed twice and incubated in fresh hypoxic or normoxic medium with 10 $\mu$M exogenous AA for 15 min. Oxygenation of the medium had no effect on the production of PGE$_2$ and TXA$_2$ (Fig. 7). These results indicated that dissolved O$_2$ at the levels of hypoxia used in this study were not rate-limiting for COX activity.

**Effect of Exogenous AA on COX-2 Activity in Hypoxia**—Monocytes were incubated with LPS for 18 h in the absence or presence of hypoxia to induce COX-2. The following day the cells were washed three times and stimulated with 200 ng/ml LPS for 30 h at 37 °C in normoxia or hypoxia. In addition, cells were hypoxic for 9 h and reoxygenated for a further 21 h. Supernatants were collected, and [3H]AA release was determined using a scintillation counter. *p < 0.05 compared with normoxic monocytes. Results are representative of three separate experiments.

**Effect of Hypoxia on Phosphorylated cPLA$_2$**. Monocytes were incubated with 1 $\mu$M A23187 under normoxic or hypoxic conditions up to 60 min. At each time point, cells were processed for Western blot analysis. Blots are representative of three separate experiments, the mean values of which are shown in the graph as percent inhibition from time 0 h. *p < 0.05 compared with normoxic monocytes.

Monocytes and Hypoxia

**Monocytes and Hypoxia**

Phosphorylated cPLA$_2$

N   H

Normoxia

Hypoxia

Time (min)

0   10  30  60

PGE$_2$ and TXA$_2$ in this study. O$_2$ in the incubation chamber was set at 1%; cf. ~20% for air at sea level. This level of hypoxia reduced dissolved oxygen in the incubation medium to 33 mm Hg. To determine whether these levels of O$_2$ were rate-limiting for eicosanoid synthesis, monocytes were first incubated in hypoxia with LPS to induce COX-2. After 18 h, cells were washed twice and incubated in fresh hypoxic or normoxic medium with 10 $\mu$M exogenous AA for 15 min. Oxygenation of the medium had no effect on the production of PGE$_2$ and TXA$_2$ (Fig. 7). These results indicated that dissolved O$_2$ at the levels of hypoxia used in this study were not rate-limiting for COX activity.

**Effect of Exogenous AA on COX-2 Activity in Hypoxia**—Monocytes were incubated with LPS for 18 h in the absence or presence of hypoxia to induce COX-2. The following day cells were washed twice and incubated with fresh normoxic or hypoxic medium and 10 $\mu$M exogenous AA for 15 min. Oxygenation of the medium had no effect on the production of PGE$_2$ and TXA$_2$ (Fig. 8). This contrasted with results above (Fig. 5) where eicosanoid synthesis from endogenous AA was reduced in hypoxia. This suggested that the reduced accumulation of eicosanoids observed in hypoxia, despite the up-regulated levels of COX, was caused by the decreased availability of endogenous AA substrate.

**Effect of Hypoxia on Endogenous AA Release**—Monocytes were incubated with [3H]AA in normoxia for 18 h at 37 °C to incorporate labeled AA into cell membranes. After 18 h, cells were washed three times and incubated with LPS in normoxia or hypoxia for 30 h. In addition, [3H]AA-prelabeled cells that
had been hypoxic for 9 h were returned to oxygenated conditions for the next 21 h. In normoxia, there was a time-dependent increase in the release of labeled AA from monocytes when stimulated with LPS (Fig. 9). By comparison, there was a marked reduction in the release of AA from monocytes stimulated with LPS in hypoxia (Fig. 9). Reoxygenation after 9 h of hypoxia resulted in a gradual restoration of AA release from cells to rates that were similar to those observed in normoxic cells (Fig. 9). Because cPLA2 is prominently involved in the release of AA from membrane phospholipids, the effects of hypoxia on cPLA2 phosphorylation were examined.

Effect of Hypoxia on the Phosphorylation of cPLA2—After stimulation with 1 μM A23187, the phosphorylation of cPLA2 appeared to be maximal at 30 min, and dephosphorylation occurred at times after 30 min (Fig. 10). In contrast, phosphorylation of cPLA2 in hypoxia appeared to be reduced at 10 min and showed an accelerated dephosphorylation of the enzyme at later times (Fig. 10). MAP kinases may regulate the phosphorylation and activation of cPLA2 (27–32). Therefore, the effects of hypoxia on cPLA2 phosphorylation were examined.

Effect of Hypoxia on the Phosphorylation of p44/42 MAPK and p38 MAPK—In normoxia, the phosphorylation of p44/42 was maximal at 30 min followed by dephosphorylation at later times (Fig. 10). MAP kinases may regulate the phosphorylation and activation of p44/42 MAPK (ERK1/2) and p38 MAPK were examined.

In normoxia, the phosphorylation of p38 MAPK was maximal at 30–60 min followed by dephosphorylation at later times. Hypoxia had no effect on the time course of phosphorylation of p38 MAPK or the time course of decay in the amount of phosphorylated enzyme (Fig. 11).
Effect of Inhibition of p44/42 MAPK Activation on AA Release in Hypoxia—Monocytes were incubated with $[^3]$H]AA in normoxia for 18 h at 37 °C for incorporation into cell membranes. After 18 h, cells were washed three times and incubated with

Effect of Hypoxia on Monocyte TNF-α Synthesis—Fresh monocytes were treated with LPS in normoxia or hypoxia, and the accumulation of TNF-α in the cell supernatants was measured. Hypoxia markedly increased TNF-α synthesis (Fig. 13). Similar time courses and increased TNF-α synthesis in hypoxia were also observed when LPS stimulation was transient (data not shown). To examine the possibility that the reduction in PGE2 synthesis in hypoxia may be related in part to the augmentation of TNF-α production, the effects of hypoxia on TNF-α synthesis were examined in the presence of COX inhibitors.

Monocytes were preincubated for 15 min at 37 °C with a selective COX-2 inhibitor, NS398 (1 μM) or a general COX inhibitor, indomethacin (10 μM) prior to LPS stimulation in normoxia and hypoxia. In normoxia and hypoxia, both NS398 and indomethacin resulted in a marked reduction in PGE2 production and a significant increase in TNF-α synthesis (Fig. 14). Furthermore, in the presence of both NS398 and indomethacin, TNF-α synthesis was similar under normoxic and hypoxic conditions (Fig. 14). In addition, exogenous PGE2 dose-dependently inhibited TNF-α synthesis in both normoxia and hypoxia (Fig. 15).

Effect of Monocyte-derived TNF-α on COX-2 Induction and Activity—Because hypoxia increased TNF-α synthesis, it was important to determine whether endogenous TNF-α could have an autocrine effect on COX-2 expression in monocytes. To examine this, fresh monocytes were preincubated with a neutralizing antibody against TNF-α or with an isotype-matched control antibody (1B5) prior to LPS stimulation in normoxia.

**DISCUSSION**

To date, in vitro studies of inflammatory mediator production by human monocytes/macrophages have been well characterized in normoxic conditions (20% O2). However, this is unlikely to reflect conditions of oxygenation which monocytes

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**FIG. 13.** Effect of hypoxia on TNF-α synthesis. Monocytes (5 × 10⁶) were stimulated with 200 ng/ml LPS for 3, 6, and 18 h in normoxia or hypoxia. Supernatants were collected and assayed for TNF-α by ELISA. *p < 0.05 compared with normoxic monocytes. Results are representative of three separate experiments.

**FIG. 14.** Effect of COX inhibitors on TNF-α synthesis. Monocytes (5 × 10⁶) were preincubated for 15 min at 37 °C with 1 μM NS398 (NS) or 10 μM indomethacin (Indo) before LPS stimulation for 18 h at 37 °C in normoxia or hypoxia. Supernatants were collected and assayed for PGE2 by RIA. TNF-α was measured by ELISA. *p < 0.05 compared with no inhibitor. a, p < 0.05 compared with equivalent treatment in normoxia. Results are representative of three separate experiments.

**FIG. 15.** Effect of exogenous PGE2 on TNF-α synthesis. Monocytes (5 × 10⁶) were preincubated for 15 min at 37 °C with 1 μM NS398 then 0–1,000 ng/ml PGE2. For LPS stimulation for 18 h at 37 °C in normoxia or hypoxia, supernatants were collected, and TNF-α was measured by ELISA and compared with nil PGE2 addition. Results are representative of three separate experiments performed in duplicate.
encounter in monocyte-containing lesions such as inflamed joints, atheromatous lesions, and solid tumors. Joints with effusions can be chronically hypoxic (14–16, 33), and dissolved O₂ levels in the range of 8–80 mm Hg have been observed (14–16). The presence of an effusion can readily increase intraarticular pressure to levels above capillary closure pressure, particularly during everyday activities such as standing, walking, and even modest flexion (33). Similarly, several studies have demonstrated a decreased oxygen concentration in the media of atherosclerotic arteries, ranging between 2 and 50 mm Hg (17, 34–36), leading to the hypothesis that hypoxia is a component of the pathology of atherosclerotic plaques (37–39). In addition, regions of reduced oxygen have been reported in cancers, including breast (7, 8), prostate (9), melanoma (10, 11), and cervical cancers (12, 13), although the oxygen levels are very heterogeneous within individual tumors. Thus examination of the effects of hypoxia on monocyte inflammatory mediator production has relevance to many pathological situations in which monocytes are present.

In this study, we incubated monocytes in 1% O₂ (v/v), which provided dissolved O₂ levels of 33 mm Hg, which is in the range observed in hypoxic joints (14–16), tumors (7–13), and arterial walls (17, 34–36, 40).

It has been demonstrated in human umbilical vein endothelial cells that hypoxia increased COX-2 expression and that an increase in transcription was involved (21). However, the effect on prostaglandin production of this COX-2 response to hypoxia was not measured (21). We observed that hypoxia caused a marked up-regulation of COX-2 mRNA and protein in fresh human monocytes and that this may be explained in part at

Fig. 16. Effect of neutralizing TNF-α on COX-2 induction and activity in normoxia. Monocytes (5 × 10⁶) were preincubated for 15 min at 37 °C with neutralizing TNF-α monoclonal antibody or isotype matched control (1B5) (8 μg/ml) prior to LPS stimulation for 18 h at 37 °C in normoxia or hypoxia. a, cell pellets were processed for Western blot analysis. The blot is representative of three separate experiments, the mean values of which are shown in the graph. Bars with different letters are significantly different from each other (p < 0.05). b, supernatants were collected, and PGE₂ and TXA₂ were measured by RIA. *p < 0.05 compared with LPS addition, no TNF-α.
least by an increase in COX-2 transcription and in COX-2 mRNA stability. The 3′-untranslated region of the COX-2 gene contains 22 copies of the AUUUA motif, which is related to mRNA stability (41–43). The response to hypoxia of COX-2 mRNA observed in this study may be a more general phenomenon because the gene for vascular endothelial growth factor also contains instability motifs in its 3′- untranslated region, and the mRNA is stabilized under hypoxic conditions (44, 45).

Although we observed an up-regulation of monocyte COX-2 in hypoxia, this was accompanied by a decrease in PGE<sub>2</sub> and TXA<sub>2</sub> production. This appears to be a paradoxical response. Rat lung exposed to hypoxia had increased COX-2 levels and increased prostaglandin production (46). Similarly, ischemia induced an increase in COX-2 mRNA and an increase in PGE<sub>2</sub> synthesis in mouse cerebral (47). Nevertheless, an increase in COX-2 expression and a decrease in PGE<sub>2</sub> synthesis were reported for the effects of hypoxia on a rabbit corneal epithelial cell line (25).

In the latter case, Bonazzi et al. (25) attribute this dissociation to increased activity of heme oxygenase in hypoxia with resultant decreased heme available for the activity of COX-2, a heme-containing protein. The inducible isoform of heme oxygenase, heme oxygenase-1, is increased in hypoxia and may be responsible for heme depletion in hypoxic cells (25, 26). However, when we repeated the procedures of Bonazzi et al. (25) of adding heme or an inhibitor of heme oxygenase, there was no restoration of prostaglandin synthesis like that reported in the rabbit corneal epithelial cell line. It is possible that this is an intrinsic difference between systems in monocytes and rabbit corneal epithelium, or it is possible that the rabbit corneal epithelial cell line is depleted in heme because of long term culture, whereas fresh human monocytes have adequate heme.

Another possible explanation for decreased synthesis in hypoxia at 1% O<sub>2</sub> is that heme can serve as a cosubstrate for COX activity and we observed that this was possible in experiments with exogenous AA, eicosanoid synthesis from reoxygenated monocytes.

We observed that AA metabolism in monocytes of hypoxia and that reoxygenation increased AA. These results correlated with decreased intracellular phosphorylation of cPLA<sub>2</sub> and of the latter enzyme in hypoxia. The latter enzyme is reportedly involved in regulating the phosphorylation of cPLA<sub>2</sub> in macrophages (33), neutrophils (29–31), and basophils (32). The involvement of the p44/42 MAPK in the reduced AA release that we observed in hypoxic monocytes is corroborated by the observation that inhibition of p44/42 MAPK activation by use of the MEK-1 inhibitor, PD98059, inhibited the restoration of AA release after reoxygenation.

Although there was reduced eicosanoid synthesis in hypoxic monocytes, there was simultaneously a marked increase in TNF-α production in hypoxia compared with normoxia. In normoxia, the increased TNF-α production in the presence of NS398 indicated that a COX-2 product is responsible for normal autocrine suppression of TNF-α synthesis. For two reasons, the responsible COX-2 product is likely to be PGE<sub>2</sub>. First, COX-2 induction in monocytes is associated with greatly increased PGE<sub>2</sub> synthesis relative to that of TXA<sub>2</sub> (48). Second, exogenous PGE<sub>2</sub> suppressed TNF-α synthesis in a dose-dependent manner. Therefore, the hypoxia-induced increase in TNF-α synthesis may be caused by the concomitant hypoxia-induced reduction in PGE<sub>2</sub> synthesis. This is not the only possible mechanism. It has been reported that hypoxia-induced increases in TNF-α synthesis in J774.1 murine macrophage cell line are attributable to hypoxia-induced mitochondrial production of reactive oxygen species (49).

Although the reduction in PGE<sub>2</sub> synthesis observed in hypoxia was caused by reduced p44/42 MAPK activation and consequent reduced AA release from membrane phospholipids, there was increased COX-2 expression. In normoxia, the suppression of LPS-stimulated COX-2 expression by the addition of neutralizing anti-TNF-α antibody indicated that endogenous TNF-α is normally involved as a mediator of COX-2 up-regulation. Therefore, the overexpression of COX-2 in hypoxia may result from increased eicosanoid production in hypoxia.

Overall, the observations in stimulated monocytes in normoxia suggested that a mechanism in which production of TNF-α up-regulates COX-2 levels of PGE<sub>2</sub>, which in turn suppresses COX-2 synthesis has been previously demonstrated previously. Although TXA<sub>2</sub> synthesis is an early COX-1-dependent response, synthesis of PGE<sub>2</sub> is delayed and is dependent on induction of COX-2 (2). Thus, it is possible that COX-2 up-regulation and consequent PGE<sub>2</sub> synthesis is a ‘self-limited’ monocyte response with respect to TNF-α synthesis (Fig. 17). This proposition is supported by the observation that TNF-α synthesis observed with COX-2 antisense oligonucleotides, the system is dysregulated with regard to this response possibly because PGE<sub>2</sub> synthesis is reduced as a result of reduced AA release, and a consequence of the unrestrained TNF-α synthesis is overexpression of COX-2 (Fig. 17).

Despite a decrease in monocyte PGE<sub>2</sub> synthesis by hypoxia, the consequences for PGE<sub>2</sub> concentrations in inflamed lesions such as a rheumatoid joint remain speculative. The rate of monocyte PGE<sub>2</sub> synthesis in a hypoxic rheumatoid joint may be reduced compared with a normoxic rheumatoid joint. However, the synovial concentration of PGE<sub>2</sub> derived from hypoxic monocytes would still be well above those in a healthy joint simply because of their presence in an inflamed synovium. In addition, there is likely to be a contribution to total joint PGE<sub>2</sub> levels from other cell types. Thus, even in a hypoxic joint, it is probable that the PGE<sub>2</sub> concentration is sufficient to contribute to the signs and symptoms of swelling and pain which are alleviated by nonsteroidal anti-inflammatory agents, including COX-2 inhibitors. Furthermore, it is possible that any increase in monocyte TNF-α synthesis which may result from reduced monocyte PGE<sub>2</sub> synthesis could be important for other joint pathology.

Whatever may be the pathological consequences, it is clear that hypoxia is an important but often neglected determinant of inflammatory mediator production and one that potentially may influence a broad range of events that occur in monocyte-containing lesions. Therefore, the effect of hypoxia on the activities of other cells at sites of inflammation or ischemia also warrants investigation.

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