

Hypoxia induces myocyte-dependent COX-2 regulation in endothelial cells: role of VEGF

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Wu, Guifu, Arjuna P. Mannam, Jiaping Wu, Simona Kirbis, Jue-Lon Shie, Christopher Chen, Roger J. Laham, Frank W. Sellke, and Jian Li. Hypoxia induces myocyte-dependent COX-2 regulation in endothelial cells: role of VEGF. *Am J Physiol Heart Circ Physiol* 285: H2420–H2429, 2003. First published July 24, 2003; 10.1152/ajpheart.00187.2003.—There is increasing evidence that cyclooxygenase (COX)-2 possess both angiogenic and cardioprotective properties. We examined the effects of hypoxic cardiac myocytes (H9c2 cells) on COX-2 expression in human umbilical vein endothelial cells (HUVECs) to determine the pathway involved in COX-2 regulation. The medium from hypoxic (<1% O₂) cardiac myocytes (HMCM) or normoxic cardiac myocytes (21% O₂) was added to HUVEC cultures. HMCM induced a transient increase of COX-2 mRNA expression at 1 and 3 h without affecting the COX-1 mRNA level. A similar effect also observed in HMCM from cultured primary cardiac myocytes (rat neonatal cardiac myocytes). The increased COX-2 mRNA was associated with a time-dependent increase in COX-2 protein expression. COX-2 was significantly induced by VEGF (4.86 ± 1.03-fold) and IL-1β (3.93 ± 0.89-fold) and slightly increased by TNF-α but not by FGF2, IGF-1, or PDGFs. Analysis of proteins secreted in HMCM showed increased levels of VEGF but not IL-1β or TNF-α. The HMCM-induced COX-2 expression was inhibited by the addition of an anti-VEGF neutralizing antibody. VEGF induced endothelial cell COX-2 expression by both increasing COX-2 transcription and prolonging the COX-2 mRNA half-life. Furthermore, staurosporine, a nonselective PKC inhibitor, prevented the induction of VEGF by hypoxia. Both a selective PKC-α and -β inhibitor and an inducible nitric oxide synthase (NOS) inhibitor decreased the induction of COX-2 by HMCM and VEGF. Finally, HMCM-induced upregulation of COX-2 was accompanied by upregulation of PGI₂ and PGE₂. These results suggest that VEGF is one of the principal factors produced by hypoxic myocytes that is responsible for the induction of endothelial cell COX-2 expression. This process likely involves both PKC and NOS pathways. Our findings have important implications regarding the cardiac protection of COX-2 in ischemic heart disease.

cyclooxygenase-2; vascular endothelial growth factor; cardiac myocytes

CYCLOOXYGENASE (COX) is a key regulatory enzyme in eicosanoid metabolism, converting free arachidonic

acid to PGH₂ (18). Prostaglandins mediate inflammation locally and modulate physiological responses systemically. Two isoforms of COX have been identified, COX-1 and COX-2, which have both common and specific roles (44). COX-1 is constitutively expressed, serving a so-called housekeeping role, in many tissues under basal conditions. For instance, COX-1 helps to maintain normal physiological functions such as mucus production in the gastric mucosa. In contrast, COX-2 is induced upon cell activation and is generally not present (or present minimally) in most tissues. Rather, its expression is more often associated with inflammation and other pathophysiological states.

Although COX-2 is widely accepted as a proinflammatory agonist and is therefore a suitable target for the treatment of chronic inflammatory disease, there is increasing evidence to suggest that COX-2 has other roles, including anti-inflammatory, antifibrotic, and antithrombotic properties (16, 17, 25). These alternative roles challenge the dogma that COX-2 is ubiquitously a foe, and, indeed, there is evidence indicating that a defect in COX-2 expression can result in pathology, as in idiopathic pulmonary fibrosis (50). It also appears that COX-2 has a dual role in inflammation: initially inducing the inflammatory process and later aiding in its resolution (16). The complexity of these mechanisms is not fully understood, and dysregulation of COX-2 expression may play a key role in COX-2-mediated pathology.

There is mounting evidence suggesting a cardioprotective effect of COX-2 and potential detrimental effects of COX-2 inhibitors on the heart (4, 6, 15, 19, 38). These studies are particularly important with the increasing use of selective COX-2 inhibitors as analgesics. COX-2 appears to mediate the cardioprotective effects in the late phase of ischemic preconditioning (42). Inhibition of COX-2 expression aggravates doxorubicin-mediated cardiac injury in vivo (11). In addition, the use of COX-2 inhibitors may result in an increased incidence of cardiovascular events and worsening heart failure (6, 15, 19, 38, 40). Thus it is imperative to understand the molecular mechanisms regulating COX-2 expression in cardiovascular disorders.

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Recent evidence suggests that COX-2 metabolic products contribute to neovascularization and may support vasculature-dependent solid tumor growth and metastasis. Selective COX-2 inhibitors are antiangiogenic (33), and COX-2-null mice are substantially protected in a genetic model of human familial adenomatous polyposis (22). COX-2 overexpression enhances the metastatic potential of CaCo-2 colon carcinoma cells through processes that are sensitive to COX-2 inhibitors (48). Coculture of endothelial cells with tumor cells promotes COX-2-dependent endothelial motility and assembly into capillary-like structures (47), an effect that is attributed to tumor cell release of angiogenic peptides and nitric oxide (NO). Alternatively, eicosanoids synthesized by endothelial COX-2 may contribute to this effect.

COX-2 is an immediate early response gene that can be induced by direct hypoxia and a variety of cytokines and growth factors (41, 51). Significant cross-regulation exists among COX-2, IL-1 β (1, 46), and VEGF (2) in endothelial cells, underscoring the probable contribution of COX-2 to angiogenesis, a process partially regulated by myocyte-endothelial interactions as a response to ischemia (10, 24, 28, 49). However, the regulation of COX-2 in hypoxia-related angiogenesis has not been fully investigated.

Therefore, we conducted the present study to elucidate the molecular mechanisms controlling the expression of COX-2 in the ischemic myocardium. We studied the ability of cardiac myocytes or cardiac myocyte-conditioned media to induce COX-2 expression in human umbilical endothelial cells (HUVECs) under normoxic and hypoxic conditions while attempting to identify the cytokines and pathways involved.

METHODS

Cell culture. Rat cardiac myocytes (H9c2 cell line, American Type Culture Collection; Manassas, VA) were cultured in DMEM (Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. HUVECs (Clonetics; San Diego, CA) were cultured in endothelial cell basal medium-2 (EBM-2) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and an EGM-2 bullet kit (Clonetics). HUVECs were grown up to 90–95% confluence and then transferred to serum and bullet kit-free medium overnight before being used for experiments.

Rat neonatal cardiac myocytes (RNCM) were prepared as described previously (37). Briefly, myocytes were isolated from heart ventricles of 1- to 2-day-old rats by mechanical and enzymatic dissociation. Cells were washed and preplated in the presence of 5% calf serum to reduce the number of contaminating nonmyocardial mesenchymal cells (NMCs). After 30 min, the still-suspended myocytes were removed from attached NMCs and diluted to 200,000 viable cells/ml in culture medium (5% serum minimum essential medium supplemented with 1.5 μ M vitamin B₁₂ and 50 U/ml penicillin). All culture dishes were kept at 37°C in humidified air with 5% CO₂. This study was approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center/Harvard Medical School (Boston, MA).

Hypoxia model. Hypoxia was induced using a Modular Incubator Chamber (Billups-Rothenberg; Del Mar, CA). The hypoxia chamber was filled with an artificial atmo-

sphere, and the concentration of oxygen (0–1%) was determined before and after incubation using an Oxygen Analyzer (Vascular Technology; Bradford, MA). The hypoxia chamber containing cell culture dishes was transferred to a culture incubator according to the time schedule of studies.

Preparation of hypoxia-conditioned medium. Hypoxic myocyte-conditioned medium (HMCM) was collected as previously described (52). Briefly, H9c2 cells were cultured to 80–90% confluence, serum-free medium was added, and cells were then incubated in the hypoxia chamber (O₂ <1%) for 24 h. HMCM was collected and transferred to the HUVEC culture. Normoxic myocyte-conditioned medium (NMCM) was prepared as control. In addition, HMCM and NMCM were also prepared from neonatal rat cardiac myocytes.

RNA isolation and Northern blotting. The cDNA probes of COX-1 and COX-2 were made using their RT-PCR products. The primers for RT-PCR were designed according to the published human cDNA sequences. The COX-1 sequences of forward primer 5'-TCATCGAGGAGTACGTGCAG-3', corresponding to bases 1080–1099, and reverse primer 5'-AGG-GACAGGTCTTGGTGTG-3', corresponding to bases 1759–1778, were used to amplify a 661-bp fragment. The COX-2 sequences of forward primer 5'-TAAACTGCGCCTTT-TCAAGG-3', corresponding to bases 781–800, and reverse primer 5'-GTGATACTTTCTGTACTGCG-3', corresponding to bases 1381–1400, were used to amplify a 620-bp fragment of COX-2. A VEGF cDNA probe was used as described previously (28).

Total RNA was obtained from cultured cells by the Tri-Reagent protocol (Sigma; St. Louis, MO). The RNA was fractionated on a 1.3% formaldehyde-agarose gel and transferred to GeneScreen Plus membranes (New England Nuclear; Boston, MA). The [α -³²P]dCTP-labeled COX-1, COX-2, and VEGF probes were hybridized in QuikHyb solution (Stratagene; La Jolla, CA). Autoradiographical signals were quantified by densitometry using ImageQuant software and adjusted by the density of 28S rRNA.

Protein extraction and Western blotting. HUVECs were lysed by RIPA solution (Boston Bioproducts; Ashland, MA) and fractionated by 10% SDS-polyacrylamide gels. Protein extracts were transferred to polyvinylidene difluoride membranes (Millipore; Bedford, MA). COX-1 and COX-2 signals were detected with anti-COX-1 (Calbiochem; La Jolla, CA) antibody and anti-COX-2 (Bio-Rad; Hercules, CA) antibody. Immunoblots were visualized by enhanced chemiluminescence Western blotting detection reagents (Amersham Life Science; Arlington Heights, IL).

Determination of proteins secreted from hypoxic cardiac myocytes. Both NMCM and HMCM were concentrated by a Centrplus Centrifugal Filter (Millipore). The final condensed medium contained molecules at a range of 10–100 kDa. Afterward, 50 μ l of condensed HMCM and NMCM were fractionated by 10% SDS-polyacrylamide gels and transferred to Immobilon-P membranes. The antibodies against VEGF (Oncogene; Boston, MA), IL-1 β (Cell Signaling Technology; Beverly, MA), and TNF- α (Biosource; Camarillo, CA) were used to detect the protein expression in conditioned medium. In addition, the levels of VEGF, IL-1 β , and TNF- α in condensed HMCM or NMCM from both H9c2 and RNCM were also determined by an ELISA kit (Chemincon; Temecula, CA) following the manufacturer's instructions. The final protein levels were normalized to cell numbers and are expressed as picograms per 10⁴ cells.

Prostanoid measurement. PGE₂ and PGI₂ levels in HUVECs treated with HMCM or NMCM were determined using an EIA kit following the manufacturer's instructions (Assay Design; Ann Arbor, MI). The selective COX-2 inhibi-

tor NS-398 (Sigma), dissolved in DMSO, was preincubated with either H9c2 cells or HMCM for 30 min before the addition to HUVECs to achieve a final concentration of 30 μ M. Data are expressed as nanograms per milligram of protein.

Growth factor stimulation studies. Selective growth factors and cytokines, including FGF-2 (25 ng/ml, Chiron; Emeryville, CA), VEGF (20 ng/ml, Genetech; Sage Brush Trail Plano, TX), TNF- α (20 ng/ml), IL-1 β (5 ng/ml), IGF-1 (50 ng/ml), PDGF-AA (20 ng/ml), PDGF-BB (20 ng/ml), and PDGF-AB (20 ng/ml, Sigma), were added. The concentration of the growth factors was in accordance with doses used in a previous study (52). Total RNA was extracted after 3 h of incubation and then subjected to Northern blotting, probed by COX-2 cDNA. To specifically block VEGF secreted in HMCM, either the monoclonal anti-human VEGF-neutralizing antibody or irrelevant murine IgG (R&D Systems; Minneapolis, MN) that served as a control, was preincubated with HMCM at a concentration of 0.2 μ g/ml at 37°C for 1 h before being added to HUVECs as described (20).

Signal pathway studies. The PKC inhibitor staurosporine (100 nM, Sigma) (8), the selective PKC- α and - β_1 inhibitor Gö6976 (500 nM, Calbiochem) (9), the NO synthase (NOS) inhibitor *N*^ω-nitro-L-arginine methyl ester (L-NAME; 500 μ M, Sigma) (9), and the selective inducible NOS (iNOS) inhibitor L-N⁶-(1-iminoethyl)lysine hydrochloride (L-NIL; 500 μ M, Sigma) (21) were used in these studies. They were added to the culture medium in the following three groups of studies: first, the inhibitors were incubated with H9c2 cells in culture during hypoxia; second, the inhibitors were added to HMCM in HUVEC culture; and third, the inhibitors and VEGF (20 ng/ml) were added to HUVEC culture. Cells were cultured for 3 h; total RNA was then extracted and used for Northern blot analysis with the COX-2 cDNA probe.

COX-2 mRNA stability assay. Actinomycin D (5 μ g/ml, Sigma) was added to HUVEC culture with or without VEGF (20 ng/ml) after overnight culture in serum and bullet kit-free EBM-2. The cells were then harvested at the indicated time points, and total RNA was extracted and subjected to Northern blot analysis using the COX-2 cDNA probe as described as in *RNA isolation and Northern blotting*. The corrected density (COX-2-to-28S rRNA ratio) was then plotted as a percentage of the control (0 h) value against time in log scale.

COX-2 transcription studies. A 933-nucleotide fragment (-830 to +103 nucleotides of the human COX-2 sequence) encompassing the basal elements of the human COX-2 promoter (a kind gift of Dr. Peter Oettgen, Beth Israel Deaconess Medical Center/Harvard Medical School) was constructed with the PXP-2 vector containing the luciferase reporter gene. The construct of the COX-2 promoter/PXP-2 fragment was transfected into HUVECs using Targefect F-2 reagent (Targeting Systems; Santee, CA). HUVECs carrying the COX-2 promoter fragment were cultured in 12-well plates while VEGF (20 ng/ml) or concentrated HMCM or NMCM were added into the culture. After 6 h of exposure, cells were lysed, and luciferase activity was determined using the Luciferase Assay System (Promega; Madison, WI).

Statistical analysis. Results are expressed as means \pm SD based on three individual experiments. All values of image densitometry studies were quantitated by ImageQuant software and adjusted by the ratio of sample loading. Data are presented as a percentage of the control value (“%control”). Statistical significance was assessed by Student's *t*-test, and *P* \leq 0.05 was considered statistical significance.

RESULTS

Expression of COX-2 in HUVECs induced by HMCM. To investigate the role of hypoxic cardiac myocytes in regulating COX-2 expression in vascular endothelial cells, we studied the ability of cardiac myocyte (H9c2)-conditioned media to induce the expression of COX-2 in HUVECs. Exposure of HUVECs to the medium conditioned by H9c2 cells cultured under normal conditions did not affect COX-1 and COX-2 expression. However, COX-2 expression in HUVECs was significantly increased by the medium conditioned by H9c2 cells cultured under hypoxic conditions for 1 h (4.86 ± 1.05 -fold) and 3 h (3.42 ± 0.7 -fold) and returned to baseline after 12 h. COX-1 mRNA did not show a significant time-dependent response to HMCM (Fig. 1A). In addition to the increase of COX-2 mRNA, expression of COX-2 protein was also increased in HUVEC incubated with HMCM from 3 to 12 h (Fig. 1B).

To avoid limitation of H9c2 cells, we duplicated the experiment in primary cardiac myocytes from neonatal rats (RNCM) under hypoxia condition. The expression of COX-2 but not COX-1 was also enhanced in HUVECs after treatment with HMCM from neonatal cardiac myocytes. The time-dependent pattern of COX-2 expression was similar with HUVECs treated with HMCM from H9c2 cells (Fig. 1C). Thus hypoxic cardiac myocytes secrete a factor or factors responsible for stimulating expression of COX-2, but not COX-1, in vascular endothelial cells.

Role of VEGF in COX-2 response to HMCM. To determine whether any known secreted factors were responsible for inducing COX-2 in response to HMCM, HUVECs were incubated with a panel of growth factors and cytokines including TNF- α , FGF2, IL-1 β , IGF-1, VEGF, PDGF-AA, PDGF-BB, and PDGF-AB for 3 h. As Fig. 2A shows, VEGF (4.86 ± 1.03 -fold) and IL-1 β (3.93 ± 0.9 -fold) significantly increased COX-2 mRNA expression (expression was slightly increased with TNF- α). Therefore, VEGF, IL-1 β , and TNF- α in HMCM were examined by Western blotting and ELISA. Compared with NMCM, the VEGF protein level was significantly increased in HMCM, whereas IL-1 β did not show any change between HMCM and NMCM (Fig. 2B). TNF- α was undetectable in either HMCM or NMCM by Western blot analysis (data not shown). Having identified the hypoxia-induced protein secretion, we were also interested in determining the concentration of the secreted factors in HMCM. As the data in Table 1 show, the level of VEGF in condensed HMCM was 68.59 ± 2.37 pg/ml from H9c2 and 75.16 ± 7.23 pg/ml from RNCM, accounting for an 8.8- and a 7.4-fold increase, respectively, compared with those in condensed NMCM (7.797 ± 3.036 pg/ml from H9c2 and 10.1 ± 2.58 pg/ml from RNCM). However, no significant differences were observed regarding IL-1 β and TNF- α in hypoxia-conditioned medium derived from either H9c2 cells or RNCM (Table 1). These results suggest that VEGF is one of the primary factors responsible for the induction of COX-2 by hypoxic cardiac

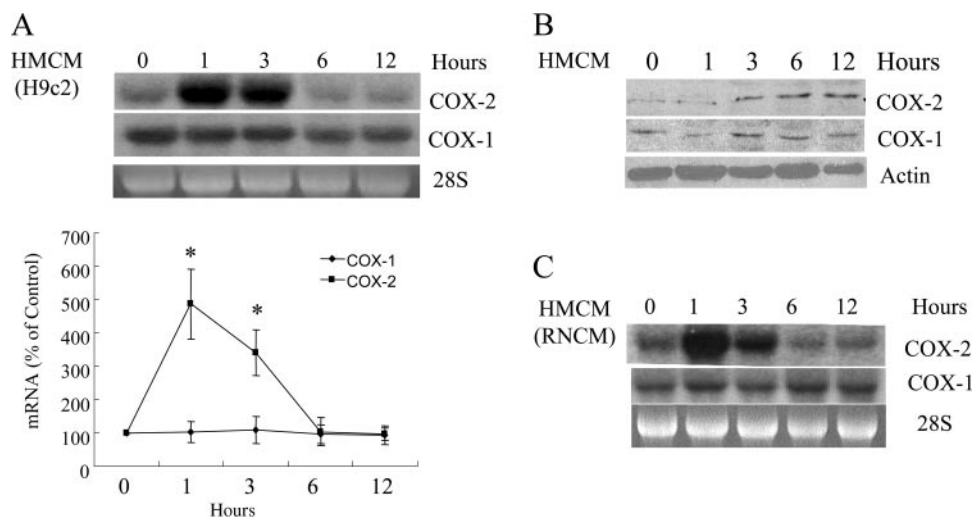


Fig. 1. Effect of myocyte-conditioned medium on cyclooxygenase (COX) expression in human umbilical vein endothelial cells (HUVECs). COX-1 and COX-2 expression in HUVECs was assessed after various durations of exposure to the medium conditioned by hypoxic cardiac myocytes (H9c2 cells) for 24 h using Northern (A, top) and Western blotting (B). As shown in the quantitative analysis in A, bottom, a significant increase in COX-2 gene expression was found at 1 h (4.86 ± 1.05 -fold) and 3 h (3.42 ± 0.7 -fold), which returned to baseline after 12 h. COX-1 mRNA expression in response to hypoxic myocyte-conditioned medium (HMCM) did not show a significant difference. Immunoblot analysis demonstrated that the delayed COX-2 response to HMCM was initiated at 3 h and kept at a high level until 12 h. COX-1 did not change at the protein level. Increased COX-2 but not COX-1 expression was also found in HUVECs treated with the medium conditioned from primary rat neonatal cardiac myocytes (RNCM) under hypoxic conditions (C). The results were quantified based on 3 experiments by Image-Quant and are presented as means \pm SD. * $P < 0.05$.

myocytes of either the H9c2 cell line or primary cardiac myocytes (RNCM).

The effect of VEGF on COX-2 expression in HUVECs was also examined. VEGF induced COX-2 mRNA expression significantly at 1 h (5.07 ± 0.8 -fold) and 3 h (3.1 ± 0.6 -fold) (Fig. 3A) and was highly consistent with HMCM-induced COX-2 expression, as shown in Fig. 1A. The induction of COX-2 expression by VEGF was dose dependent (Fig. 3B). As little as 0.1 ng/ml VEGF was able to increase COX-2 mRNA level in HUVECs. VEGF at 20 ng/ml resulted in maximal stimulation. In addition, hypoxia-induced VEGF expression was seen in both types of cardiac myocytes, H9c2 cells and RNCM, as shown on Fig. 3C.

Further evidence to support the notion that VEGF plays a key role in the hypoxic myocyte-dependent induction of COX-2 is shown in Fig. 3D. The addition of anti-VEGF-neutralizing antibody to HMCM before the media was applied to HUVEC culture decreased the level of COX-2 mRNA by $>50\%$ compared with HMCM treated with general murine IgG. Thus VEGF seems to be the predominant factor in HMCM that induces COX-2 gene expression in vascular endothelial cells.

Signaling pathways of HMCM-induced COX-2 expression in endothelial cells. After the demonstration of VEGF's key role in hypoxia-induced COX-2 expression, the signaling pathways involved were investigated. PKC and NOS are the major signaling pathways that mediate the activation of COX-2 by growth factors and hypoxia (5, 12, 23, 32, 43). Therefore, these two signaling pathways were examined by inhibitor studies as

follows: 1) adding the inhibitors in cardiac myocytes culture undergoing hypoxia and then taking medium for HUVEC culture; 2) adding the inhibitors in HUVEC culture with HMCM taken from cardiac myocytes; and 3) adding the inhibitors with exogenous VEGF together in HUVEC culture. The effect of the inhibitors in HMCM-induced COX-2 expression is shown on Fig. 4A. The addition of staurosporine (a PKC nonselective inhibitor) or Gö6976 (a selective PKC- α and - β isoform inhibitor) to H9c2 cells during hypoxia blocked COX-2 expression by 20–30%, whereas the addition of L-NAME (a NOS inhibitor) and L-NIL (an iNOS inhibitor) resulted in no significant changes (Fig. 4A, top). When the same group of inhibitors was added to HMCM incubated with HUVECs, HMCM-induced COX-2 expression was not inhibited by staurosporine or L-NAME but was inhibited by Gö6976 (60%) or L-NIL (40%) (Fig. 4A, middle). Furthermore, VEGF-induced expression of COX-2 was significantly inhibited by Gö6976 or L-NIL (Fig. 4A, bottom). These findings suggest that PKC is involved in the induction and secretion of VEGF, and PKC- α , PKC- β , and iNOS pathways seem essential for the induction of COX-2 by causing the secretion of VEGF from cardiac myocytes under hypoxic conditions. In addition, the significant reduction of hypoxia-induced VEGF expression by staurosporine in cardiac myocytes (H9c2 cells) was observed in RNA and protein levels, further supporting that PKC but not the NOS pathway is involved in the response to VEGF expression induced by hypoxia (Fig. 4, B and C).

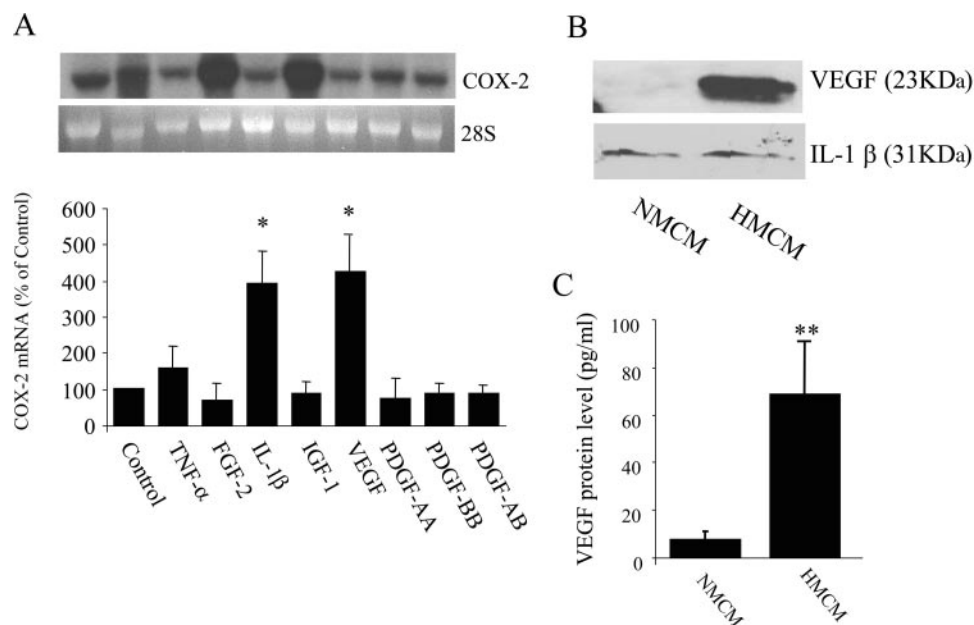


Fig. 2. Role of VEGF in the COX-2 response to HMCM. *A*: cytokine and growth factor stimulation of COX-2 expression in HUVECs. *Top*, Northern blot. Subconfluent serum-starved HUVECs were treated with the following factors for 3 h: TNF- α (20 ng/ml), FGF2 (25 ng/ml), IL-1 β (5 ng/ml), IGF-1 (50 ng/ml), VEGF (20 ng/ml), PDGF-AA (20 ng/ml), PDGF-BB (20 ng/ml), and PDGF-AB (20 ng/ml). Compared with the vehicle (control), IL-1 β and VEGF strongly promoted the expression of the COX-2 gene in HUVECs, whereas TNF- α only slightly changed the COX-2 expression. *Bottom*, bar graph of quantitative analysis. Data were collected by 3 independent investigators. *B*: Western blot analysis of the secreted VEGF and IL-1 β proteins in the medium conditioned by H9c2 cell culture under normal and hypoxia conditions. The increased presence of VEGF in the medium conditioned by hypoxia (HMCM) was compared with the medium under normal condition (NMCM). There was no difference in the IL-1 β protein level in either HMCM or NMCM; TNF- α was not detectable by Western blot in either HMCM or NMCM (data not shown). Note both HMCM and NMCM are concentrated from the myocyte-conditioned medium, and the same amount of condensed HMCM and NMCM was fractionated by 10% SDS-polyacrylamide gels. The blot was stripped and reblotted for VEGF, IL-1 β , and TNF- α , respectively. *C*: VEGF protein level in HMCM determined by ELISA. There is an 8.8-fold increase of VEGF in HMCM compared with NMCM. ** $P < 0.01$.

Mechanism of the VEGF-mediated increase of COX-2 expression. To determine whether VEGF regulates COX-2 gene expression by transcriptional or posttranscriptional mechanisms, the steady-state level of COX-2 mRNA was measured via a COX-2 mRNA half-life assay in the presence of actinomycin D. In addition, COX-2 promoter activity stimulated by VEGF or HMCM was also studied. The COX-2 mRNA half-life was 2.1 h in the absence of VEGF and 3.9 h in the

presence of VEGF (20 ng/ml) (Fig. 5A). Thus the increased level of COX-2 mRNA in HUVECs appeared to be due in part to an increase in the stability of mRNA. To assess the effect of VEGF on COX-2 gene transcription, the activities of a luciferase construct under the control of a human COX-2 promoter were measured. Exposure of HUVECs transfected with this construct to VEGF at 20 ng/ml led to a threefold increase in luciferase activity. In addition, exposure of HUVECs transfected with the COX-2 promoter construct to concentrated HMCM revealed a 1.6-fold increase (Fig. 5B). These results imply that VEGF regulates COX-2 expression at both transcriptional and posttranscriptional levels.

Regulation of prostanoid synthesis by HMCM-induced COX-2. To determine whether HMCM-induced COX-2 expression in endothelial cells was associated with increased COX-2 enzymatic activity, the endothelial contents of major arachidonic acid metabolites, PGE₂ and 6-keto-PGF_{1 α} (a stable metabolite of PGI₂), were measured using EIA in HUVECs cultured with myocyte-conditioned medium. The effect of COX-2 on endogenously derived prostanoid synthesis in HUVECs treated with HMCM is shown in Table 2. Compared with the NMCM group, the level of PGE₂ in HUVEC treated with HMCM increased by 3.5-fold

Table 1. Comparison of proteins secreted by hypoxic myocytes

	H9c2		RNCM	
	NMCM	HMCM	NMCM	HMCM
VEGF	7.99 \pm 3.04	68.59 \pm 22.37*	10.1 \pm 2.58	75.16 \pm 7.23*
IL-1 β	28.52 \pm 6.34	26.93 \pm 8.41	29.44 \pm 8.65	31.87 \pm 7.23
TNF- α	5.06 \pm 0.89	6.397 \pm 1.023	6.43 \pm 0.83	7.26 \pm 0.923

Values (in pg/10⁴ cells) are means \pm SD of triplet samples. Both H9c2 and rat neonatal cardiac myocytes (RNCM) were incubated in either normoxia or hypoxia conditions for 24 h. Medium was collected and concentrated for protein measurement by ELISA. Robust secretion of VEGF was observed in hypoxia-conditioned medium (HMCM) from both H9c2 and RNCM. There were no significant differences in secreted IL-1 β and TNF- α in conditional medium between hypoxia and normoxia conditions in both cell types. NMCM, medium under normoxic conditions. * $P < 0.05$.

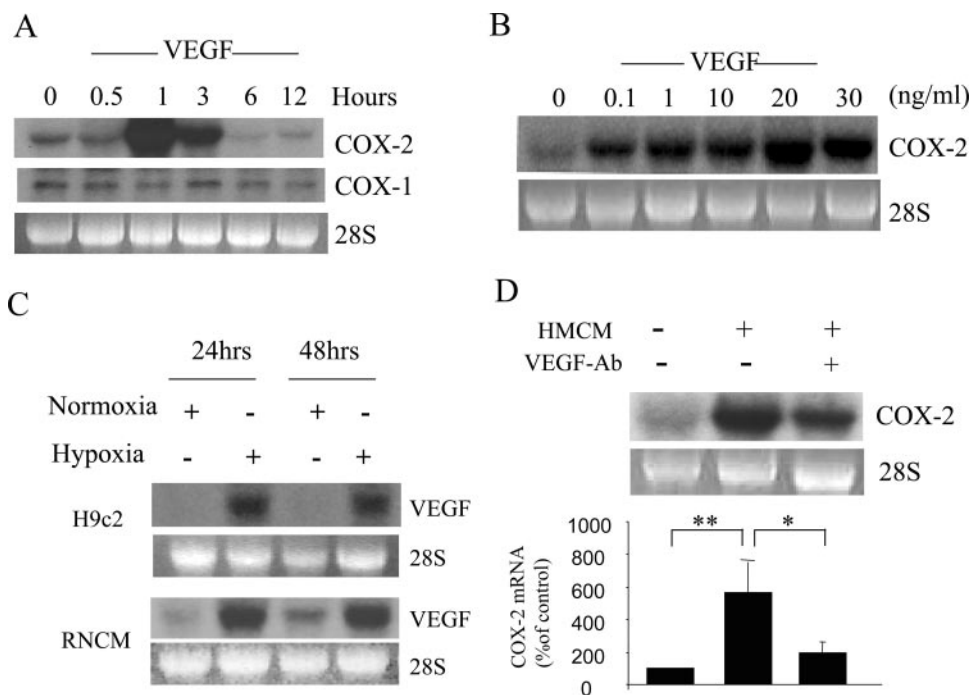


Fig. 3. VEGF produced by hypoxic myocytes is the dominant factor responsible for induction of COX-2 expression. **A:** time-dependent response of COX-1 and COX-2 in HUVECs in response to VEGF. HUVECs were treated with VEGF (20 ng/ml) for various time periods (0, 0.5, 1, 3, 6, and 12 h). Northern blot analysis indicated that COX-2 expression was induced by VEGF at 1 h (5.07 ± 0.8 -fold) and 3 h (3.1 ± 0.6 -fold). The increased COX-2 expression by VEGF was identical to the HMC-induced COX-2 expression (Fig. 1A). **B:** dose response of VEGF in COX-2 expression. HUVECs were incubated for 1 h with the indicated concentrations of VEGF. As little as 0.1 ng/ml of VEGF was able to increase the COX-2 mRNA level in HUVECs. VEGF at 20 ng/ml reached maximal stimulation. **C:** Northern blot analysis of VEGF expression in cardiac myocytes (H9c2 cells and RNCM) before and after exposure to 24 and 48 h of hypoxia. The results showed that VEGF was induced by hypoxia in the myocytes after 24 and 48 h. **D:** induction of COX-2 expression by HMC was inhibited by anti-VEGF antibody (Ab). *Top*, HMC was collected from H9c2 cells incubated in hypoxia for 24 h and preincubated with anti-VEGF-neutralizing antibody (0.2 μ g/ml) or irrelevant murine IgG as a control for 30 min. The treated HMC was applied to the HUVECs and incubated for 3 h. HMC-induced COX-2 expression was blocked by an anti-VEGF antibody (5.68 ± 2.5 - vs. 2.31 ± 1.2 -fold). *Bottom*, quantitative analysis based on 3 repeat experiments by ImageQuant and presented as means \pm SD. ****** $P < 0.01$; ***** $P < 0.05$.

(1.89 ± 0.74 vs. 6.68 ± 1.46) and the level of 6-keto-PGF_{1 α} in HUVECs increased by 2.1-fold (6.83 ± 3.14 vs. 14.52 ± 2.26) (Table 2). Moreover, the increase in PGE₂ and 6-keto-PGF_{1 α} was completely inhibited when the HMC or H9c2 cells were preincubated with the selective COX-2 inhibitor NS-398 (Table 2). The dosage of NS-398 (30 μ M) used in the present study was determined by a previous report (27) demonstrating that the range of 1–50 μ M of NS-398 caused the inhibition of synthesis of arachidonic acid metabolites through COX-2. Therefore, it appears that NS-398 was effective in blocking the increase in COX-2 activity associated with myocyte-dependent, hypoxia-induced COX-2 expression in endothelial cells.

DISCUSSION

Gene regulation and cross talk between myocytes and endothelial cells are not completely understood. However, it is clear from the proximity of these cells and the interactions described to date that this cross-talk provides key regulation of inflammation and angiogenesis. In the experiments presented here, we demonstrated the effect of HMC on COX-2 regulation

in HUVECs. We identified a specific growth factor, VEGF, induced by hypoxia from cardiac myocytes and secreted into culture media that affects COX-2 regulation in endothelial cells. In fact, among the selective cytokines/growth factors, VEGF and IL-1 β directly up-regulate COX-2 but not COX-1 mRNA by more than threefold. However, an elevated level of VEGF was detected in HMC, whereas the level of IL-1 β was not different between HMC and NMC. A number of findings in our study point to VEGF as the key molecule responsible for the ability of HMC to induce COX-2 expression. First, VEGF is capable of directly stimulating COX-2 mRNA expression in the same pattern as HMC. Second, VEGF is in high concentration in the HMC. Finally, HMC pretreated with an antibody against VEGF is unable to induce COX-2 expression.

Several studies (28, 29), including the present study, have described as playing a VEGF a critical role in myocardial inflammation and angiogenesis. The regulation of VEGF and COX-2 are closely related. VEGF is upregulated by the PGE series through COX-2 in several cell types (14). On the other hand, in response to

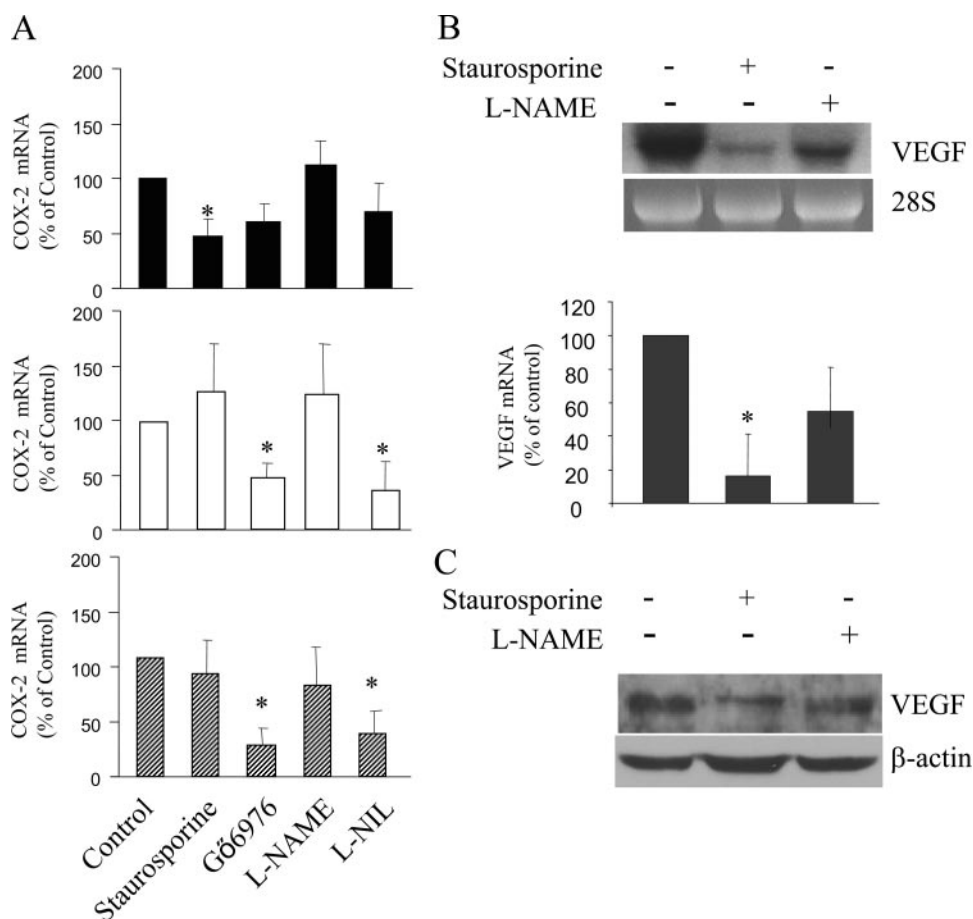


Fig. 4. Potential signaling pathway of the COX-2 response to HCMC in HUVECs. **A:** COX-2 expression was examined in HUVECs by Northern blot analysis in 3 groups of experiments with the addition of inhibitors including staurosporine (100 nM), Gö6976 (500 nM), *N*^ω-nitro-L-arginine methyl ester (L-NAME; 500 μM), and L-*N*⁶-(1-iminoethyl)lysine hydrochloride (L-NIL; 500 μM) to the culture medium. The signaling pathways were examined by inhibitor studies as follows: 1) adding the inhibitors to the medium of H9c2 cell cultures during 24 h of hypoxia and then transferring to HUVECs (*top*); 2) adding the inhibitors to the HCMC after hypoxia and then culturing HUVECs (*middle*); and 3) adding the inhibitors into HUVEC culture in which VEGF (20 ng/ml) was administered simultaneously (*bottom*). HUVECs were harvested after 3 h of incubation. HCMC- or VEGF-induced COX-2 expression was used as a control. In addition, staurosporine and Gö6976 blocked COX-2 expression in HUVECs when they were added to H9c2 cells during exposure to hypoxia. No significant changes were observed when L-NAME and L-NIL were added (*top*). HCMC-induced COX-2 expression was not inhibited by staurosporine and L-NAME but by Gö6976 (60%) and L-NIL (40%) (*middle*). VEGF-induced expression of COX-2 was inhibited by Gö6976 and L-NIL (*bottom*). **B:** VEGF mRNA expression was measured by Northern blot analysis in hypoxic H9c2 cells incubated with staurosporine (100 nM) and L-NAME (500 μM). The increased VEGF was inhibited completely by staurosporine but not by L-NAME. **C:** Western blot analysis confirmed the protein level of VEGF that was significantly blocked by the PKC inhibitor staurosporine. The experiments were repeated 3 times and quantified by ImageQuant and are presented as means ± SD. **P* < 0.05.

VEGF, both COX-2 protein and its activity increase in a dose-dependent manner in vascular endothelial cells (2). Tamura et al. (45) have shown that the promoter region of the COX-2 gene contains a GATA *cis*-acting element that is essential for VEGF-induced COX-2 promoter activity in human microvascular endothelial cells. In this study, the stability of COX-2 mRNA under VEGF stimulation was not tested. We found that VEGF-dependent activation of COX-2 expression involves not only transcriptional but posttranscriptional events by prolonging the COX-2 mRNA half-life. Although several lines of evidence suggest that COX-2 is a factor responsible for induction of VEGF in tumor angiogenesis, our report is one of few studies (2, 45)

that raises the possibility that COX-2 is a downstream target for VEGF-induced angiogenesis, suggesting that these two genes could be mutually regulated.

The pathway of VEGF induced by hypoxia is not fully elucidated. Hypoxia-inducible factor (HIF)-1 α is a major factor involved in hypoxia-induced VEGF expression (34, 36). The PKC- δ isozyme acts as a shared component in transmitting hypoxia-induced signals to HIF-1 (3), whereas hyperglycemia-induced VEGF expression is HIF-1 α dependent and requires PKC (39). To elucidate the factors that may inhibit hypoxic myocyte-dependent COX-2 expression induced by VEGF, we determined that activation of VEGF expression from hypoxic cardiac myocytes appears to depend on

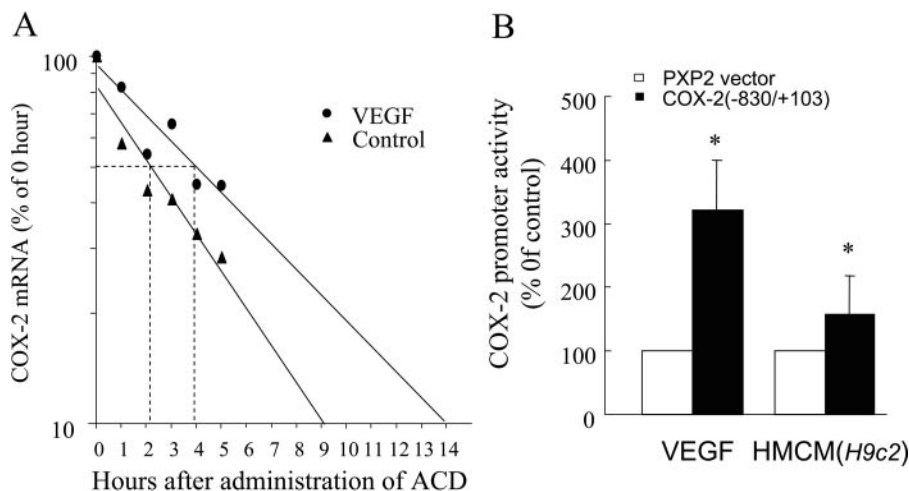


Fig. 5. Mechanism of VEGF-mediated increased COX-2 expression in HUVECs. *A*: to assess the effect of VEGF administration on the COX-2 mRNA half-life, HUVECs were exposed to either vehicle (control) or VEGF for 1 h before the administration of actinomycin D (ACD; 5 μ g/ml). After total RNA extraction at the indicated times after ACD administration, COX-2 expression was detected by Northern blot analysis. The signal was quantified using ImageQuant software with RNA loading adjusted by the values for 28S rRNA and then plotted as a percentage of the 0-h value against time. VEGF extended the half-life of COX-2 mRNA from 2.1 to 3.9 h. *B*: to clarify the effect of VEGF administration on COX-2 promoter activity in HUVECs, we conducted an analysis of luciferase activity in HUVECs, which were transfected with a construct containing a human COX-2 promoter fragment (nucleotides -830 to +103) linked to a luciferase reporter gene. There was a 3-fold increase in luciferase activity after VEGF stimulation and a 1.4-fold increase in COX-2 promoter activity by HMCM versus NMCM. The experiments are presented as means \pm SD. * P < 0.05.

the PKC signaling pathway, because staurosporine at low concentrations blocked hypoxia-induced activation of VEGF. This observation is in line with previous studies (13, 26) that have suggested PKC involvement in the control of VEGF expression. In addition, our observations have also demonstrated that VEGF-dependent regulation of COX-2 is PKC- α , PKC- β , and iNOS dependent because both Gö6976 and L-NIL could significantly block COX-2 expression induced by VEGF and COX-2 expression induced by HMCM. PKC has also been suggested to mediate IL-1 β and PMA-induced COX-2 expression in pulmonary epithelial cells

(30). Accordingly, we found that pretreatment with the PKC- α and - β inhibitor Gö6976 in HMCM completely blocked the ability of VEGF to induce COX-2 expression in HUVECs. There is evidence indicating that endothelial NOS and iNOS, targets of VEGF (31), may serve to increase the expression of COX-2. L-NAME, as a nonselective NOS inhibitor, could inhibit increased COX-2 expression induced by HMCM and VEGF, supporting the hypothesis that VEGF induced by hypoxia stimulated COX-2 expression via a NO-dependent pathway. This is consistent with previous data showing that COX-2 activity in preconditioned myocardium is mediated by iNOS (5).

Table 2. Effect of HMCM on HUVEC content of PGE₂ and PGI₂

	PGE ₂	6-keto-PGF ₁
NMCM	1.89 \pm 0.74	6.83 \pm 3.14
HMCM	6.68 \pm 1.46*	14.52 \pm 2.26†
HMCM + NS-98	0.56 \pm 0.02	3.94 \pm 0.31
H9c2 + NS-98	0.64 \pm 0.17	4.88 \pm 0.07

Values (in ng/mg protein) are means \pm SD of triplet measurements. Human umbilical vein endothelial cells (HUVECs) were cultured in serum-free medium for overnight before incubation with HMCM or NMCM as controls for 24 h. NS-398 (30 μ M) was either preincubated with HMCM for 30 min before the addition of HMCM to the HUVEC culture or incubated with cardiac myocytes (H9c2) during hypoxia for 24 h before the collection of HMCM to treat HUVECs. The HUVEC content of PGE₂ and 6-keto-PGF_{1 α} were measured by ELISA. The levels of PGE₂ (P < 0.05) and 6-keto-PGF_{1 α} (P < 0.01) were significant higher in HMCM-than NMCM-treated HUVECs, and these increases were abrogated when NS-398 was given, indicating that HMCM-induced HUVEC cyclooxygenase-2 (COX-2) expression associated with an increase in COX-2 activity. * P < 0.05; † P < 0.01.

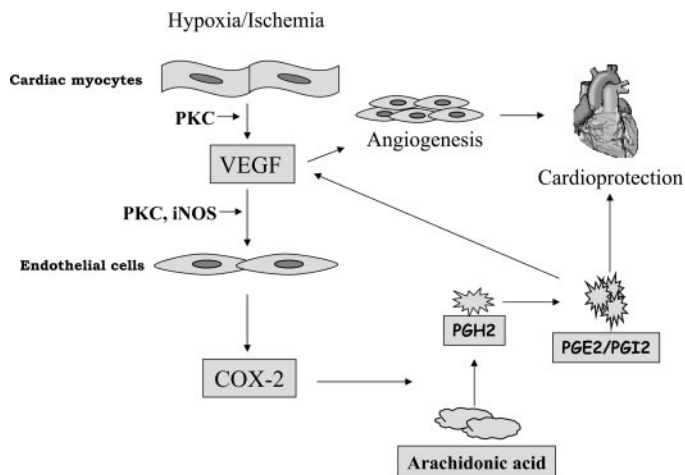


Fig. 6. Proposed pathway of hypoxic myocyte-induced COX-2 gene regulation. iNOS, inducible nitric oxide synthase.

Upregulation of COX-2 in endothelial cells strongly influences cellular COX-2 activity (7). This results in an increase in prostanoid synthesis that includes thromboxane A₂ (TXA₂), PGI₂, and PGE₂. In endothelial cell studies, COX-1 was linked to TXA₂ production, whereas the induction of COX-2 shifted prostanoid synthesis to favor PGE₂ and PGI₂ production (7). Expression of prostaglandin synthesis can be stimulated by various physical and chemical agents in human endothelial cells, such as shear stress and hypoxia (41). Hypoxic human endothelial cells in culture produce increased prostacyclin, and hypoxia increases expression of the COX-2 gene in human endothelial cells independent of other stimuli. We have demonstrated in the present study that the upregulation of HMCM-induced endothelial COX-2 was accompanied by the upregulation of terminal products, such as PGI₂ and PGE₂, by about twofold. Therefore, hypoxic myocyte-dependent endothelial COX-2 seems to directly stimulate prostanoid synthesis and expression.

One important aspect of the present study is that it defines the role of VEGF, which not only serves as an angiogenic factor but also serves as a signal regulator, inducing COX-2 expression in endothelial cells. No information is currently available regarding this issue. To exclude the possibility that this VEGF effect is unique to the H9c2 cardiac cell line, we confirmed our experiment in primary cultured RNCM. Hypoxia-induced VEGF expression and secreted VEGF to HMCM were observed in both types of cardiac myocytes. As previously reported, VEGF expression in myocardium is increased in the setting of myocardial infarction, hypoxia, and ischemia (28, 29). However, these studies did not identify the involvement of VEGF in biological communication between cardiac myocytes and endothelial cells when exposed to hypoxic conditions to, furthermore, induce angiogenesis. This activity of VEGF potentiates the angiogenesis cascade, additionally; the present study suggests that VEGF provides further cardiac protection through COX-2 (Fig. 6).

The induction of COX-2 appears to be cardioprotective, which was proven by the fact that selective inhibition of COX-2, as used clinically, is associated with cardiovascular events (11), and upregulated COX-2 in preconditioned myocardium in vivo mediated the anti-stunning and anti-infarct effects of late preconditioning (42). In addition, COX-2 increases vascular prostacyclin production in endothelial cells, resulting in vasodilation, and decreased vascular resistance of the ischemic bed, improving blood flow (35). In turn, this may improve cardiac function. VEGF and COX-2 could be mutually regulated to become an internal regulation cycle to strength both angiogenesis and cardiac function in the ischemic heart.

In summary, the present study has elucidated molecular events underlying COX-2 gene expression resulting from the interaction of hypoxic myocytes and vascular endothelial cells. VEGF appears to play an important role as a bridge factor between the two cell types in inducing the expression of COX-2 under hypoxic conditions. Our findings show that PKC is one of

the key factors in response to hypoxic myocytes secreting VEGF, and both PKC and iNOS are involved in VEGF inducing COX-2 expression (Fig. 6). However, the regulation of COX-2 by VEGF and the contribution of COX-2 to the inflammatory response after myocardial ischemia or in preconditioning in vivo will require further investigation.

DISCLOSURES

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