Expression and Regulation of Adhesion Molecules in Cardiac Cells by Cytokines

Response to Acute Hypoxia

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Abstract—Adhesion molecules mediate inflammatory myocardial injury after ischemia/reperfusion. Cytokine release and hypoxia are features of acute ischemia that may influence expression of these molecules. Accordingly, we studied intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) responses to cytokines and acute hypoxia in cultured myocardial cells. Northern blot analysis and immunoassay showed that the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α stimulated concentration-dependent increases in ICAM and VCAM mRNA and protein. In both cardiac myocytes and fibroblasts, pretreatment with a specific inhibitor of nuclear transcription factor-κB (NF-κB) prevented cytokine induction of both molecules. We also found that inhibition of tyrosine kinase and p38/RK (stress-activated protein kinase) pathways prevented IL-1β–induced ICAM and VCAM protein synthesis, whereas extracellular signal–regulated protein kinase (ERK1/ERK2) inhibition did not. Neither hypoxia (0% O_2 for 6 hours) alone nor hypoxia/reoxygenation had any significant effect on ICAM and VCAM mRNA. However, hypoxia did enhance IL-1β–induced ICAM mRNA expression in myocytes. As a possible mechanism of this synergistic action on CAM expression, hypoxia induced a time-dependent increase in the DNA binding activity of both NF-κB and activator protein-1 (AP-1), two transcription factors important for cell adhesion molecule expression. In contrast to the enhanced ICAM mRNA induced by IL-1β during hypoxia, however, protein levels for this adhesion molecule were unchanged beyond IL-1β–stimulated levels, suggesting posttranscriptional and/or posttranslational control mechanisms. We conclude that cytokines regulate ICAM and VCAM mRNA and protein in both cardiac myocytes and fibroblasts. Furthermore, adhesion molecule induction requires translocation of at least two transcription factors, NF-κB and AP-1. **(*Circ Res*. 1998;82:576-586.)**

Key Words: cytokine ■ hypoxia ■ cell adhesion molecule ■ mRNA ■ signal transduction

Cell adhesion molecules are surface proteins involved in modulating intercellular communication among a wide variety of different cell types. Several major families of adhesion molecule receptors have been identified and characterized; these include the integrins, cadherins, selectins, membrane-associated proteoglycans, and the immunoglobulin superfamily members. VCAM-1 (or CD106) and ICAM-1 (or CD54) are two members of the immunoglobulin gene superfamily that are critical in the recruitment and infiltration of inflammatory cells to sites of injury. VCAM-1 binds circulating monocytes and lymphocytes expressing the integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$,[1–3] whereas ICAM-1 is the counterreceptor for several leukocyte β_2 integrins (eg, lymphocyte function–associated antigen [CD11a/CD18] and Mac-1 [CD11b/CD18]). The interaction of ICAM-1 with leukocyte integrins also plays an important role in leukocyte trafficking and the initiation of antigen-specific immune responses.[4,5]

Myocardial CAM gene expression is upregulated in inflammatory states such as ischemia/reperfusion and myocarditis.[5–9] Elevated CAM expression is temporally associated with leukocyte sequestration and infiltration into myocardial tissues. In cardiac inflammation, resident cells (eg, endothelial cells, myocytes, fibroblasts, and smooth muscle cells) and infiltrating leukocytes release cytokines capable of transcriptionally activating CAM genes and, as a consequence, promote leukocyte sequestration and transmigration. The importance of myocyte ICAM expression in neutrophil adherence and subsequent myocardial injury has been shown by several groups.[10–12] Although ICAM and VCAM are constitutively expressed in a few cell types, they are readily induced by proinflammatory stimuli such as IL-1, TNF, LPS, and phorbol esters.[1,3,5,13,14]

Control of CAM expression is largely due to an increase in mRNA production, and several specific mechanisms responsible for the transcriptional activation of CAM genes have been investigated.[14–17] Both ICAM and VCAM genes contain sequences in their promoter regions that are recognized by the NF-κB/rel and AP-1 transcription factor families.[16,17] Deletion analysis has demonstrated that these sites are necessary for both cytokine or LPS induction of these adhesion molecules.[16,17]

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Selected Abbreviations and Acronyms

AMCA	= 7-amino-4-methylcoumarin-3-acetic acid
AP-1	= activator protein-1 (Fos/Jun transcription factor)
CAM	= cell adhesion molecule
CREB	= cAMP-responsive element–binding protein
DB	= dissociation buffer
EMSA	= electrophoresis mobility shift assay
ERK	= extracellular signal–regulated protein kinase
FCM	= flow cytometry
FITC	= fluorescein isothiocyanate
GRE	= glucocorticoid response element
ICAM	= intercellular adhesion molecule
IκB	= inhibitory binding protein of NF-κB
IL	= interleukin
iNOS	= inducible NO synthase
LPS	= lipopolysaccharide
MAP kinase	= mitogen-activated protein kinase
NF-κB	= nuclear transcription factor-κB
p38/RK	= a MAP kinase called stress-activated protein kinase (SAPK) or reactive kinase (RK)
PBS/T	= PBS/Tween 0.05
PDTC	= pyrrolidine dithiocarbamate
PI	= propidium iodide
PKA	= cAMP-dependent kinase
PKC	= protein kinase C
PMA	= phorbol 12-myristate 13-acetate
Sp1	= stimulatory protein-1 (transcription factor)
TNF-α	= tumor necrosis factor-α
VCAM	= vascular cell adhesion molecule

Although CAMs have been extensively investigated in both vascular tissue and in endothelial cells, there are few reports available concerning CAM regulation by cytokines in myocardial cells (myocytes and nonmyocytes). Furthermore, in the investigations that have been reported, the signal transduction pathways underlying CAM induction have not been identified. Previous reports indicate that cytokines such as TNF-α and monocyte chemotactic protein-1 induced a dose-dependent induction of ICAM mRNA and protein in neonatal cardiac myocytes.[12,13] Among the important stimuli in the production and release of cytokines in several cell types is hypoxia.[18–21] Moreover, hypoxia and/or reoxygenation have been shown to induce ICAM and E-selectin in endothelial cells.[19,22–24]

However, little is known about (1) the mechanisms of adhesion molecule regulation in cardiac myocytes and fibroblasts by hypoxia and cytokines and (2) the potential interaction of these two variables that are known to be involved in inflammatory myocardial injury. Because hypoxia and cytokine release are components of acute and chronic myocardial ischemia, we investigated the induction and the regulation of cardiac myocyte and fibroblast ICAM and VCAM expression by hypoxia and cytokines in an established cell culture model.

Materials and Methods

Tissue culture media and additives were from GIBCO Life Technologies and the University of California at San Francisco cell culture facility. Human recombinant IL-1β and TNF-α were from R & D Systems. LPS, PMA, forskolin, and PDTC were from Sigma Chemical Co. Tyrphostin, SB203580, and PD98059 were from Calbiochem. Mouse VCAM-1 (monoclonal antibody against rat VCAM-1) was from Babco. Mouse ICAM (monoclonal antibody against rat ICAM-1) was from R & D Systems. MF-20–FITC mouse monoclonal antibody against striated muscle myosin was from the University of Iowa hybridoma bank. AMCA-conjugated affiniPure goat anti-mouse IgG was from Accurate Chemical and Scientific Corp. Normal goat serum was from Jackson Laboratories. [γ-^{32}P]ATP was from Amersham. Poly(dI-dC).poly(dI-dC) was from Pharmacia Biotech Inc. cDNA for murine ICAM was from American Tissue Culture Collection (ATCC, clone No. 63044), and human cDNA for VCAM was the kind gift of Dr Tucker Collins (Brigham and Women's Hospital, Boston, Mass). The 28S oligoprobe was from Clontech. NF-κB, AP-1, GRE, CREB, and Sp1 consensus oligonucleotides used for gel-shift assays were from Promega. All reagents used were certified free from endotoxin by the manufacturer.

Cell Culture

Primary myocyte cell cultures were prepared by enzymatic dissociation of ventricular tissue from 1-day-old Sprague-Dawley rat pups according to methods described previously.[25,26] Myocytes were seeded onto 100-mm plastic dishes at a final density of 150 to 200 cells/mm^2. Through culture day 3, cells were kept in DMEM containing 5% bovine calf serum supplemented with 1.5 mmol/L B_{12}, 50 U/mL penicillin, and 0.1 mmol/L bromodeoxyuridine to prevent low-level nonmyocardial cell proliferation as previously described.[25,26] On day 3, cells were placed in serum-free medium containing 10 μg/mL insulin and 10 μg/mL transferrin. Nonmyocytes (mostly fibroblasts) were separated from cardiomyocytes by 1 hour of preplating, during which cardiomyocytes did not attach to the culture plates. Cells were grown at 37°C in humidified air with 1% CO_2. Confluent fibroblasts were passaged on day 1, seeded in 100-mm dishes, and refed with growth medium (MEM/5% fetal calf serum) every 48 hours. Under the above conditions, myocyte cultures showed $<$10% contamination with other myocardial cell types. All experiments were initiated on day 4, 24 hours after the change to serum-free conditions.

Cell Treatments and Induction of Hypoxia

After overnight incubation, serum-free medium was removed, and fresh medium was added. Cytokines (IL-1β and TNF-α), LPS, PMA, forskolin, PDTC, tyrphostin, SB203580, PD98059, or vehicle was then added, and cells were returned to the incubator or placed in the hypoxia chamber.

Experiments at low oxygen tension were performed in an airtight Plexiglas humidified chamber (Anaerobic Environment, Sheldon), which was maintained at 37°C and continuously gassed with a mixture of 99% N_2/1% CO_2/0% O_2.[26–28] Cells were placed into the hypoxia chamber on culture day 4 and remained for 6 to 12 hours. Maintenance of the desired O_2 concentration was routinely monitored during incubation using an oxygen electrode (Controls Katharobic System). For concurrent normoxic conditions, cells were placed in a Forma Scientific incubator (gassed with 99% air/1% CO_2 at 37°C).

Northern Blot Analysis of CAM mRNA Expression

Total RNA was extracted from the cells by the guanidinium thiocyanate–phenol–chloroform method of Chomczynski and Sacchi.[29] For Northern hybridization, 20 μg of total RNA was size-fractionated on a 1% agarose gel containing 2.2 mol/L formaldehyde. Gels were photographed and rinsed in 10× SSC (1× SSC contains 150 mmol/L NaCl and 15 mmol/L trisodium citrate), and RNA was transferred to a Hybond-N membrane (Amersham Inc) by blotting using 20× SSC buffer. Thereafter, filters were air-dried and UV–cross-linked (Stratalinker). Blots were hybridized at 55°C in a buffer containing 0.25 mol/L sodium phosphate, 7.5% dextran, and 7% SDS. cDNA probes used were murine ICAM (1.2-kb *Xho*I-*Eco*RI fragment) and human VCAM (0.8-kb *Eco*RI-*Hin*dIII fragment). Probes were labeled by random priming (Decaprime, Ambion) to a specific activity of 10^9 cpm/μg DNA using [^{32}P]dCTP (6000 Ci/mmol). After the labeling reaction, probes were purified using a Biospin column (Bio-Rad) to remove unincorporated radioactive [^{32}P]dCTP. Filters were washed at room temperature for 5 minutes in 2× SSC and 0.1% SDS and at 55°C in 0.1× SSC containing 0.1 SDS for 20 minutes. Subsequently, filters were wrapped in plastic wrap and exposed to Kodak X-OMAT films at −80°C for 1 to 2 days. A 28S oligoprobe (Clontech) was used to control for variations in RNA loading. Several exposures of the Northern blot were taken to ensure that quantification of the hybridization signal for each gene was expressed in the linear range and normalized by dividing the optical density of the hybridization signal for each gene by the optical density of the corresponding 28S signal.

ELISA of VCAM and ICAM

Total cellular expression of VCAM and ICAM in myocytes and fibroblasts was analyzed by an ELISA after cell fixation. For these experiments, cells were seeded onto 96-well flat-bottomed microtiter plates in 100 μL DMEM. After overnight incubation in serum-free medium, cells were treated with cytokines for the designated time period. After treatment, cells were washed twice with DMEM and then fixed at room temperature for 15 minutes with 1% paraformaldehyde dissolved in PBS. After four washes with PBS/T, cells were incubated for 30 minutes in PBS/T containing 2% BSA to block nonspecific binding. Then cells were washed and incubated with specific mouse monoclonal antibodies against ICAM and VCAM at a dilution of 1:500. After 1 hour at 37°C, cells were washed four times with PBS/T and incubated with goat anti-murine IgG coupled to horseradish peroxidase (1:1000). After 1 hour, cells were washed four times with PBS/T, and peroxidase substrate solution (1 μg/mL *o*-phenylenediamine in citrate buffer, pH 5, containing 0.015% H_2O_2) was added for 10 minutes. The reaction was stopped by adding 2 mol/L N_2SO_4. Optical density was read in a multichannel spectrophotometer at 492 nm. Each experiment was performed in quadruplicate. Data were corrected for the blank values obtained without use of the primary antibody.

FCM Analysis of Myocardial CAM Expression

Myocytes plated as described above were treated with IL-1β (1 ng/mL) for 24 hours in serum-free medium. At this time, cells were trypsinized for 10 minutes with 500 μL of 0.75 mg/mL trypsin (1:250, Difco) and DNAse I (250 μg/mL) in DB containing 137 mmol/L NaCl, 5.36 mmol/L KCl, 0.81 mmol/L $MgSO_4 \cdot 7H_2O$, 5.55 mmol/L dextrose, 0.44 mmol/L KH_2PO_4, 0.34 mmol/L $Na_2HPO_4 \cdot 7H_2O$, 20 mmol/L HEPES, and 1% BSA. The remaining attached cells (mostly nonmyocytes) from the same dish were removed by a second more aggressive trypsinization step with 2 mg/mL trypsin and 0.2% EDTA along with gentle scraping. All cells were pooled in DB containing a final concentration of 10% bovine calf serum and collected by centrifugation (500*g* for 5 minutes). Cell pellets were fixed by resuspension in 500 μL of 25% ethanol/15 mmol/L $MgCl_2$ on ice for 30 minutes, followed by a second centrifugation (500*g* for 5 minutes). Fixed cells were resuspended in 100 μL DB containing 10% normal goat serum (to block nonspecific binding of antibody), 0.1% Triton X-100, and 1:50 dilution of monoclonal antibody to either VCAM-1 or ICAM-1. After overnight incubation at 4°C, the cells were washed with 3 mL DB on ice and incubated in 100 μL DB containing 0.1% Triton X-100 and 1:100 goat anti-mouse AMCA for 1 hour on ice. Cells were then washed two times with DB and incubated overnight at 4°C in DB containing 0.1% Triton X-100 and MF-20–FITC (1:300). Cells were then washed again in DB, filtered through a 70-mm nylon mesh, and resuspended in 400 μL of DB containing 10 μg/mL PI and 2 μg/mL RNAse in preparation for FCM. FCM was performed on a dual-laser FACStar Plus (Becton Dickinson Immunocytometry Systems) flow cytometer. The primary laser was set at the 488-nm line of an argon-ion laser for excitation of FITC and PI. FITC emission was detected through bandpass filter at 530±30 nm and PI emission at >620 nm. The second laser was set at the UV lines for excitation of AMCA. AMCA emission was detected at 425±50 nm. Thresholding was performed using PI intensity to observe only nucleated cells, omitting debris. A minimum of 30 000 events were collected for postacquisition processing using CellQuest software (Becton Dickinson Immunocytometry Systems).

Electrophoresis Mobility Shift Assay

Nuclear extracts were prepared according to Dignam et al[30] as previously described.[26] Nuclei were resuspended in 40 μL of 20 mmol/L HEPES, pH 7.9, 400 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride, rocked for 20 minutes at 4°C, and centrifuged at 14 000 rpm for 5 minutes. Supernatants containing nuclear proteins were snap-frozen in liquid nitrogen and stored at −80°C.

Oligonucleotide probes for the NF-κB and AP-1 consensus sequences (Promega Inc) were end-labeled with [γ-^{32}P[ATP by incubation with T4 polynucleotide kinase at 37°C for 10 minutes. The labeled probe was separated from unincorporated nucleotide using a spin column (Bio-Rad). Nuclear protein content was determined by the bicinchoninic acid method (BCA, Pierce). EMSA experiments were performed by incubating 10 μg of nuclear extracts in 20 μL of binding buffer containing 50 mmol/L Tris, pH 7.5, 250 mmol/L NaCl, 2.5 mmol/L dithiothreitol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 0.25 mg/mL poly(dI-dC), and 10% glycerol for 10 minutes at room temperature. For competition experiments, an excess of unlabeled NF-κB and nuclear protein were preincubated in the binding buffer for 10 minutes. ^{32}P-labeled oligonucleotide probe (20 000 to 50 000 cpm) was then added, and the reaction mixture was incubated for 20 minutes at room temperature. The reaction was stopped by adding 2 μL of 10× loading buffer (250 mmol/L Tris, pH 7.5, 0.2% bromophenol blue, and 0.2% xylene cyanole). Samples were electrophoresed in native 4% polyacrylamide gels in running buffer (0.5× TBE) at 100 V for 3 hours. The gels were then dried and exposed to autoradiographic x-ray film (X-Omat AR-5, Eastman Kodak Co) with an intensifying screen for 6 to 12 hours at −80°C.

Data Analysis

Hybridization signals on autoradiographs were quantified by scanning densitometry (NIH image). Values are expressed as mean±SEM. Differences in the means among the groups were tested by ANOVA. If the F test showed overall significance, comparison among groups was performed by the Student-Newman-Keuls test. Values of $P<.05$ were considered statistically significant.

Results

ICAM-1 and VCAM-1 mRNA Expression

Time and Concentration Dependence

Although unstimulated cardiac myocytes constitutively expressed low levels of ICAM and VCAM mRNA, the proinflammatory cytokines IL-1β and TNF-α induced both concentration- and time-dependent increases in CAM mRNA expression (Fig 1A). A plateau was observed as early as 1 hour for ICAM; the peak expression for VCAM was at 6 hours. After 24 hours, the expression of both ICAM and VCAM mRNA declined but was still significantly increased above control (Fig 1B). LPS also induced VCAM and ICAM mRNA expression (data not shown). Similar induction of VCAM and ICAM mRNAs was seen in cardiac fibroblasts treated with IL-1β or TNF-α (Fig 2).

Effects of Hypoxia

Given our previous findings that IL-1β–induced iNOS mRNA and protein expression were inhibited by prolonged hypoxia,[26] we asked whether hypoxia modulates ICAM and VCAM mRNA expression. We found that neither hypoxia alone (0% for 6 hours) nor hypoxia followed by reoxygenation for 1 to 24 hours had any significant effect on ICAM-1 and VCAM-1 mRNA in either myocytes (n=10) or fibroblasts (n=3) (Figs 2 and 3). In contrast, hypoxia did enhance ICAM mRNA expression induced by IL-1β in myocytes, although this observation did not extend to the fibroblasts (n=6) (Figs 2 and 3). Hypoxia did not affect VCAM mRNA expression in either myocytes or fibroblasts (Figs 2 and 3).

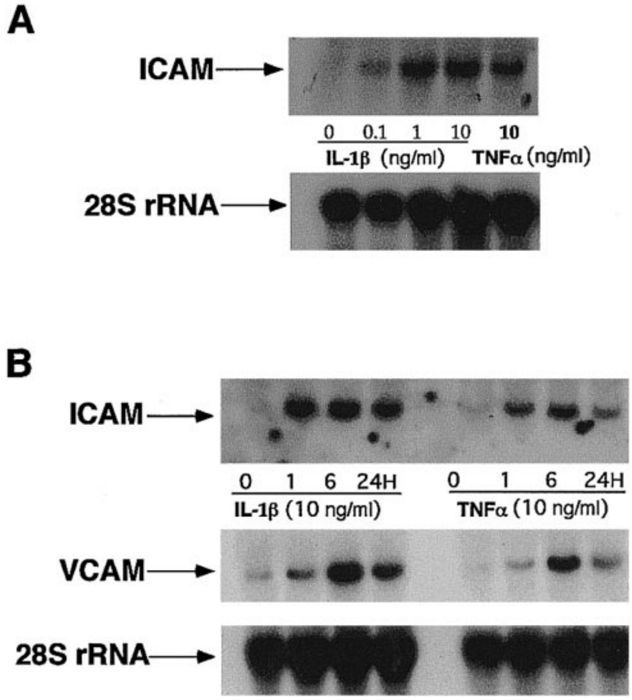

Figure 1. Concentration dependence and time course of cytokine-induced ICAM and VCAM mRNA in cardiac myocytes. Neonatal cardiac myocytes were incubated with vehicle (PBS/BSA), IL-1β (0.1 to 10 ng/mL), or TNF-α (10 ng/mL) for 6 hours. Total RNA (20 μg/lane) was isolated, and ICAM-1 mRNA levels were determined by Northern blotting (top blots of both panels). rRNA (28S) levels were used as an internal standard to confirm equal loading (bottom blots of both panels). A, ICAM-1 mRNA is induced by IL-1β in a concentration-dependent manner up to 10 ng/mL. B, ICAM and VCAM mRNA were induced in a time-dependent fashion by IL-1β and TNF-α. This experiment was repeated with same result.

ICAM and VCAM Protein Expression

ELISA

Consistent with the mRNA analysis, ELISA showed that both ICAM and VCAM were constitutively expressed in myocytes and fibroblasts. These adhesion molecules were further upregulated by incubation with the proinflammatory mediators IL-1β, TNF-α, and LPS, peaking at 8 to 12 hours for all agents tested (Fig 4). Moreover, we also found a time-dependent increase of soluble ICAM in cell culture medium (data not shown).

Flow Cytometry

Since primary cultures of cardiac myocytes are always "contaminated" to some degree by cardiac nonmyocytes, it was critical to confirm that our observations of CAM expression in myocytes were due to the expression of CAMs by myocytes themselves.

FCM is a technique that takes advantage of the differential staining characteristics of cultured cells to make unambiguous observations on the expression of various cell surface and/or intracellular proteins. FCM is an important tool in the determination of cell cycle events and has also been used to measure the expression of cytokines by mixed populations of cells. Using MF-20, the well-described antibody to sarcomeric myosin heavy chain, we have been able to use FCM to distinguish cardiac myocytes from a nonmyocyte population present in our culture system.

Previous work with nonmyocytes has indicated that these cells are largely fibroblast in origin[31] and that primary cultures of cardiac myocytes contain $\approx$15% "contaminating" fibroblasts. In an effort to delineate further the cell specificity of our observations of CAM expression in our cultured cells, we used FCM to compare the expression of ICAM-1 and VCAM-1 on the two cell types and their responses to IL-1β. As indicated in Fig 5, MF-20 staining separates cells into two distinctive populations of the appropriate composition, ie, 80% MF-20 positive (myocytes) and 20% MF-20 negative (nonmyocytes).[31] By staining with antibody to CAM, we have found that, under control conditions, most of the cells are located in the negative–CAM-staining quadrants. After stimulation with IL-1β, however, there is a clear shift of both myocytes and nonmyocytes into the quadrants that indicate an increase in CAM staining. Although the data shown are for VCAM-1, identical results were also obtained with the antibody to rat

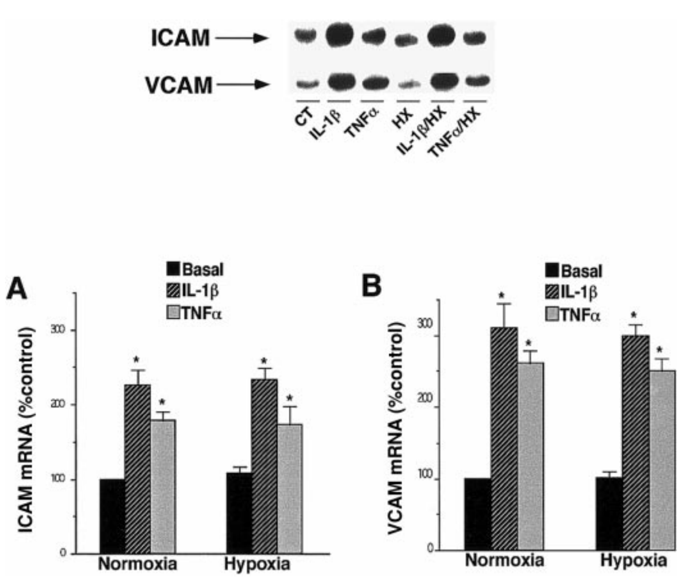

Figure 2. Acute hypoxia has no effect on cytokine-induced ICAM (A) and VCAM (B) mRNA in cardiac fibroblasts. Neonatal cardiac fibroblasts were treated with vehicle (PBS/BSA), IL-1β (10 ng/mL), or TNF-α (10 ng/mL) for 6 hours in the presence or absence of hypoxia (0% O_2), and cells were analyzed for ICAM-1 and VCAM-1 mRNA expression by Northern blot analysis (top) by hybridization with specific cDNA probes. Quantitative analysis was performed by densitometric scanning of the blots and normalized with respective 28S rRNA. Values are expressed as percentage of control. Each bar represents the mean$\pm$SEM of three separate experiments (A and B). *$P<.05$ vs basal.

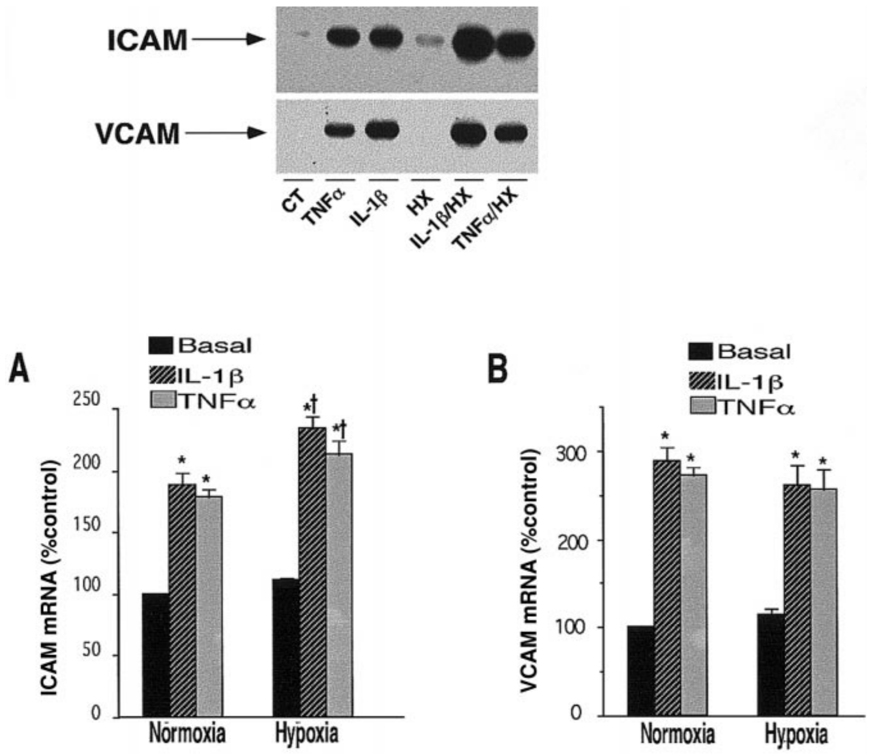

Figure 3. Effect of hypoxia on ICAM and VCAM expression stimulated by cytokines in cardiac myocytes. The Northern blots at the top are from neonatal cardiac myocytes that were treated for 6 hours with vehicle (control [CT]), TNF-α (10 ng/mL), or IL-1β (10 ng/mL) under normoxic or hypoxic (HX) conditions (0% O_2 for 6 hours). The blots were performed as described in "Materials and Methods." The data shown are representative of eight separate experiments involving IL-1β and TNF-α with or without HX. Panels A and B show densitometric analyses of acute hypoxia on cytokine-induced ICAM (A) and VCAM (B) mRNA. Myocytes were stimulated with vehicle (control [basal]), IL-1β (10 ng/mL), or TNF-α (10 ng/mL) for 6 hours in presence or absence of hypoxia. Total cellular RNA was isolated and analyzed for the expression of ICAM and VCAM mRNA by hybridization with specific cDNA probes. Rehybridization with a 28S probe was performed to control for RNA loading. Quantitative analysis was performed by densitometric scanning of the blots and was normalized with respective 28S rRNA. Values are expressed as percentage of control. Each bar represents the mean±SEM of eight separate experiments. **P*<.05 vs basal; †*P*<.05 vs normoxia.

ICAM-1. These observations are consistent with both the RNA and protein data, suggesting that IL-1β increases the expression of both CAMs in both cell populations. In our myocyte cultures, it is the myocytes that are responsible for the bulk of CAM expression, which is, in turn, in proportion to their preponderance in these cultures.

Effect of Hypoxia

To determine whether hypoxia modulates ICAM mRNA and protein expression in a parallel fashion, we investigated the effect of hypoxia on ICAM protein. We found that hypoxia did not affect baseline levels of ICAM protein. Rather, we found that despite enhanced ICAM mRNA expression stimulated by cytokines during hypoxia, the increased protein levels after cytokine stimulation under normoxic conditions were unchanged by hypoxia, suggesting that additional posttranscriptional as well as posttranslational mechanisms may operate under hypoxic conditions (Fig 4).

Mechanisms Regulating ICAM and VCAM mRNA and Protein

Regulation by PKC and cAMP

Although the intracellular signals that mediate the induction of CAMs in cardiac cells have not been clearly identified, both PKC and PKA have been implicated in the regulation of cytokine-induced CAM expression in other cell types. To further analyze these signal transduction pathways, we initially used the phorbol ester PMA and the cAMP stimulatory agent forskolin. PMA, 100 nmol/L for 6 hours, induced ICAM mRNA expression but was less efficacious than either IL-1β or TNF-α (Fig 6). However, depletion of PKC by 24 hours of pretreatment with 500 nmol/l PMA inhibited ICAM mRNA induction by PMA without an effect on VCAM mRNA, implicating PKC as a dominant signal pathway in ICAM mRNA induction (Fig 6). In contrast to ICAM induction by PMA, however, neither the PKC inhibitor staurosporine (100 nmol/L) nor PKC depletion prevented ICAM and VCAM mRNA induction by IL-1β (n=3, data not shown). Forskolin (10 μmol/L for 6 hours), which activates adenylate cyclase and promotes cAMP synthesis, had no effect on either CAM mRNA expression; however, in the presence of hypoxia, forskolin did induce ICAM mRNA expression in cardiac myocytes (n=2, data not shown).

Role of NF-κB and AP-1

Since NF-κB binding motifs have been identified in VCAM and ICAM promoters, we sought to evaluate the role of NF-κB activation in CAM induction in cardiac myocytes. We used PDTC, a radical oxygen species scavenger and metal chelator, as an inhibitor of radical oxygen species and/or NF-κB activation. Pretreatment with PDTC (50 μmol/L) inhibited ICAM-1 and VCAM-1 mRNA upregulation induced by IL-1β and TNF-α (Fig 6). These observations suggest that the transcription factor NF-κB is involved in the activation of adhesion molecule gene expression in cardiac cells.

In our previous report on the induction of iNOS gene expression in response to moderate prolonged hypoxia (1% O_2 for 48 hours), we found no activation of NF-κB.[26] In contrast, cells subjected to more severe hypoxia (0% O_2 for 1 to 6 hours) exhibited time-dependent increases in the DNA binding activities of both NF-κB and AP-1 as shown by gel shift analysis (Figs 7A and 7B). The increases in NF-κB and AP-1 DNA binding in response to acute hypoxia appear to be specific, because DNA binding for the unrelated transcription factors CREB, GRE, and Sp1 do not increase in nuclear extracts prepared from hypoxic myocytes (Fig 7C).

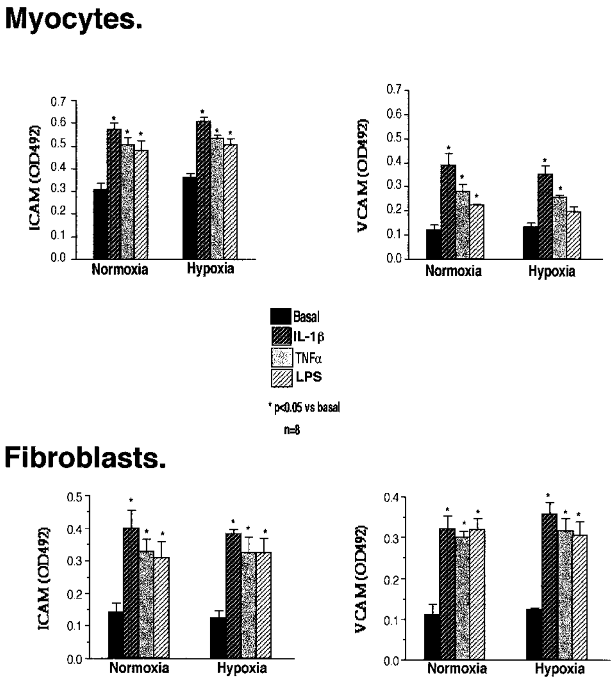

Figure 4. Top, ICAM and VCAM protein expression in cardiac myocytes: effect of cytokines, LPS, and/or hypoxia. Cardiac myocytes seeded in 96-well plates were either left untreated or stimulated with IL-1β (10 ng/mL), TNF-α (10 ng/mL), or LPS (1 μg/mL) for 6 to 12 hours at 37°C under normoxic or hypoxic conditions. Cell surface levels of ICAM-1 and VCAM-1 were measured by ELISA using specific antibodies for ICAM-1 and VCAM-1 as described in "Materials and Methods." Data are presented as optical density value at 492 nm. Test and control samples were analyzed in triplicate in each experiment. Each bar represents the mean±SEM of eight separate experiments. *P<.05 vs basal. Bottom, ICAM and VCAM protein expression in cardiac fibroblasts: effect of cytokines, LPS, and/or hypoxia. Cardiac fibroblasts seeded in 96-well plates were either left untreated or stimulated with IL-1β (10 ng/mL), TNF-α (10 ng/mL), or LPS (1 μg/mL) for 6 to 12 hours at 37°C under normoxic or hypoxic conditions. Cell surface levels of ICAM-1 and VCAM-1 were measured by ELISA using specific antibodies for ICAM-1 and VCAM-1 as described in "Materials and Methods." Data are presented as optical density value at 492 nm. Test and control samples were analyzed in triplicate. Each bar represents the mean±SEM of eight separate experiments. *P<.05 vs basal.

In a previous report, we demonstrated a link between a cytokine-inducible gene (iNOS) and activation of the transcription factor NF-κB in cardiac myocytes.[26] In the present study, we extend this observation and find an induction of DNA binding activity of the transcription factors AP-1 and NF-κB by IL-1β, LPS, and PMA both in cardiac myocytes and fibroblasts (Fig 8). Similar to the results described earlier on the PDTC inhibition of CAM mRNA expression, NF-κB mobilization was inhibited by cotreatment with this agent (Fig 8). Furthermore, PDTC also blocked DNA binding activity of AP-1 induced by IL-1β and PMA (n=4, data not shown). These observations implicate a redox-sensitive pathway in both NF-κB and AP-1 induction.

We also found that PDTC blocked the increase in ICAM and VCAM protein expression stimulated by IL-1β in both myocytes and fibroblasts, consistent with the requirement for an oxidative pathway in cytokine-induced CAM expression in cardiac cells (Fig 9). Since cytokines activate a broad spectrum of signaling molecules, we investigated additional second-messenger pathways implicated in cytokine-induced ICAM and VCAM expression. Our data showed that tyrphostin, a tyrosine kinase inhibitor, prevents IL-1β induction of both ICAM and VCAM (Fig 9). In a similar fashion, SB203580, a specific inhibitor of p38/RK stress kinase, blocked IL-1β–induced ICAM and VCAM expression, whereas PD98059, an inhibitor of ERK1/ERK2, did not (Fig 9).

We considered the possibility that during hypoxia there might be extensive cell death such that stable levels of cytokine-stimulated protein expression actually represent an increase in protein content per cell. To investigate this question, we used the viability/cytotoxicity kit (Live/Dead assay, Molecular Probes) to determine the percentage of cell death during 6 hours of 0% O_2. Control cultures routinely contained 5% to 10% dead cells, and hypoxia increased this proportion to 24±6% (n=4). Thus, there was a 15% to 20% decrease in the number of the living cells in hypoxic cultures, consistent with a modest proportional increase in protein expression per cell, which was far less than the 2-fold increase in mRNA expression that occurred after cytokine stimulation.

Discussion

ICAM-1 and VCAM-1 mRNA and Protein Expression

Expression of CAMs by myocardial cells is a major determinant of injury after ischemia/reperfusion. The importance of adhesion molecules in the inflammatory process has been confirmed by several in vitro and in vivo studies. Specifically, monoclonal antibodies and antisense oligonucleotides to adhesion molecules and their ligands, as well as the absence of CAM expression in knockout mice, prevent tissue injury associated with both acute and chronic inflammation.[32–38] Modulation of the expression and function of cardiac genes by hypoxia and cytokines may be two important mechanisms through which inflammatory signals initiate and propagate tissue injury after ischemia/reperfusion. Our data provide unambiguous evidence that cytokines cause a significant induction of CAM mRNA and protein in both cardiac myocytes and fibroblasts. Previous studies have shown the induction of VCAM mRNA in rat cardiac myocytes[13] and the expression of VCAM protein in murine hearts with acute myocarditis.[8] Our data are the first to demonstrate both constitutive and increased levels of VCAM protein in cardiac myocytes. Our data also indicate that myocardial CAMs exhibit cell-specific expression patterns, because hypoxia, an important component of ischemic myocardial injury, differentially regulates cytokine-induced CAM mRNAs between these two cell types.

A major aspect of the present work is the utilization of FCM to establish the specificity of CAM expression in cytokine-treated myocardial cells. It is well recognized that the lack of myocardial cell lines requires that in vitro investigations of gene expression must use primary cells that are, by necessity, cocultures consisting of both cardiac myocytes and nonmyocytes. As such, it was critical to confirm that our observations on CAM expression in IL-1β–treated myocytes were due to expression of the CAMs of interest by the myocytes themselves. A possible explanation of our results is that the increase

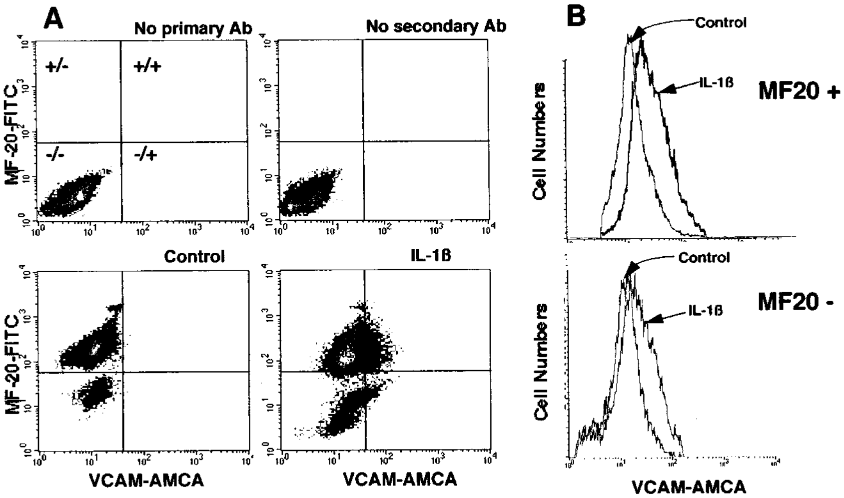

Figure 5. FCM analysis of myocardial VCAM expression. Cardiac myocytes were treated with IL-1β (1 ng/mL) or vehicle for 24 hours and analyzed by FCM as indicated in "Materials and Methods." A, Representative "quadrant" analysis of data obtained from bivariate analysis of VCAM expression in myocyte cultures divided into two populations (MF-20–negative fibroblasts and MF-20–positive myocytes). Background immunofluorescence was determined by analysis of cells incubated with primary or secondary antibodies (Abs) alone (upper panels). As indicated, the upper left quadrant represents cells that are MF-20 positive (myocytes) but VCAM negative (+/−); the lower left quadrant contains cells that are MF-20 negative (fibroblasts) and VCAM negative (−/−). The upper right quadrant contains cells that are MF-20 positive and VCAM positive (+/+); the lower right quadrant contains cells that are MF-20 negative but VCAM positive (−/+). B, Plots derived from data in panel A showing the shift in VCAM-AMCA fluorescence intensity between control and IL-1β–treated myocytes (MF-20+) and fibroblasts (MF-20−). Given the logarithmic scale, the shift observed represents a doubling of VCAM intensity of treated cells. Each experiment was repeated twice more with identical results.

in CAM mRNA and protein in the myocyte cultures was due to an exuberant response of the contaminating nonmyocyte population. Although the use of in situ hybridization could identify cell-specific responses at the mRNA level, the critical question we addressed was whether IL-1β induces CAM protein expression. FCM established that the bulk of the increase in CAM protein in the myocyte cultures was the result of enhanced myocyte expression. The maintenance of cytokine-induced adhesion molecule mRNA and protein levels during acute hypoxia is a new finding suggesting that hypoxia does not depress cellular mechanisms implicated in myocyte damage.

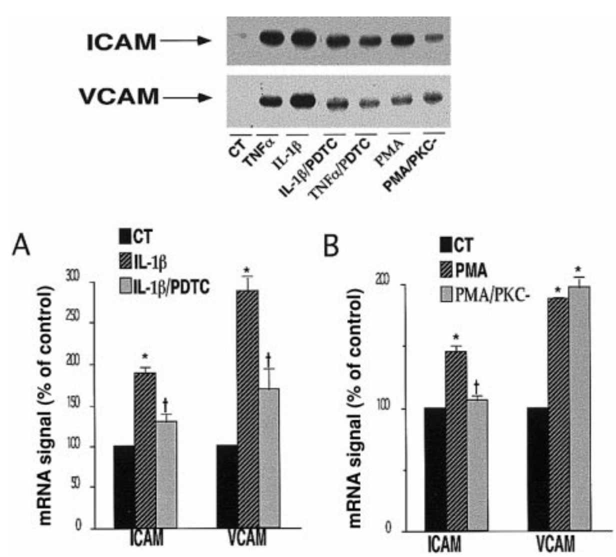

Figure 6. Effects of PDTC cotreatment and PKC depletion on cytokine- and PMA-induced ICAM and VCAM mRNA in myocytes. Cytokine-induced ICAM and VCAM mRNA was inhibited by cotreatment with the antioxidant PDTC (50 μmol/L). The phorbol ester PMA (100 nmol/L) alone stimulated ICAM and VCAM mRNA, an effect that was blocked for ICAM mRNA by prior PMA treatment (500 nmol/L for 24 hours), which depletes PKC (PMA/PKC−). A and B, Densitometric analyses of PDTC and PKC depletion on ICAM and VCAM mRNA expression. Bar graphs show the effect of PDTC (50 μmol/L) and PKC depletion (24 hours of treatment with 500 nmol/L PMA) on IL-1β (10 ng/mL)–induced and PMA (100 nmol/L)–induced ICAM and VCAM mRNA. Values represent densitometric quantification of signal intensity after Northern blotting (top). Each scanning value was normalized to its corresponding 28S value and expressed as percentage of control (CT). Data represent the average of five separate experiments. **P*<.05 vs CT; †*P*<.05 vs IL-1β or PMA.

Signaling Pathways Regulating ICAM and VCAM mRNA and Protein

Intracellular signal transduction required for ICAM and VCAM expression in response to cytokines may involve pathways that include the intracellular second messengers PKC,[39] cAMP,[40,41] and Ca^{2+},[42] as well as proteasome activation.[43] In cultured neonatal rat myocardial cells, we have shown that the PKC activator PMA induces ICAM expression in both myocytes and fibroblasts. This effect is PKC dependent, since PKC downregulation prevented ICAM induction by PMA. However, PKC inhibition did not prevent CAM induction by cytokines, suggesting that PKC is not the main pathway by which IL-1β stimulates ICAM and VCAM mRNA. Although the cAMP-PKA pathway is another signal transduction cascade that has been implicated in CAM induction, we found that under normoxic conditions, forskolin had no effect on ICAM expression in these cells.

In additional experiments, we studied signaling pathways implicated in cytokine-induced CAM expression. Consistent with our previous data in which the tyrosine kinase inhibitor genistein blocked IL-1β–induced NF-κB translocation and iNOS expression,[26] we found that tyrphostin, another potent tyrosine kinase inhibitor, significantly reduced IL-1β–stimulated ICAM and VCAM protein expression in both myocardial cell types.

We also asked whether there is a connection between myocardial CAM expression and the MAP kinases and their homologues, namely, the stress-activated protein kinases (c-*jun* N-terminal kinases and p38/RK, the mammalian homologue of the yeast HOG1 kinase).[44,45] p42/p44 MAP kinases, also known as ERKs, and their upstream activator, MAP kinase

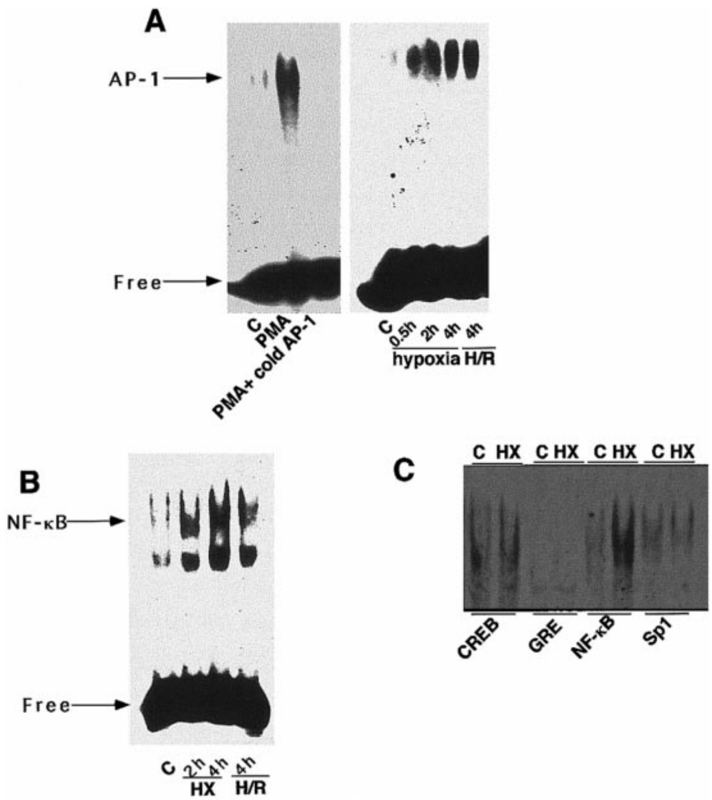

Figure 7. Effect of acute hypoxia on activation of the transcription factors AP-1 and NF-κB. DNA binding activity for AP-1 and NF-κB was determined by a gel shift assay using cardiac myocyte nuclear extracts as described in "Materials and Methods." A, Left, AP-1 competition binding assay. The specificity of the DNA binding complex was evaluated by competition with excess unlabeled AP-1 oligonucleotide. Lane 1 is control (C); lane 2, PMA (100 ng/mL, 2 hours); and lane 3, PMA+100× excess unlabeled AP-1, which displaced labeled AP-1 binding. A, Right, Acute hypoxia-induced time-dependent AP-1 DNA binding activity. Lane 1 is control (C); lanes 2, 3, 4, and 5, 0.5-, 2-, and 4- hour hypoxia (0% O_2) and 4-hour hypoxia+2-hour reoxygenation (H/R), respectively. B, Acute hypoxia (HX)-induced time-dependent NF-κB DNA binding activity. Lane 1 is control (C); lane 2, 2-hour HX (0% O_2); lane 3, 4-hour HX (0% O_2); and lane 4, 4 hour HX+2-hour reoxygenation (H/R). Free indicates free probe. C, Blot showing that acute HX has no effect on Sp1, CREB, or GRE DNA binding activities. Lanes 1, 3, and 5 are control (C); lanes 2, 4, and 6, 4-hour HX. NF-κB is shown as a positive control. Data are representative of five separate experiments.

kinase, were among the first kinases implicated in IL-1β signaling.[46] Using PD98059, a potent inhibitor of ERK signaling, we could not block IL-1β–induced CAM expression. However, using SB203580, a specific inhibitor of p38/RK stress kinase,[47,48] we found that cytokine-induced CAM expression was abolished in both cardiac myocytes and fibroblasts. Therefore, our data implicate the stress kinase cascades in cytokine induction of adhesion molecule expression in cardiac cells.

Role of NF-κB and AP-1 in Regulating ICAM and VCAM mRNA and Protein

As noted above, changes in the expression of VCAM and ICAM genes during myocardial ischemia/reperfusion involve a complex program of intracellular signal transduction processes and transcription events. A common feature, however, is the presence of a κB DNA-regulatory element interacting with the transcription factor NF-κB. The NF-κB system represents the most important transcription-regulatory element in the CAM promoters and plays a key role in regulating cytokine-induced leukocyte adhesion.[16,17,49,50]

We used inhibitor experiments with the metal chelator and radical-scavenging antioxidant PDTC to assess activation of endogenous gene expression in our system. PDTC potently and specifically inhibits NF-κB activation in response to various stimuli by suppressing the release of IκB in intact cells.[16,17] We demonstrated that PDTC pretreatment prevents ICAM-1 and VCAM-1 mRNA expression in IL-1β–and TNF-α–stimulated myocytes. Thus, this redox-sensitive control mechanism appears to operate for VCAM-1 and ICAM-1 mRNA induction by cytokines in cardiac myocytes as previously described for VCAM in endothelial cells.[51,52]

A second key component of the CAM transcriptional machinery involves the transcription factor AP-1, a two-subunit DNA binding protein composed of heterodimers or homodimers of the c-*fos* and c-*jun* proto-oncogene families. The specificity of AP-1 for activating various genes depends on its composition and degree of phosphorylation.[53,54] It has been shown that oxidative stress induced by H_2O_2 upregulates ICAM expression in endothelial cells via activation on the AP-1/ets transcription factor complex, whereas TNF-α–induced ICAM expression is not due to AP-1 activation but rather to NF-κB translocation.[55] Using gel shift assays, we found that PMA, which induces ICAM mRNA expression, also activates AP-1 DNA binding activity. We also found that DNA binding activities of both of these transcription factors are increased in cardiac cells subjected to acute hypoxia. The activation of both NF-κB and AP-1 DNA binding activity was time dependent, with AP-1 activation peaking very quickly at 30 minutes. This activation is consistent with the hypoxia-induced activation of immediate-early response genes, such as c-*fos* and c-*jun* as previously described,[56] that then transcribe AP-1 binding factors/activators.

Previous reports have shown that NF-κB and AP-1 DNA binding are important for CAM induction in other cell types.[16,17,55] We found that although hypoxia alone induces an early activation of NF-κB and AP-1 DNA binding in cardiac myocytes, there was no subsequent increase in CAM expression. IL-1β also induced DNA binding of these transcription factors as well as induction of ICAM and VCAM mRNA, whereas hypoxia enhanced ICAM but not VCAM induction by cytokines. It should be noted that responses may differ depending on the duration of exposure to hypoxia and the

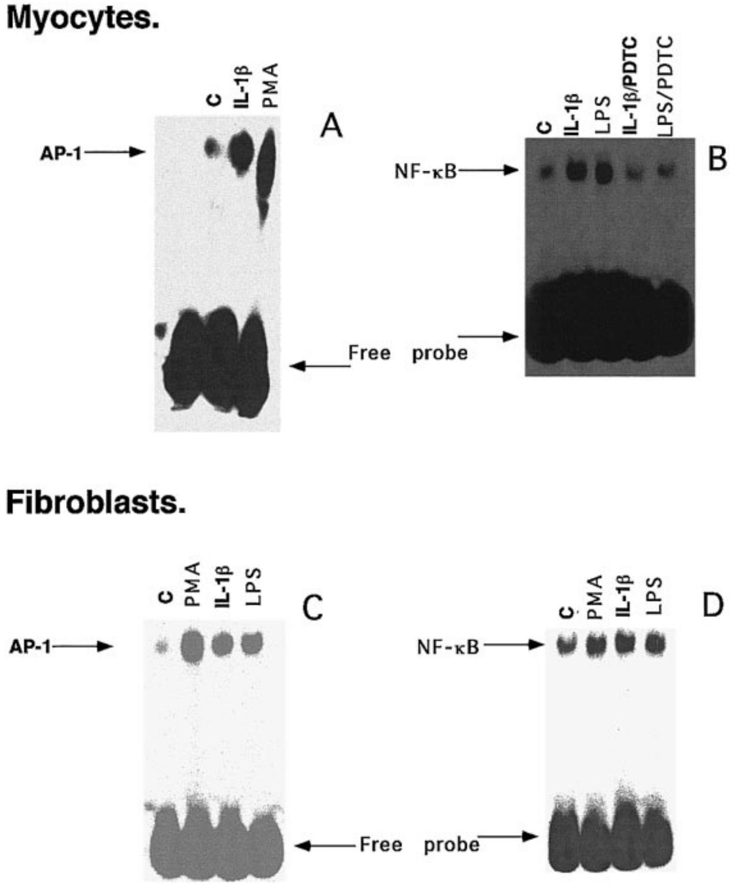

Figure 8. Induction of AP-1 and NF-κB activity by IL-1β, LPS, and PMA and inhibition by PDTC. A, Myocytes were stimulated with vehicle (control [C]), IL-1β (10 ng/mL), or PMA (100 nmol/L) for 2 hours. Nuclear extracts were prepared and analyzed for AP-1 binding using EMSA. B, PDTC blocks NF-κB binding activity induced by IL-1β and LPS in cardiac myocytes. Lane 1 is control (C); lane 2, IL-1β (10 ng/mL for 2 hours; lane 3, LPS (1 μg/mL); lane 4, IL-1β+PDTC (50 μmol/L) for 2 hours; and lane 5, LPS+PDTC (50 μmol/L) for 2 hours. C, Induction of AP-1 binding activity in cardiac fibroblasts. Lane 1 is control (C); lane 2, PMA (100 nmol/L) for 2 hours; lane 3, IL-1β (10 ng/mL) for 2 hours; and lane 4, LPS (1 μg/mL) for 2 hours. D, NF-κB DNA binding activity in cardiac fibroblasts. Lane 1 is control (C); lane 2, PMA (100 nmol/L) for 2 hours; lane 3, IL-1β (10 ng/mL) for 2 hours; and lane 4, LPS (1 μg/mL) for 2 hours. Data are representative of three to six separate experiments.

level of hypoxia to which cells are exposed. For example, we recently reported that IL-1β induces iNOS gene expression, de novo synthesis of iNOS protein, and NO generation in neonatal rat cardiomyocytes and that chronic hypoxia (1% O_2 for 48 hours) decreased or abolished these IL-1β–stimulated responses.[26] During prolonged 1% hypoxia, there was no induction of NF-κB,[26] whereas in the present study, 4 hours of 0% O_2 induced NF-κB activity (Fig 7C).

Although a direct connection between the transcription factors AP-1 and NF-κB and CAM induction by hypoxia and/or cytokines is not conclusively proven by our data, the presence of NF-κB and AP-1 sites in the upstream regulatory sequences and the lack of induction of several other transcription factors (eg, Sp1, CREB, and GRE) during hypoxia suggests a close relationship. Moreover, our observations suggest that although activation of either of these two transcription factors may be necessary for CAM mRNA induction during hypoxia alone, they are not sufficient. We speculate that additional as-yet-undefined signals are required for further induction of these genes during cytokine stimulation. These factors presumably play a role in the synergistic interaction of hypoxia and cytokines on adhesion molecule mRNA expression in our cells.

These data raise the possibility that myocardial CAM expression during ischemia-associated inflammation is modulated by both cytokines and the cellular oxygen environment (ie, redox state), which function in concert to determine specific CAM expression. Ultimately, CAM induction under these pathological circumstances may involve specific interaction of transcriptional factors (such as NF-κB and AP-1), which may be required to regulate complex patterns of CAM gene expression.

Our studies indicate that CAM mRNAs and protein were upregulated in parallel in both cardiac myocytes and fibroblasts in response to cytokines and LPS. We found that despite enhancement of cytokine-induced ICAM expression in hypoxic myocytes, ICAM protein expression was not similarly augmented. To our knowledge, the posttranscriptional events in ICAM expression have not been established, but variability in mRNA translation, although less well characterized than the influences controlling gene transcription, is now recognized as a major determinant in the regulation of gene expression. Moreover, during hypoxia an increase of adhesion-processing enzymes such as chedases[57] may be an alternative explanation of our results. Our observation of a disparity between ICAM mRNA and protein induction during hypoxia suggests that additional posttranscriptional regulation mechanisms that prevent ICAM protein enhancement may be activated during hypoxia.

The augmented response of ICAM mRNA to cytokine stimulation during hypoxia contrasts with the VCAM mRNA response. This observation suggests that hypoxia differentially regulates cardiac cell responses, with a permissive effect on cytokine-induced ICAM mRNA expression in myocytes but not in fibroblasts. Taken together, these data reflect a cell type–specific effect and identify differential regulatory path-

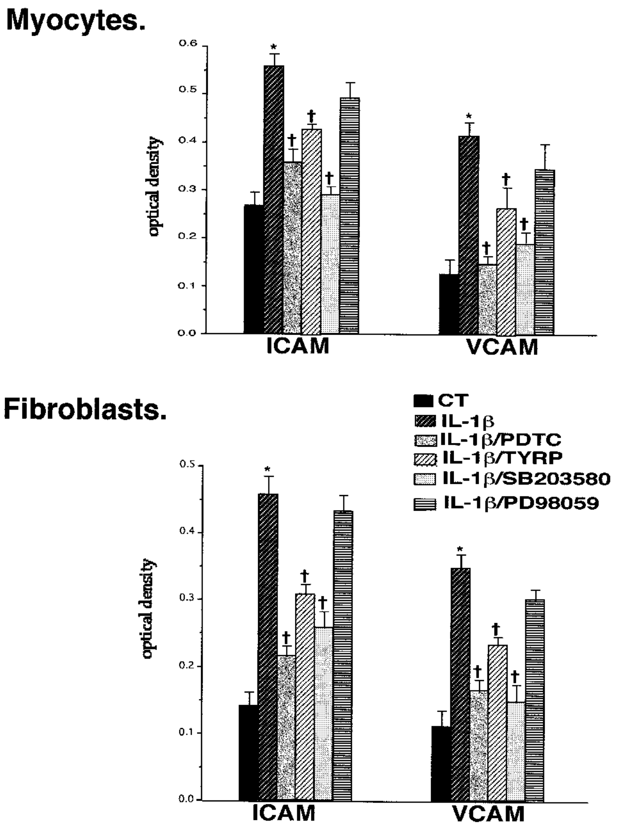

Figure 9. Inhibition of increased ICAM and VCAM protein expression stimulated by IL-1β in cardiac myocytes and fibroblasts. Cardiac myocytes or fibroblasts were treated with 10 ng/mL IL-1β alone, with 10 ng/mL IL-1β+50 μmol/L PDTC, with 10 ng/mL IL-1β+10 μmol/L tyrphostin (TYRP), with 10 ng/mL IL-1β+10 μmol/L SB203580, or with 10 ng/mL IL-1β+10 μmol/L PD98059 for 6 hours. ICAM and VCAM were analyzed by ELISA as described in "Materials and Methods." Test and control (CT) samples were analyzed in triplicate. Each bar represents the mean±SEM of five to eight separate experiments. *P<.0.5 vs control; †P<.05 for PDTC vs IL-1β.

ways that control gene expression of ICAM and VCAM in response to hypoxia and/or cytokines in cells resident in the heart. Our results also provide new insights into the factors involved in cell-specific CAM induction in the heart and may provide additional targets for therapeutic intervention.

Acknowledgments

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