Cytokines induce HIF-1 DNA binding and the expression of HIF-1-dependent genes in cultured rat enterocytes

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Submitted 20 February 2002; accepted in final form 30 September 2002

Scharte, Marion, Xiaonan Han, Daniel J. Bertges, Mitchell P. Fink, and Russell L. Delude. Cytokines induce HIF-1 DNA binding and the expression of HIF-1-dependent genes in cultured rat enterocytes. Am J Physiol Gastrointest Liver Physiol 284: G373–G384, 2003. First published October 2, 2002; 10.1152/ajpgi.00076.2002.—Cellular adaptation to hypoxia depends, in part, on the transcription factor hypoxia-inducible factor-1 (HIF-1). Normoxic cells exposed to an inflammatory milieu often manifest phenotypic changes, such as increased glycolysis, that are reminiscent of those observed in hypoxic cells. Accordingly, we investigated the effects of cytomix, a mixture containing IFN-γ, TNF, and IL-1β on the expression of HIF-1-dependent proteins under normoxic and hypoxic conditions. Incubation of intestine-derived epithelial cells (IEC-6) under 1% O2 increased HIF-1 DNA binding and expression of aldolase A, enolase-1, and VEGF mRNA. Incubation of normoxic cells with cytomix for 48 h also markedly increased HIF-1 DNA binding and expression of mRNAs for these proteins. Incubation of hypoxic cells with cytomix did not inhibit HIF-1 DNA binding or upregulation of HIF-1-dependent genes in response to hypoxia. Neither cytomix nor hypoxia increased steady-state levels of HIF-1α mRNA. Incubation of IEC-6 cells with cytomix induced nitric oxide (NO)-biosynthesis, which was blocked if the cultures contained L-N(G)-l-arginine hydrochloride (L-NIL). Treatment with L-NIL, however, failed to significantly alter aldolase A, enolase-1, and VEGF mRNA levels in normoxic cytomix-treated cells. Proinflammatory cytokines activate the HIF-1 pathway and increase expression of glycolytic genes in nontransformed rat intestinal epithelial cells, largely through an NO-independent mechanism.

nitric oxide; glycolysis; epithelium; intestinal; aldolase A; enolase-1; vascular endothelial growth factor; hypoxia-inducible factor-1; deoxyribonucleic acid

IN MAMMALIAN CELLS, ADAPTATION to reduced oxygen availability includes the upregulation of specific genes involved in angiogenesis, erythropoiesis, glucose transport, and glycolysis. A number of different transcription factors and signaling molecules have been implicated as being important in this adaptive response, including various Smad proteins (1) and endothelial Per-ARNT-Sim (PAS) domain protein-1/hypoxia-inducible factor-2 (EPAS-1/HIF-2) (34). The heterodimeric protein HIF-1, however, is the most thoroughly studied hypoxia-responsive transcription factor (40). HIF-1, a member of the basic helix-loop-helix/PAS (PAS homology domain) protein family, binds to a core DNA sequence within hypoxia response elements. HIF-1 consists of HIF-1α and the arylhydrocarbon nuclear translocator (ARNT) (49). ARNT is constitutively expressed, whereas HIF-1α expression is increased under hypoxic conditions. HIF-1α stability is regulated posttranscriptionally by the ubiquitin-proteasome system (22, 36). Under normoxic conditions, HIF-1α binds to the von Hippel-Lindau tumor suppressor protein (pVHL) and is targeted for destruction by an E3 ubiquitin ligase complex (18, 20, 30). When oxygen partial pressure is low, HIF-1α does not bind pVHL and therefore is not degraded. As a result, HIF-1α accumulates and forms heterodimers with ARNT that translocate to the nucleus and upregulate transcription of HIF-1-responsive genes.

Although signaling through the HIF-1 pathway has been studied primarily in the context of cellular adaptation to hypoxia, accumulating data suggest that proinflammatory mediators also can upregulate HIF-1α protein expression and HIF-1 DNA-binding activity. Cytokines, such as IL-1β and TNF and/or IFN-γ, have been shown to increase HIF-1 DNA-binding activity in human hepatoma cells (15), human renal tubular epithelial cells (12), and transformed human intestinal epithelial cells (7). In addition, several in vitro studies have demonstrated that another proinflammatory mediator, nitric oxide (NO), can promote HIF-1 activation under normoxic conditions (25, 37).

When normoxic cells are exposed to an inflammatory milieu in vivo or in vitro, they often manifest phenotypic changes reminiscent of those that are observed when cells are subjected to hypoxia. For example, Bagby et al. (4) reported several years ago that the rate of glycolysis is increased in muscle tissue harvested from endotoxemic rats. More recently, L’Her and Sibert (26) showed that the rate of anaerobic glycolysis is significantly increased and the rate of mitochondrial

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respiration is significantly decreased in normoxic myocytes obtained from septic rats compared with sham-operated controls. Our laboratory recently reported that the cellular respiration rate is decreased, and the lactate production rate is increased when cultured normoxic Caco-2 cells are studied after being incubated for 24 h with cytomix, a mixture of the proinflammatory cytokines TNF, IL-1β, and IFN-γ (23). Along similar lines, Benigni et al. (6) reported that both TNF and macrophage inhibitory factor promote lactate production by cultured rat myoblasts, and Albina and coworkers (2, 29) showed that the rate of glucose flux through the glycolytic pathway is markedly increased in immunostimulated macrophages.

Because the HIF-1 pathway can be activated by proinflammatory mediators, we were prompted to hypothesize that HIF-1-mediated signaling is important for the development of a hypoxic phenotype when normoxic cells are exposed to an inflammatory environment. Remarkably little is known, however, about changes in the expression of key glycolytic enzymes in normoxic or hypoxic cells exposed to proinflammatory cytokines. Accordingly, in the present study, we investigated the effects of cytomix on the expression of the HIF-1-dependent glycolytic enzymes enolase-1 and aldolase A, as well as the HIF-1-dependent cytokine vascular endothelial growth factor (VEGF), under normoxic and hypoxic conditions in nontransformed small intestine-derived epithelial cells (IEC-6) from rats. We chose a nontransformed cell line, because constitutive upregulation of HIF-1α has been reported in different malignant tissues, including colon (45, 52).

**MATERIALS AND METHODS**

Cell culture and reagents. All reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Cell culture media and reagents were from BioWhittaker (Walkersville, MD). The IEC-6 cell line (CRL-1592), a nontransformed small intestine epithelial cell line from rats, was obtained from the American Type Culture Collection (Manassas, VA). Cells (passage 17–52) were grown and maintained as confluent monolayers on Biocoat collagen I-coated plates (Becton Dickinson, Franklin Lakes, NJ) in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD), 45 U/ml penicillin, 9 μg/ml streptomycin, and 2 mM L-glutamine. Cells were fed biweekly and kept in a humidified atmosphere of 5% CO₂ in air at 37°C.

Experimental conditions. Cell culture media were changed immediately before the beginning of an experiment and, in the case of all of the 48-h experiments, 24 h later. Cells were left untreated or exposed to a combination of proinflammatory cytokines (1,000 U/ml IFN-γ, 10 ng/ml TNF, and 1.0 ng/ml IL-1β; Endogen, Woburn, MA) called cytomix. After 24 h, the culture medium was changed, and fresh cytomix was added. Some cells were maintained under 21% O₂ (normoxia + cytokine; NC + NC); others were exposed to hypoxia (H; 1% O₂) for an additional 24 h (NC + HC). Some cells were exposed to cytomix only during the second 24 h and kept under 21% O₂ (NC) or 1% O₂ (HC). For hypoxic stimulation, dishes were placed in a humidified incubator with an atmosphere composed of 1% O₂, 5% CO₂, and 94% N₂ for 24 h. The oxygen level was maintained with a PROOX 100 oxygen-regulating system (Reming Instruments, Redfield, NY), which purges the chamber with N₂ gas and maintains the PO₂ at the set level. Cells not exposed to cytomix were maintained under 21% O₂ (N) or 1% O₂ (H).

**RNA isolation.** RNA was isolated from fresh cultures by using TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. RNA was resuspended in 50 μl of RNAsecure (Ambion, Austin, TX), incubated at 60°C for 10 min, and purified with RNeasy columns (Qiagen, Valencia, CA) according to the manufacturer’s protocol. To remove potential contamination with genomic DNA, the RNA was treated with DNase (DNA-free, Ambion, Austin, TX) according to the manufacturer’s protocol. The RNA concentration was determined by measuring the optical density (OD₂₆₀) with a spectrophotometer. OD₂₆₀/OD₂₈₀ ratios of >1.8 were obtained for all samples, indicating high purity. RNA was diluted with nulease-free water to 40 ng/μl and stored at −80°C. All solutions were made by using diethyl pyrocarbonate (DEPC)-treated water.

**Reverse transcription.** Reverse transcription (RT) was carried out in a 100-μl reaction volume containing 1× PCR buffer II (PE Biosystems, Foster City, CA), 125 units of M-MLV reverse transcriptase (Promega, Madison, WI), 200 ng of total RNA. RT was performed by incubating the samples at 25°C for 10 min, 48°C for 40 min, and 95°C for 5 min in a PerkinElmer GeneAmp PCR System 9700. “No RT” controls were carried out in all cases by using the same RT reaction mixture but substituting DEPC-H₂O for the RT. For all quantitative analyses, two RT reactions were carried out for each RNA sample by using 200 and 50 ng/100 μl RT reaction mixture. The “no RT” controls were carried out with 200 ng of RNA.

**Real-time quantitative PCR.** Quantitative real-time PCR is based on the detection of a fluorescent signal that increases linearly with accumulating amplification product during the PCR reaction. A dual-labeled fluorogenic oligonucleotide probe (TaqMan probe) consists of a short 20–25 base oligodeoxynucleotide, which anneals to the target sequence between the forward and reverse primers. The probe is labeled with two different fluorescent dyes, a reporter dye (6-carboxyfluorescein) and a quencher dye (6-carboxytetramethylrhodamine), which suppresses the reporter fluorescence activity by energy transfer within the intact probe. During the extension phase of PCR, the probe is cleaved by the endogenous 5′-nuclease activity of AmpliTaq Gold polymerase, which cleaves the reporter chromophor from the TaqMan probe, leading to an increase in the intensity of reporter fluorescence. The increase in fluorescence (ΔRn) signal is continuously measured and plotted vs. PCR cycle number, reflecting the amount of PCR amplification products. The ABI Prism 7700 detection system software calculates the ΔRn by subtracting the fluorescence signal of the baseline emission during cycles 3 to 6 from the fluorescence signal of the product at any given time. A threshold was set at the early log phase of product accumulation. The threshold cycle number value (CT) is the cycle number at which each sample’s amplification plot reaches this threshold.

Three HIF-1-responsive genes were studied: aldolase A, enolase-1, and VEGF. 18S ribosomal RNA served as a reference gene. PCR primers and fluorogenic probes (TaqMan) were designed by using Primer Express software (PE Biosystems); primer and probe sequences for 18S were kindly provided by Professor Tony Godfrey (University of Pittsburgh). All primer sequences are given in Table 1. Wherever possible,
primers and probes were designed to span introns in the genomic DNA, thereby minimizing the potential for confounding of the signal by contaminating genomic DNA. Probes were obtained from Integrated DNA Technologies (Coralville, IA). Primers were obtained from Life Technologies. Quantitative RT-PCR was performed for each sample with two RNA concentrations (50 and 200 nM), each in duplicate. PCR reactions were performed in 50-μl volumes consisting of 1× PCR buffer A (PE Biosystems), 3.5 mM MgCl₂, 300 μM each of dATP, dCTP, dGTP, and dTTP, 1.25 units AmpliTaq Gold (PE Biosystems), and 5 μl of the appropriate RT reaction product. The enolase-1, aldolase A, and VEGF amplification primers were present at 300 nM, and the TaqMan probe was present at 200 nM. The 18S amplification primers were each at 100 nM, and the TaqMan probe was at 100 nM. Amplification and detection were carried out by using an ABI 7700 detection system as follows: 1 cycle at 95°C for 12 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. At the end of the PCR, baseline and threshold values were set in the ABI 7700 Prism software. The calculated Cₜ values were analyzed in Microsoft Excel.

Calculation of relative expression. Relative mRNA expression of target genes was calculated with the comparative Cₜ method. The amount of target gene was normalized to the endogenous 18S control gene to control for quantity of RNA input. Difference in Cₜ values was calculated for each mRNA by taking the mean Cₜ of duplicate reactions and subtracting the mean Cₜ of duplicate reactions for reference RNA measured on an aliquot from the same RT reaction. CT(target gene) - CT(18S).

Assessment of HIF-1α mRNA expression by using semi-quantitative RT-PCR. Total RNA was treated with DNAfree (Ambion, Houston, TX) as instructed by the manufacturer by using 10 units of DNase I/10 μg RNA. The RNA was reverse transcribed in a 20 μl reaction volume containing 0.5 μg of oligo(dT)₁₅ (Promega, Madison, WI), 1 mM of each dNTP (Promega), 15 units avian myeloblastosis virus RT (Promega), and 1 U/μl of recombinant RNasin ribonuclease inhibitor (Promega) in 5 mM MgCl₂, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, and 0.1% Triton X-100. The mixture was incubated at 42°C for 25 min and then heated to 99°C for 5 min to terminate the reaction. A 20-μl PCR reaction mixture was assembled by using 2 μl of cDNA template, 10 units AdvanTag Plus DNA polymerase (Clontech, Palo Alto, CA), 1.5 mM MgCl₂, and 1.0 μM of each of primer in 1× AdvanTag Plus PCR buffer. The primer pairs for 18S ribosomal RNA were as follows: upper primer, 5′-GCC GGG GAG GTA GTG ACG AAA AAT-3′; and lower primer, 5′-GCC CCG CTC CCA AGA CCC ACG AAC TAC-3′. The HIF-1 primers were as follows: upper primer, 5′-TAC TGG GTT TCA TGA TTA TTA TGG-3′; and lower primer 5′-ACT TCA GGA ACC GCC GTG GAT TTA-3′. PCR was performed by heating the samples at 94°C for 2 min, followed by 94°C for 45 s, 64°C for 45 s, and 68°C for 90 s for 25 cycles (18S) or 30 cycles (HIF-1), and then 68°C for 5 min. Fifteen microliters of each PCR reaction product was electrophoresed on a 2% agarose gel.

HIF-1 immunoprecipitation and Western blotting. IEC-6 cells were grown in 10-cm dishes then lysed in 1 ml of RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1.0 mM sodium orthovanadate, and 1× mammalian protease inhibitor cocktail). The lysate was transferred to a 1.5-ml Microfuge tube and sonicated for 30 s on ice by using a 0.1-W Fisher Scientific sonic dismembrator fitted with a microtip on power setting 3. The lysate was incubated for 10 min on ice followed by centrifugation at 10,000 g for 15 min at 4°C. The supernatant was transferred to a new tube, and the total protein concentration was determined by using the Bio-Rad (Heracles, CA) protein reagent.

The whole protein lysate (100 μg) was mixed with 0.25 μg of nonimmune murine IgG and 2 μl of protein A/G agarose (resuspended volume; Santa Cruz Biotech, Santa Cruz, CA) and incubated at 4°C for 2 h. The beads were removed by centrifugation at 1,000 g for 5 min at 4°C, and the supernatant was combined with 26 μg anti-mouse HIF-1 monoclonal antibody (NOVUS Biologicals, Littleten, CO) and incubated at 4°C for 2 h. A 20-μl aliquot of suspended agrose A/G beads was added, and the tubes were incubated with rocking overnight at 4°C. The beads were washed five times with 1 ml of RIPA buffer. The final pellet was dissolved by boiling for 10 min in Laemmli buffer, consisting of the following reagents at the indicated final concentrations: 20% glycerol, 10% β-mercaptoethanol, 5% SDS, 0.2 M Tris-HCl (pH 6.8), and 0.04% bromophenol blue. The protein samples were centrifuged for 10 s, and the supernatants were electrophoresed at 100 mA for 40 min on 7.5% precast SDS-polyacrylamide gels (Bio-Rad).

Size-fractionated proteins were electroblotted onto a hydro-P polyvinylidene difluoride membrane (Amersham...
Pharamacia Biotech, Leicester, Denmark), and blocked with Blotto (1× TBS, 5% milk, 0.05% Tween 20, and 0.2% NaCl) for 60 min. The membrane was then incubated at room temperature for 1 h with anti-HIF-1 monoclonal antibody diluted 1:2,000 in PBST (1× PBS and 0.02% Tween 20). After being washed three times in 1× PBST, immunoblots were exposed at room temperature for 1 h to a 1:20,000 dilution of horseradish peroxidase-conjugated anti-mouse secondary antibody (Sigma-Aldrich). After three washes in PBST and two washes in PBS, the membrane was impregnated with the enhanced chemiluminescence substrate (Amersham, Leicester, England) that was used to expose X-ray film according to the manufacturer's instructions. The autoradiographs were captured by using a Hewlett Packard (Palo Alto, CA) ScanJet 6300s.

Measurement of nitrate/nitrite and other biochemical parameters. Supernatants from cultures of IEC-6 cells were stored at −20°C. Nitrate/nitrite (NOx) measurements were performed by using a colorimetric assay (Bioxytech NO assay; Oxis International, Portland, OR) according to the manufacturer's instructions. Briefly, nitrate was reduced to nitrite by an enzyme reduction before quantification of nitrite by using Griess reagent. Glucose and lactate concentrations in culture supernatants were measured by using an auto-analyzer (model ABL-725; Radiometer, Copenhagen, Denmark). Glucose consumption was determined by subtracting the final glucose concentration from the concentration of glucose at the beginning of the incubation period and dividing by 24 h. Lactate production was determined by subtracting the starting lactate concentration from the concentration of lactate at the end of the incubation period and dividing by 24 h.

Nuclear extract preparation. The day before each experiment, cells were plated at a density of 0.8–1.0 × 106 cells per well in six-well Biocoat tissue culture plates (Becton Dickinson). Nuclear extracts were prepared by using the following modification of a previously published method (11, 43). All steps were performed on ice, and centrifugation steps were performed in an Eppendorf microcentrifuge (model 5417R; Brinkmann, Pittsburgh, PA) at 4°C. Cells were removed from the incubator and immediately placed on ice, washed once with 1 ml of PBS, and then harvested in 1 ml of PBS by using a rubber policeman. The cells were transferred to a 1.5-ml tube and centrifuged at 14,000 g for 10 s. The cell pellet was resuspended in 400 μl of buffer I (in mM) 10 Tris-HCl (pH 7.8), 10 KCl, 1.5 MgCl2, 1.0 sodium orthovanadate, 1.0 di-thiothreitol, plus 0.3 M sucrose, 500 μM phenylmethylsulfonyl fluoride, and 1× mammalian protease inhibitor cocktail (catalog number P-8340; Sigma-Aldrich) and incubated for 15 min. We then added (octylphenoxy)polyethoxyethyl (IGEPAL CA-620; NP-40) to 0.5% (25 μl of a 10% vol/vol stock) while the tube was vortexed at half speed for 10 s. Nuclei were isolated by centrifugation at 500 g for 2 min. The supernatant was aspirated, and the nuclear pellet was gently resuspended in 60 μl of buffer II [10 mM Tris-HCl (pH 7.8), 420 mM KCl, 1.5 mM MgCl2, 20% glycerol]. After a 15-min incubation period, the nuclear extracts were cleared by centrifugation at 14,000 g for 10 min. The supernatant was transferred to a new tube, and the protein concentration was determined by using a commercially available Bradford assay (Bio-Rad protein assay, Hercules, CA). Nuclear extracts were then refrozen and stored at −70°C.

EMSA. Oligonucleotides were synthesized by Life Technologies. The sequence of the double-stranded oligonucleotide from the human erythropoietin 3' enhancer containing the HIF-1 binding sequence was as follows: sense 5′-AGC TGG CCC TAC GTG CTG TCT CAG-3′ and antisense 5′-AAT TTC GAG ACA GCA CGT AGG GCA-3′ (49). Boldface nucleotides indicate the consensus HIF-1 binding site. The double-stranded wild-type oligonucleotide was end-labeled with γ-[32P]ATP (New England Nuclear, Boston, MA) by using T4 polynucleotide kinase (Promega, Madison, WI). Nuclear proteins (~5 μg/reaction) were incubated with radiolabeled probe in bandshift buffer (in mM) 10 Tris-HCl (pH 7.5), 50 KCl, 50 NaCl, 1.0 MgCl2, 1.0 EDTA, and 5% glycerol in the presence of 0.5 ng of calf thymus DNA. For competition reactions, a 3- to 100-fold molar excess of unlabeled wild-type HIF-1 or NF-κB (Promega) specific oligonucleotide was added simultaneously with the labeled HIF-1 oligonucleotide probe. Supershift assays were performed by incubating nuclear extracts with 2 μl of anti-HIF-1 monoclonal antibody (Novus Biologicals, Littleton, CO) for 20 min before the addition of the radiolabeled oligonucleotide. An equivalent amount of mouse anti-human CD25 monoclonal antibody (Caltag, Burlingame, CA) was used as an isotype control. Reactions were size-fractionated on 4% nondenaturing polyacrylamide gels at 120 V in 0.25% Tris-borate-EDTA buffer for 1 h at room temperature. The gels were dried and used to expose Biomax films (Kodak, Rochester, NY) at −70°C overnight by using an intensifying screen. Figures shown are representative of experiments repeated on at least five separate occasions.

Data analysis. In one experiment, VEGF mRNA expression was sevenfold greater than the mean value for this parameter measured under the same conditions in numerous other experiments. Accordingly, results from this single experiment were not included in the analyses of the results. Data are expressed as means ± SE. The quantitative real-time RT-PCR assays were carried out on multiple days by using samples collected over a period of many months from multiple cell cultures growing on multiwell plates. In each individual experiment, we always included control wells (i.e., cells grown under normoxia in the absence of cytokines) among the wells used to study the experimental conditions. Accordingly, we used paired-sample t-tests to evaluate contrasts between the various experimental conditions and the control condition. The analyses among the other experimental conditions were carried out by using ANOVA followed by Fisher’s protected least-squares difference test. Because the variances among the data for the various experimental groups tended to be proportional to the means, the results were logarithmically transformed before carrying out the statistical analyses to detect differences among groups. Glucose consumption and lactate production rate data were analyzed by using ANOVA followed by Dunnett’s test. NOx concentrations in culture supernatants were logarithmically transformed, resulting in a normal distribution of variances, before ANOVA followed by Dunnett’s test. The null hypothesis was rejected for P < 0.05.

RESULTS

Glucose consumption and lactate production by cytokine-stimulated IEC-6 cells. IEC-6 monolayers were incubated under the following conditions: normoxia for 24 h in control medium (N); normoxia in the presence of cytomix for 24 h (NC); normoxia with cytomix for 24 h followed by changing the media and adding fresh cytomix and incubating for an additional 24 h under normoxia (NC + NC); hypoxia (1% O2) for 24 h (H); hypoxia with cytomix for 24 h (HC), or normoxia for 24 h in the presence of hypoxia followed by changing the media and then hypoxia for 24 h in the presence of...
cytomix was added. The cells were then maintained under 21% O₂ medium was changed after 24 h of exposure to cytomix and fresh the NC twice during successive 24-h periods of incubation (i.e., stimulated with fresh cytomix-containing medium consumption more than doubled when IEC-6 cells were exposed to cytomix. Although glucose consumption increased when the cells were incubated under hypoxic conditions or under hypoxia (1% O₂). Other cells were challenged with cytomix for 24 h (H), hypoxia for 24 h in the presence of cytomix (HC), or normoxia for 24 h in the presence of cytomix followed by changing the media and then hypoxia for 24 h in the presence of fresh cytomix (NC + HC). As shown in Fig. 2, the concentration of NO₃/NO₂ in culture supernatants was significantly greater than the control value (N) after exposure to cytokines (i.e., NC or NC + NC). Similarly, levels of NO₃/NO₂ were greater than the control condition (H) when hypoxic cells were exposed to cytomix (HC or NC + HC). Treatment of normoxic immunostimulated cells with an isoform selective iNOS inhibitor, 20 μM L-NAME (1-iminoethyl)lysine hydrochloride (L-NIL), significantly blunted the release of NO (NCNC + L-NIL in Fig. 2).

Effect of proinflammatory cytokines on HIF-1 DNA-binding activity under normoxic and hypoxic conditions. We used EMSA to assess DNA-binding by HIF-1. The identity of the HIF-1-DNA complex was confirmed by performing competition studies by using specific (HIF-1) and nonspecific (NF-κB) unlabeled oligonucleotide probes. As expected, HIF-1 DNA binding was not detected when cells were cultured in normal medium fresh cytomix (NC + HC). As shown in Fig. 1, glucose consumption more than doubled when IEC-6 cells were stimulated with fresh cytomix-containing medium twice during successive 24-h periods of incubation (i.e., the NC + NC condition). Glucose consumption also increased when the cells were incubated under hypoxic conditions, irrespective of whether the cells were also exposed to cytomix. Although glucose consumption increased substantially when cells were stimulated with cytomix or incubated under 1% O₂, the glucose concentration in samples of media at the end of the final incubation period was never <6.5 ± 0.8 mM.

NO production by cytomix-stimulated IEC-6 cells. Our laboratory previously reported that incubation with cytomix increases inducible NO synthase (iNOS) expression and NO production by cultured Caco-2 (transformed human enterocytic) cells (9). Potoka et al. (35) reported that cytomix increases iNOS expression and NO- production by cultured (nontransformed) IEC-6 cells. To confirm and extend these findings, IEC-6 monolayers were incubated under the following conditions: normoxia for 24 h in control medium (N), normoxia in the presence of cytomix for 24 h (NC), normoxia with cytomix for 24 h followed by changing the media and adding fresh cytomix and incubating for an additional 24 h under normoxia (NC + NC), hypoxia (1% O₂) for 24 h (H), hypoxia for 24 h in the presence of cytomix (HC), or normoxia for 24 h in the presence of cytomix followed by changing the media and then hypoxia for 24 h in the presence of fresh cytomix (NC + HC). As shown in Fig. 2, the concentration of NO₃/NO₂ in culture supernatants was significantly greater than the control value (N) after exposure to cytokines (i.e., NC or NC + NC). Similarly, levels of NO₃/NO₂ were greater than the control condition (H) when hypoxic cells were exposed to cytomix (HC or NC + HC). Treatment of normoxic immunostimulated cells with an isoform selective iNOS inhibitor, 20 μM L-NAME (1-iminoethyl)lysine hydrochloride (L-NIL), significantly blunted the release of NO (NCNC + L-NIL in Fig. 2).

Fig. 1. Glucose consumption (A) and lactate production (B) by normoxic or hypoxic intestine-derived epithelial cells (IEC-6) incubated in the presence or absence of cytomix. Glucose and lactate concentrations in media were measured before and after the final 24 h of incubation to calculate glucose consumption and lactate production rates. Several different conditions were investigated. Some cells were incubated in the absence of added cytokines under normoxic (N) conditions or under hypoxia (H; 1% O₂). Other cells were challenged with cytomix under 21% O₂ (NC) or 1% O₂ (HC). In some cases, the medium was changed after 24 h of exposure to cytomix and fresh cytomix was added. The cells were then maintained under 21% O₂ (NC + NC) or at 1% O₂ (NC + HC) for an additional 24 h. *P < 0.05 vs. N.

Fig. 2. Nitric oxide (NO) production by IEC-6 cells. In all cases, nitrate/nitrite (NOₓ) concentrations were determined after cells were incubated with fresh medium for 24 h. Thus the measured NOₓ concentrations are indicative of total NO production by the cells during their final 24 h in culture. Several different conditions were investigated. The conditions labeled N, NC, NC + NC, H, HC, and NC + HC are the same as those described in the legend for Fig. 1. In addition, some cells were treated with cytomix for 24 h under normoxic conditions. The original medium was discarded, and the cells were incubated under normoxic conditions for an additional 24 h with fresh medium containing cytomix and 20 μM L-NAME (1-iminoethyl)lysine hydrochloride (L-NIL; NCNC + L-NIL). Results are expressed as means ± SE for at least 4 determinations. *P < 0.05 vs. N.
Incubation under 1% O₂ for 24 h (H) induced the formation of an HIF-1 DNA complex. HIF-1 DNA binding also was induced by incubation with cytomix for 24 h under normoxic conditions (NC) or by incubating with cytomix under normoxic conditions for 24 h followed by changing the media and adding fresh cytomix and incubating for 24 h more under normoxic conditions (NC/H). Furthermore, cytomix exposure did not inhibit formation of an HIF-1 DNA complex in response to hypoxia. HIF-1 DNA binding was most evident when IEC-6 cells were pretreated with cytomix for 24 h under 21% O₂ and then after changing the culture media reexposed to cytomix for a further 24 h under 1% O₂ (NC/H).

Effect of proinflammatory cytokines on HIF-1α gene expression under normoxic and hypoxic conditions. As expected, HIF-1α protein expression increased in IEC-6 cells after incubation under 1% O₂ for 24 h (H) (Fig. 4A). HIF-1α protein levels also were increased after incubation with cytomix for 48 h under normoxic conditions (NC + NC) or under normoxic conditions for 24 h followed by hypoxic conditions for 24 h (NC + HC). In contrast to these observed changes in HIF-1α protein expression, steady-state levels of HIF-1α mRNA were not appreciably different for cells incubated under these four conditions (Fig. 4B).

Effect of cytomix on expression of aldolase A, enolase-1, and VEGF mRNA. When IEC-6 cells were incubated with cytomix for 24 h under 21% O₂ (NC in Fig. 5), steady-state mRNA levels for three HIF-1-responsive genes, aldolase A, enolase-1, and VEGF, all increased significantly relative to the values measured in normoxic control cells (N). When cells were stimulated with cytomix for 24 h under 21% O₂ and then incubated for 24 h more with fresh medium containing cytomix under the same concentration of O₂ (NC + NC), there was further upregulation of the steady-state levels of the mRNAs encoding all three proteins relative to cells incubated with cytomix under normoxic conditions for just 24 h (P = 0.026 vs. NC for aldolase A, P = 0.024 vs. NC for enolase-1, P = 0.11 vs. NC for VEGF).

Effect of inhibiting NO• production with l-NIL on the expression of aldolase A, enolase-1, and VEGF mRNA in cytomix-stimulated IEC-6 cells. Because NO• can promote HIF-1 activation under normoxic conditions (25, 37), and cytomix can induce iNOS expression and NO• production in IEC-6 cells (see Fig. 2), we sought to
determine whether increased NO production was responsible for cytomix-induced upregulation of aldolase A, enolase-1, and VEGF gene expression in this cell line. Accordingly, some monolayers were exposed to cytomix for 24 h under normoxic conditions, and after being changed, the media were reexposed to cytomix for another 24 h (NC + NC). Other monolayers were treated identically, except during the second 24-h incubation period with cytomix the cells were also treated with 20 μM L-NIL. Although treatment with L-NIL markedly decreased cytomix-induced NO production (Fig. 2), treatment with this agent had a relatively small and inconsistent effect on the upregulation

Fig. 6. Steady-state aldolase A (A), enolase-1 (B), and VEGF (C) mRNA in IEC-6 cells exposed to cytomix in the presence or absence of pharmacological inducible NO synthase (iNOS) inhibition. Untreated control cells were maintained under 21% O2 (N). Some cells were incubated with cytomix for 24 h (NC). Other cells were exposed to cytomix for 24 h, at which time the medium was changed and fresh cytomix was added. The cells were then incubated under 21% O2 for an additional 24 h (NC + NC). Other cells were exposed to conditioned media for 24 h under 21% O2 (CM). The conditioned media was obtained after the second 24-h incubation for cells treated like those in the NC + NC group. RNA was isolated and subjected to real-time RT-PCR. Results are expressed as mean ± SE for at least 10 replicates. *P < 0.05 vs. N; **P < 0.05 vs. NC.
of steady-state aldolase A, enolase-1, and VEGF mRNA levels after exposure to the cytokine cocktail (Fig. 6). Probability values for the contrasts between the NC + NC condition and the NCNC + L-NIL condition ranged from 0.12 (aldolase A) to 0.63 (enolase-1).

We wondered whether different results would be obtained if iNOS activity was inhibited during the entire 48 h period of cytomix exposure. Therefore, we carried out two pilot experiments in which some monolayers were exposed to cytomix for 24 h under normoxic conditions and after changing the media, were reexposed to cytomix for another 24 h (NC + NC). Other monolayers were treated identically, except 20 μM L-NIL was also present during both incubation periods. By using this design, we observed no differences in the expression of aldolase A, enolase-1, or VEGF mRNA among cells cultured in the presence or absence of the iNOS inhibitor (data not shown).

Effect of conditioned media from cytomix-stimulated cells on expression of aldolase A, enolase-1, and VEGF mRNA. Results presented in Fig. 5 indicate that cytomix increased the expression of several HIF-1-responsive genes in normoxic IEC-6 cells. Moreover, these results suggest that this effect was more pronounced after 48 h of exposure to cytomix (NC + NC) than after 24 h of exposure to the cocktail of proinflammatory cytokines (NC). Although one explanation for the difference between the results obtained for the NC + NC condition and for the NC condition might simply be related to the kinetics of the cytokine-induced response in IEC-6 cells; another possible explanation is that cytomix-stimulated cells release one or more factors into the medium that act in an autocrine and/or paracrine fashion to promote upregulation of HIF-1-responsive genes. To test this hypothesis, conditioned media were obtained from IEC-6 cells incubated under the NC + NC condition; that is, the cells were exposed to cytomix for 24 h under 21% O2, and the cells were then incubated under normoxia for a second 24-h period in fresh medium containing cytomix. The conditioned medium was harvested after the second 24-h incubation period. When naive IEC-6 cells were incubated with the conditioned medium for 24 h under 21% O2 (CM in Fig. 5), they expressed significantly higher steady-state aldolase A, enolase-1, and VEGF mRNA levels than did normoxic control cells (N in Fig. 5). Of course, conditioned medium contained residual cytokine. Accordingly, it is important to note that steady-state aldolase A, enolase-1, and VEGF mRNA levels were significantly greater in cells exposed to conditioned medium than in cells incubated with fresh cytomix for the same duration (24 h) (NC). This latter observation supports the view that conditioned medium contained a factor or factors in addition to those comprising cytomix (i.e., IL-1β, TNF, and IFN-γ) that promoted increased expression under normoxia of several HIF-1-responsive genes.

Effect of combining hypoxia plus cytomix stimulation on aldolase A, enolase-1, and VEGF mRNA expression. Steady-state levels of aldolase A, enolase-1, and VEGF mRNA increased significantly when IEC-6 cells were incubated under hypoxic conditions (H vs. N in Fig. 7). When the hypoxic cells were also stimulated with cytomix, whether just during the period of incubation under 1% O2 (HC) or before and during the period of incubation under 1% O2 (NC + HC), there was no change in the expression of aldolase A and enolase-1 mRNA relative to that observed for cells challenged with hypoxia alone (Fig. 7, A and B). In contrast, VEGF mRNA expression, which was increased by hypoxia alone, was increased still further by prior and concomitant exposure of cells to cytomix (Fig. 7C).

DISCUSSION

HIF-1, as suggested by its name, was originally identified as a transactivating factor that regulates the transcription of a number of genes in response to diminished ambient Po2 (50). Under hypoxic conditions, active HIF-1 heterodimers accumulate in the nucleus where they bind to cis-acting elements in the regulatory regions of HIF-1-responsive genes, enhancing the rate of transcription of these genes. Although the importance of HIF-1-mediated signaling in the adaptive response of cells to hypoxia is firmly established, it is becoming increasingly apparent that certain conditions can lead to HIF-1 DNA binding (12, 14, 15, 39, 46) and, in some cases, expression of HIF-1-responsive genes (5, 12, 13, 21, 27), even in the absence of cellular hypoxia. For example, a number of studies have documented HIF-1 DNA binding in various cell types after incubation with certain proinflammatory cytokines (notably, TNF and IL-1β) (12, 14, 15, 39, 46), Fewer studies, however, have demonstrated increased expression of hypoxia-responsive genes. Thus El Awad et al. (12) showed that incubating human renal epithelial cells with IL-1β produces a small (~50%) but significant increase in VEGF protein expression. However, these investigators found that VEGF mRNA expression did not change significantly after exposure of renal epithelial cells to IL-1β. Other investigators have shown that IL-1β increases VEGF expression in smooth muscle cells and fibroblasts (5, 21, 27), TNF increases VEGF expression in keratinocytes (13), and IL-6 and IL-1β induce VEGF expression in A-431 epidermoid carcinoma cells (10). In contrast to these findings, Hellwig-Burgel et al. (15) reported that incubating HepG2 (human hepatoblastoma) cells with either IL-1β or TNF decreased production of erythropoietin and had no effect on the production of VEGF protein or the steady-state level of VEGF mRNA. Expression of genes encoding both of these proteins is regulated by HIF-1.

Aldolase A and enolase-1 are important glycolytic enzymes. Aldolase catalyzes the conversion of fructose-1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Enolase catalyzes the penultimate step in glycolysis, which is the dehydration of 2-phosphoglycerate to form phosphoenolpyruvate. The promoter regions for the genes that encode these enzymes include hypoxia-responsive elements that contain functionally active binding sites for HIF-1
of HIF-1α mRNA, a finding entirely consistent with the accepted notion that posttranslational events are largely responsible for the regulation of HIF-1-dependent signaling in response to changes in oxygen availability (22, 36). When normoxic IEC-6 cells were incubated with cytomix, a cocktail of cytokines containing TNF, IL-1β, and IFN-γ, we again observed increased cellular levels of HIF-1α protein and HIF-1 DNA binding, and, as was true for hypoxia, these changes were not accompanied by increased steady-state levels of HIF-1α mRNA. Thus as in the case of the HIF-1 response to hypoxia, the increase in HIF-1 DNA binding induced by cytomix appears to occur as a result of posttranslational events. When normoxic cells were incubated with cytomix, we also observed increased steady-state mRNA levels for three HIF-1-responsive genes: aldolase A, enolase-1, and VEGF. Therefore, our findings are consistent with observations made by other investigators, who examined the effects of proinflammatory cytokines on HIF-1 DNA binding and/or transcriptional activation in other cell types (12, 14, 15, 39, 46). However, our observations are also novel, because, to our knowledge, we are the first to document that proinflammatory cytokines induce transcriptional activation of the glycolytic HIF-1-responsive genes, aldolase A and enolase-1. These observations are important for two reasons. First, our findings may account, at least in part, for the development of a hypoxic phenotype, when normoxic cells or tissues are exposed to an inflammatory milieu (2, 6, 23, 26, 29). Second, unlike many previous studies that focused on changes in expression of VEGF, a gene that is also regulated by transcription factors other than HIF-1, we studied three different HIF-1-regulated genes. Because the expression of all three genes moved in parallel in all of our studies, we believe that it is likely that the cytomix-induced alterations in the expression of these genes was mediated through activation of the HIF-1 signaling pathway.

Our data are insufficient to exclude the possibility that although exposure to cytomix promoted HIF-1 DNA binding and increased expression of three HIF-1-responsive genes, these cellular events were not causally related. In other words, it is possible that factors other than (or in addition to) HIF-1-dependent transcriptional activation may be important in causing upregulation of aldolase A, enolase-1, and VEGF expression in cytomix-stimulated IEC-6 cells. An elegant way to directly test this hypothesis would be to deplete HIF-1α by using an antisense approach as described recently by Caniggia et al. (8). We attempted to carry out similar studies. We prepared the same phosphothioate oligonucleotide described by Caniggia et al. (8), and successfully introduced it into IEC-6 cells. Unfortunately, we did not observe decreased expression of HIF-1 as revealed by EMSA. Thus we were unable to unambiguously confirm that increased expression of aldolase A, enolase-1, and VEGF in cytomix-stimulated IEC-6 cells is mediated via signaling through the HIF-1 pathway.
Exposure of normoxic cells to compounds that release NO into solution, such as S-nitroso-N-acetylpenicillamine, NOC5, and S-nitrosoglutathione, has been shown to promote HIF-1 DNA binding (24, 33, 38, 39) and enhanced transcription of HIF-1 responsive genes (24). Furthermore, cytomix was previously shown to induce iNOS expression and NO bioynthesis by cultured enterocytes (9), an observation confirmed in the present series of experiments. Accordingly, we hypothesized that increased production of NO is an intermediate step in the process leading to induction of aldolase A, enolase-1, and VEGF expression when IEC-6 cells are incubated with cytomix. To test this hypothesis, we incubated the cells with cytomix in the absence or presence of L-NIL, a potent isoform selective iNOS inhibitor (32). Although we confirmed that L-NIL blocked NO production by cytomix-stimulated IEC-6 cells, we also showed that this agent had minimal effects on the induction of aldolase A, enolase-1, and VEGF mRNA expression after incubation with cytomix. These findings, which are consistent with recently reported observations from another laboratory (3), suggest that one or more NO-independent pathways are responsible for induction of these genes when normoxic IEC-6 cells are exposed to cytomix. One such pathway is suggested by findings obtained by Haddad and Land (14), who showed that the oxidant species, hydrogen peroxide and hydroxyl radical, are involved in TNF-dependent activation of HIF-1 in normoxic cells. Another potential pathway is suggested by findings reported by Sandau et al. (39). These investigators showed that wortmannin blocked HIF-1α accumulation in TNF-stimulated normoxic LLC-PK1 (transformed renal) epithelial cells, suggesting that the phosphatidylinositol-3-kinase (PI3-kinase) pathway is involved in this phenomenon (38). This view is strengthened by studies showing that TNF-induced HIF-1α accumulation is blocked when cells were transfected with a plasmid encoding a dominant negative form of the p85 regulatory subunit of PI3-kinase (39).

The effect of NO on HIF-1 DNA binding and HIF-1-mediated transcriptional activation also has been studied in a hypoxic milieu. Various NO donors have been shown to inhibit HIF-1 DNA-binding activity in response to hypoxia or incubation with CoCl2 (17, 37, 44), the latter condition being an hypoxia-independent perturbation that stabilizes HIF-1α and promotes HIF-1 binding to the hypoxia-responsive elements in target genes. Furthermore, in some studies, incubating hypoxic cells with an NO donor was shown to attenuate induction of either a reporter construct (44, 51) or an HIF-responsive reporter gene (17, 28) in response to hypoxia. Because cytomix-stimulated cells produce NO, we hypothesized that incubation of hypoxic IEC-6 cells with cytomix would blunt the DNA binding of HIF-1 and the upregulation of several hypoxia-responsive genes normally observed when the cells are incubated under an atmosphere containing 1% O2. Contrary to our expectations, however, we observed no diminution in HIF-1 DNA binding when cells were incubated with cytomix for 24 h under normoxic conditions and were then incubated for 24 h more with cytomix under hypoxic conditions. Indeed, if there was any change at all, it was in the direction of a further increase in HIF-1 DNA binding relative to that observed with either hypoxia alone or cytomix alone. One possible explanation for our observations is suggested by the data shown in Fig. 2. Hypoxia appeared to decrease the production of NO by immunostimulated IEC-6 cells. This finding, which is consistent with previously reported observations from studies that used other cell types (16, 31), suggests the possibility that the amount of NO produced during hypoxia was simply inadequate to overcome the effects of hypoxia and/or cytomix exposure that promotes modulation of gene expression through the HIF-1 pathway. Although this idea is plausible, previously published data regarding the concentration of NO that is necessary to inhibit HIF-1 activity in response to hypoxia are inconsistent; in some studies (25), inhibition of HIF-1 activity was detected only when high concentrations of NO donors were employed, but in other studies (37), the same effect was observed only at low concentrations of NO donors. In any event, it is important to point out that previous studies of the effect of NO on hypoxia-induced HIF-1-dependent gene expression were carried out by using exogenous sources of NO. To our knowledge, our study is the first to investigate the effect of endogenously produced NO on HIF-1-dependent gene expression in hypoxia. The effect of endogenously derived NO on HIF-1 activity in hypoxia might differ from that produced by exogenous NO, possibly because of subtle differences in the actual molecular species involved (e.g., nitroxy equivalents vs. nitrosonium equivalents) (33).

Cytomix treatment for 24 h under normoxic conditions resulted in a statistically significant, but still relatively minor, increase of HIF-1 target gene expression. However, when cells were treated with two 24-h applications of cytomix, aldolase A and enolase-1 mRNA levels were significantly higher than when the cells were exposed to cytomix for only 24 h. A similar effect was also observed for VEGF mRNA expression, although in this instance the difference between a single 24-h treatment and two 24-h-long treatments with cytomix did not quite achieve statistical significance (P = 0.11). As noted above, Hellwig-Burghel et al. (15) found that incubating HepG2 cells with either IL-1β or TNF had no effect on steady-state levels of VEGF mRNA. In view of our findings showing that prolonged exposure to cytomix is necessary to observed marked upregulation of VEGF gene expression, the failure of Hellwig-Burghel et al. (15) to observe this effect was probably due to the short period of incubation (4 h) used in their studies.

Although the greater degree of gene induction after prolonged incubation with cytomix might simply reflect the kinetics of this transcriptional activation pathway, our data suggest that another mechanism might also be important. Specifically, we found that conditioned medium obtained from cytomix-stimulated IEC-6 cells was a potent inducer of aldolase A, enolase...
lase-1, and VEGF mRNA expression in naive cells. Because the conditioned medium may have contained one or more of the cytokines present in cytomix, we carried out control experiments in which cells were exposed to cytomix for the same period of time (24 h). Conditioned medium was significantly more potent than cytomix in these assays, suggesting that immunostimulated IEC-6 cells release one or more factors that can initiate transcription of HIF-1-responsive genes. Further studies will be necessary to elucidate the identity of the factor(s) present in conditioned medium.

Two considerations prompted us to use a combination of three proinflammatory cytokines (TNF, IL-1β, and IFN-γ) rather than just a single cytokine, such as TNF or IL-1β, for the studies presented here. It is likely that multiple cytokines are involved when a systemic inflammatory process, such as occurs in sepsis, or a more localized form of inflammation, such as the case in Crohn’s disease or ulcerative colitis, affects gut mucosal function (see, for example, Ref. 47 for a review of intestinal epithelial hyperpermeability associated with inflammatory conditions). Second, as noted above, we sought to determine whether endogenous NO production contributes to cytokine-induced modulation of expression of the glycolytic enzymes aldolase A and enolase-1. Although incubating immortalized enterocyte-like cells with IFN-γ alone is sufficient to induce iNOS expression and increased NO production (9, 48), the effect is considerably more robust when the cells are exposed to IFN-γ plus TNF and IL-1β (9).

The overarching rationale for the type of studies described herein was to take advantage of the relative simplicity of an in vitro system to model clinical situations in which the intestinal epithelium is exposed to an inflammatory milieu under normoxic or hypoxic conditions. In studies comparing the effects of hypoxia alone with hypoxia plus cytomix, we observed no differences whatsoever on the expression of aldolase A and enolase-1. In contrast, we observed that VEGF mRNA expression is increased in the HC condition relative to the H condition by ~50% (P = 0.15) and by ~100% in the NC + HC condition (P = 0.047). These findings support the view that the modulation of VEGF expression by the combination of hypoxia plus cytomix is different than the regulation of the other two genes. The data presented herein are insufficient to resolve the molecular mechanism(s) that form the basis for this difference.

In summary, we showed herein that proinflammatory cytokines induce the expression of several HIF-1-dependent genes in normoxic IEC-6 cells. Two of these genes, aldolase A and enolase-1, encode important enzymes in the glycolytic pathway. Thus our findings might explain, at least in part, why cells manifest an hypoxic phenotype when they are subjected to an inflammatory milieu.

This study was supported by Deutsche Forschungsgemeinschaft Grant SCHA915/1–1 and National Institute of General Medical Sciences Grants GM-58484 and GM-37631.

REFERENCES


Cytokines and HIF-1


