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Depletion of Intracellular GTP Results in Nuclear Factor-κB Activation and Intercellular Adhesion Molecule-1 Expression in Human Endothelial Cells

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ABSTRACT

The expression of the intercellular adhesion molecule 1 (ICAM-1) on the surface of endothelial cells plays an important role in immune-mediated processes. The induction by the proinflammatory cytokine interleukin (IL)-1β is regulated by nuclear transcription factor κB (NF-κB). We studied the effect of an inosine-5′-monophosphate dehydrogenase (IMPDH) inhibitor, mycophenolic acid (MPA), on constitutive and IL-1β–induced expression of ICAM-1 in human umbilical vein endothelial cells (HUVECs). Unexpectedly, pretreatment with MPA enhanced the constitutive expression and potentiated the induction of ICAM-1 by IL-1β, as detected by flow cytometry. Northern blot analysis revealed an increase in ICAM-1 mRNA levels in cells treated with MPA. This was associated with an increase in phosphorylation of IκB-α (an inhibitor of NF-κB), nuclear translocation of the NF-κB subunits p50 and p65 and their binding to DNA as detected by Western blotting, confocal microscopy, and electrophoretic mobility shift assay. The upregulation of ICAM-1 by MPA was prevented by high doses (100 μM) of guanine or guanosine but not by physiological doses (0.1 μM), indicating that guanylates are involved in endothelial responses to IL-1β. Cultivation of HUVECs in the absence of guanine enhanced further ICAM-1 expression during IMPDH inhibition. These results demonstrate that cytokine-mediated endothelial ICAM-1 expression can be modulated by IMPDH inhibition. We believe this represents a novel interaction between endothelial guanylate metabolism, NF-κB activation, and adhesion molecule expression.

The expression of adhesion molecules is critically involved in the initiation of rejection after solid organ transplantation. Most of the clinical pathological findings during rejection can be attributed to abnormalities in vascular endothelial activation or dysfunction. It was shown that the release of soluble intercellular adhesion molecule 1 (ICAM-1) starts 6 days before biopsy-proven cardiac allograft rejection; peak concentrations were measured 3 days before rejection (Weigel et al., 2000). The activation of nuclear transcription factor κB (NF-κB) is a potential mechanism for vascular endothelial activation during rejection. The expression of ICAM-1, which plays a crucial role in the recruitment of leukocytes during rejection, is also regulated by NF-κB. In unstimulated cells, NF-κB predominantly exists as a heterodimer, composed of p50 and p65 subunits, that resides in the cytoplasm associated with several inhibitory molecules called IκBs, whose major isoforms are IκB-α and IκB-β (Thompson et al., 1995; Verma et al., 1995; Wulczyn et al., 1996). NF-κB activity can be induced in most cell types upon exposure to stimuli, including cytokines (interleukin-1, tumor necrosis factor-α), endotoxin, and oxidative stress.

In response to interleukin (IL)-1β, tumor necrosis factor receptor-associated factor 6 is recruited to the intracellular domain of the IL-1 receptor, which subsequently interacts with NF-κB–inducing kinase. This leads to IκB-α phosphorylation at serine 32 and 36 by activation of a kinase complex containing IκK-α and IκK-β, which leads to polyubiquitination at lysines 21 and 22 and then degradation by a proteolytic complex (Chen et al., 1995b, 1996; Read et al., 1995). The free NF-κB is then able to translocate to the nucleus and induce transcription of genes that bear 10-base pair recognition sequences (κB sites) found in the 5′-flanking regions (Voraberger et al., 1991; Ledebur and Parks, 1995). Importantly, NF-κB not only leads to transcriptional activation but

G.W. and P.B. contributed equally to first authorship.

ABBREVIATIONS: ICAM-1, intercellular adhesion molecule 1; FCS, fetal calf serum; MPA, mycophenolic acid; IL, interleukin; NF-κB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate; EMSA, electrophoretic mobility shift assay; IMPDH, inosine-5′-monophosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; IκB, inhibitor of NF-κB; pIκB, phosphorylated IκB; TPCK, N^{α}-tosylphenylalanyl-chloromethylketone; MG-132, carbobenzoxyl-leucinyl-leucinyl-leucinal-*H*; PMSF, phenylmethylsulfonylfluoride; DTT, dithiothreitol; PBS, phosphate-buffered saline; SSC, standard saline citrate; pcv, packed cell volume(s); RT, room temperature; TBS, Tris-buffered saline.

also controls the expression of its own inhibitor by inducing the IκB-α gene, leading to replenishment of IκB-α protein, which then complexes remaining cytoplasmic NF-κB and thus down-regulates the activation process (Müller et al., 1993; Read et al., 1994; Collins et al., 1995; Baeuerle and Baltimore, 1996).

Previous investigations provided evidence that inhibition of inosine-5′-monophosphate dehydrogenase (IMPDH) depletes intracellular GTP not only in leukocytes but also in endothelial cells and leads to an increased content of UTP. Treatment of endothelial cells with the IMPDH inhibitor mycophenolic acid (MPA) led to a statistically significant decline of intracellular GTP from 2.2 to 0.8 nmol/10^6 cells (Bertalanffy et al., 1999). Unexpectedly, it was discovered that these metabolic changes are accompanied by a strongly enhanced ICAM-1 surface expression in endothelial cells. In an attempt to dissect the level at which the effect of IMPDH inhibition on endothelial cells occurs, we have investigated the effectiveness of high and low doses of guanine for preventing the MPA-induced surface expression of ICAM-1; the influence of MPA on transcription and stability of ICAM-1 mRNA; and the effects of MPA on the activation of NF-κB in cultured human umbilical vein endothelial cells (HUVECs) (phosphorylation and degradation of IκB-α and nuclear translocation of the subunits p50 and p65 and their binding to DNA).

Herein, we demonstrate that IMPDH inhibition and consecutive depletion of guanine nucleotides leads to a higher responsiveness against stimulation with IL-1β in endothelial cells through activation of NF-κB. Endothelial cells provide a large surface in the body, and their enhanced activation by IL-1β during IMPDH inhibition might result in profound complications during therapy with the IMPDH inhibitor MPA.

Materials and Methods

Reagents. Methanol was used as vehicle for preparing a stock solution of MPA (Sigma-Aldrich, St. Louis, MO), which was further diluted with RPMI 1640 medium containing GlutaMAX (Invitrogen, Paisley, UK) and 10% fetal calf serum (FCS) (PromoCell, Heidelberg, Germany) to yield final concentrations of 1, 5, 10, 20, and 30 μM. IL-1β (R & D Systems, Minneapolis, MN) was dissolved in RPMI 1640 medium at a final concentration of 100 pg/ml. Pyrrolidine dithiocarbamate (PDTC) (Sigma-Aldrich) and guanine (Sigma-Aldrich) were dissolved in distilled water and diluted with RPMI 1640 medium to final concentrations of 100 or 0.1 μM, respectively. N^{α}-tosylphenylalanyl-chloromethyl-ketone (TPCK) (Sigma-Aldrich) and curcumin (Sigma-Aldrich) were dissolved in ethanol and further diluted with RPMI 1640 medium to yield final concentrations of 25 (TPCK) or 20 μM (curcumin), respectively. Carbobenzoxyl-leucinyl-leucinyl-leucinal-*H* (MG-132) (Calbiochem, Bad Soden, Germany) was dissolved in dimethyl sulfoxide and further diluted with RPMI 1640 medium to yield a final concentration of 20 μM. Control experiments were performed with RPMI medium containing GlutaMAX and 10% FCS, and the equivalent concentrations of the solvents were used for the incubations.

Isolation, Characterization, and Culture of HUVECs. HUVECs were isolated from fresh-term umbilical cords as described previously (Bertalanffy et al., 1999). In brief, both ends of the umbilical cord were cannulated with one-way stopcocks, and the lumen was perfused with PBS. The vein was filled with PBS containing 0.1% collagenase (*Clostridium histolyticum* type II; Invitrogen) and incubated at RT for 10 min. The collagenase solution was flushed into a tube by using an equal volume of Medium 199 (Invitrogen) containing 20% FCS (pH 7.4). Cells were pelleted by centrifugation at 200*g* for 5 min and resuspended in Medium 199 containing 20% FCS, 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 100 U/ml low-molecular-weight heparin (Sigma-Aldrich), and 30 μg/ml bovine hypothalamic growth factor (Upstate Biotechnology, Lake Placid, NY) and plated into 75-cm^2 culture flasks precoated with 2 μg/cm^2 human fibronectin (Upstate Biotechnology).

Cells were maintained in a humidified incubator at 37°C and 5% CO_2 until confluence. The confluent primary and single donor HUVEC monolayers were washed, trypsinized, and subcultured in Medium 199 with the same supplements as above. Only cells from these first subcultures were used for the experiments described below. Purity of the cells was evaluated by factor VIII (FVIII:vWF) staining (Jaffe et al., 1973), transmission electron microscopy (presence of Weibel-Pallade bodies), and expression of CD62E (E-selectin). No contamination by myocytes or fibroblasts was detected.

Experimental Protocols. Flow charts of the experimental protocols A and B are depicted in Fig. 1. After trypsinization of primary HUVEC monolayers, cells were subcultured in Medium 199 (guanine content: 1.6 μM) or RPMI 1640 medium (guanine content: 0.1 μM) containing the supplements described in the cell culture section until confluence. Afterward, the culture medium was removed, and the cells were covered with RPMI 1640 medium containing 10% FCS and 0.1 μM guanine. The cells were then incubated with MPA (15 μM) for 24 h and activated with IL-1β (100 pg/ml). EMSA, confocal microscopy, and immunoblotting were performed 3 h after IL-1β activation, and Northern blot analysis was performed 4 h and flow cytometry 24 h after IL-1β activation (Fig. 1, Protocol A). Time-matched controls were treated with neither MPA nor IL-1β. Further experimental groups consisted of cells that had been treated with MPA or IL-1β alone. These experiments were repeated in cells that had been coincubated with MPA and either 0.1 or 100 μM of guanine (GUA) (Fig. 1, Protocol B). All experiments were repeated in cells that had been pretreated with substances shown to interfere with NF-κB activation. The antioxidant PDTC (100 μM) was added 90 min before MPA treatment. The protease inhibitor TPCK (25 μM) and the proteasome inhibitor MG-132 (20 μM) were added 60 min before MPA treatment. The antioxidant curcumin (20 μM) was added 30 min before the incubation with MPA.

Flow Cytometry for ICAM-1. HUVECs were grown to confluence and treated as shown in Fig. 1. After being washed with PBS containing 5% FCS, 0.1% sodium azide, and 5 mM D-glucose, the cells were released with the addition of 0.25 mM EDTA (Merck, Darmstadt, Germany) and gentle scratching on ice. After centrifugation at 200*g* for 5 min, the cells were resuspended in PBS containing 5% FCS, 0.1% sodium azide, and 5 mM D-glucose and stained with 2 μg/ml of a fluorescein-isothiocyanate–labeled monoclonal anti-CD54 (ICAM-1) antibody (R & D Systems) or with an isotype-matching nonspecific antibody for 30 min on ice. Propidium iodide (20 μg/ml) was used to gate out dead cells from the flow analysis. The cells were analyzed with a Beckman Coulter EPICS XL-MCL flow cytometer (Fullerton, CA) by using the same settings for all samples. Gated cells were acquired (5000 events), and markers were set according to negative control values to quantitate the percentage of positively stained cells.

RNA Extraction and Northern Blot Analysis. For RNA analysis, cells were grown to confluence, stimulated, and total RNA was extracted with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Total RNA (20 μg) was separated on 1.5% (w/v) agarose-formaldehyde gels by electrophoresis. RNA was blotted overnight onto a positively charged nylon membrane (Ambion, Austin, TX) by capillary action in a buffer containing 5× SSC and 10 mM NaOH (pH 11.0). The RNA was fixed on the membrane by baking at 80°C for 1 h and hybridized to radiolabeled cDNA probes of human ICAM-1. ICAM-1 cDNA was obtained by reverse transcription-polymerase chain reaction. The primers for ICAM-1 bind to mRNA positions 795 to 814 and 1526 to

1544, respectively, giving a product length of 750 bp. They read as follows: 5′-CAC AGT CAC CTA TGG CAA CG-3′ and 5′-TTC TTG ATC TTC CGC TGG C-3′. Reverse transcription-polymerase chain reaction products were gel purified from a low-melting agarose gel. Purified cDNA (25 ng) was labeled with a random priming kit (DECAprime DNA labeling system; Ambion) and [α-^{32}P]dCTP (ICN Pharmaceuticals, Costa Mesa, CA). Labeled cDNA was then purified from unincorporated nucleotides by CHROMA SPIN +TE-30 columns (BD Biosciences Clontech, Palo Alto, CA) and measured in a beta counter; 5×10^5 counts/ml were used for hybridization. Prehybridization was carried out for 1 h at 65°C, and hybridization was allowed to proceed overnight at 65°C. Prehybridization and hybridization buffers consisted of 6× SSC, 0.01 M EDTA (pH 8.0), 5× Denhardt's solution, 100 μg/ml sheared denatured salmon sperm DNA (Ambion), and 0.5% SDS (Bio-Rad Laboratories Inc., Hercules, CA). Membranes were washed with 2× SSC for 15 min at 50°C and twice with 2× SSC and 0.1% SDS at 50°C for 15 min, followed by two washes with 0.15× SSC at 50°C for 15 min. An 18S rRNA template (Ambion) was used to monitor lane loadings and transfer efficiency. For rehybridization, membranes were rinsed five times with a stripping solution containing 0.15× SSC, 1% (w/v) SDS, and 40 mM Tris at 80°C. Bands obtained by autoradiography were quantitated with a densitometer (PDI, Huntington Station, NY). All Northern blot experiments were carried out twice and gave comparable results.

mRNA Stability Assay. HUVECs were left untreated or incubated with 15 μM MPA for 24 h and then stimulated with 100 pg/ml IL-1β. Four hours after stimulation with IL-1β, actinomycin D (Sigma-Aldrich) was added at a final concentration of 10 μg/ml. At various time points (0, 30, 60, 120, and 240 min), cells were harvested, total RNA was isolated, and 20 μg of each sample was subjected to Northern blot analysis as described above. Blots were then stripped and rehybridized for 18S rRNA. Autoradiographic signals for ICAM-1 mRNA were quantitated by densitometry and normalized to 18S rRNA signals.

Assay of NF-κB Activity (EMSA): Preparation of Cytoplasmic and Nuclear Extracts. Cells were grown in 75-cm^2 flasks and exposed to vehicle, agents, or IL-1β as appropriate (Fig. 1), and reactions were terminated by washing cells twice with ice-cold PBS containing 1 μg/ml each of leupeptin and aprotinin (ICN Pharmaceuticals) and 0.5 mM phenylmethylsulfonylfluoride (PMSF) (Sigma-Aldrich). Cells were then removed by scraping and transferred to Eppendorf tubes. The cellular material was recovered by centrifugation (500*g* for 4 min at 4°C), and the supernatant was aspirated; the pellet was washed with 5× packed cell volume (pcv) cold hypotonic buffer A consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.5 mM PMSF (Sigma-Aldrich), and 0.5 mM dithiothreitol (DTT) (Sigma-Aldrich). Thereafter, the cells were resuspended in 3× pcv buffer A supplemented with 0.25% Nonidet P-40 (ICN Pharmaceuticals) and incubated on ice for 15 min. Nuclei were pelleted at 500*g* and 4°C for 4 min. The supernatant was carefully removed and immediately frozen in liquid nitrogen, and the nuclear pellet was resuspended in 1× pcv cold

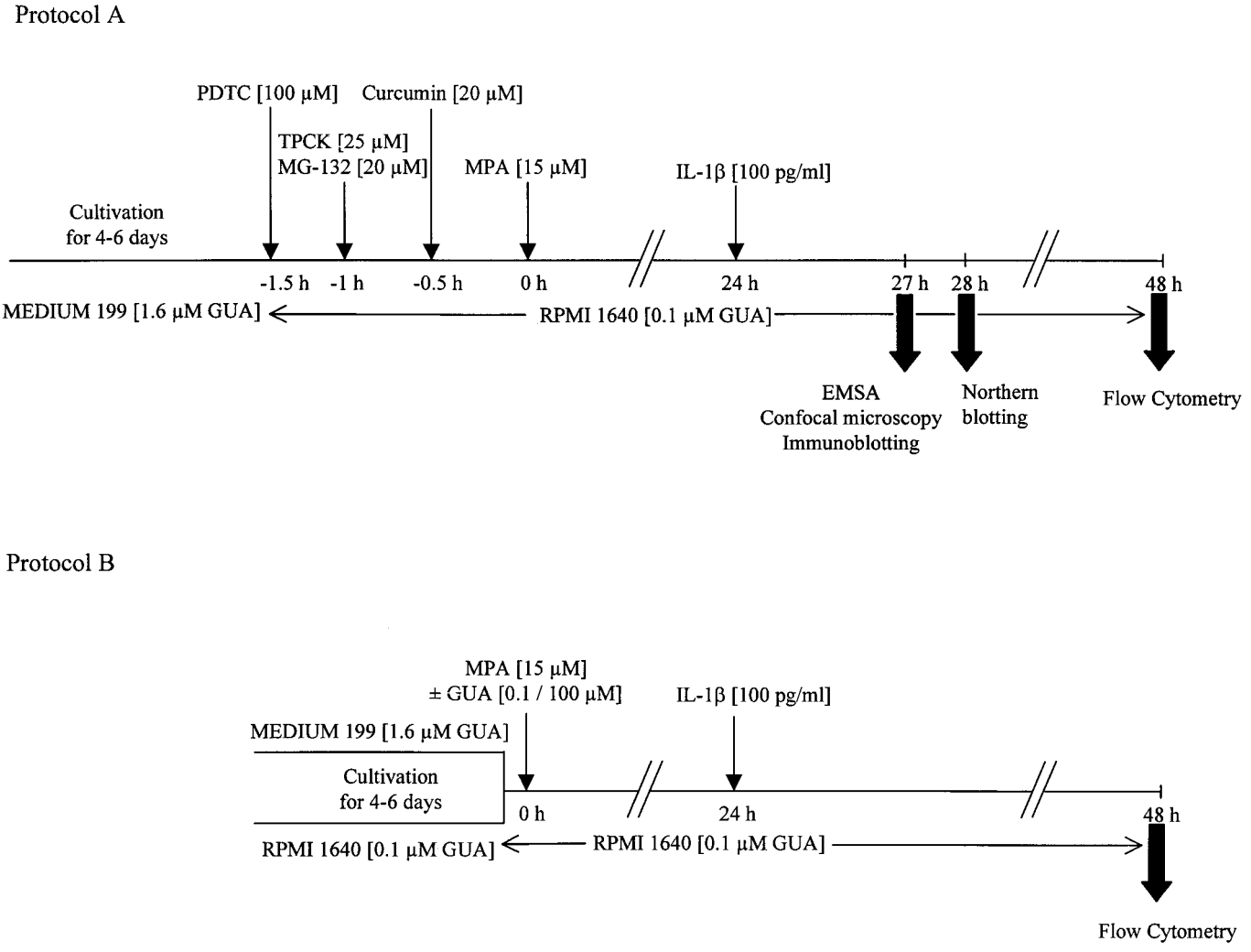

Fig. 1. Flow diagram of the various experimental protocols. A, standard protocol; B, different cultivation conditions.

extraction buffer C (20 mM HEPES, pH 7.9, 0.45 M NaCl, 1 mM EDTA, 1 μg/ml each of leupeptin and aprotinin, and 0.5 mM each of PMSF and DTT) and incubated on ice on a rocking platform for 15 min. The samples were then sonicated on ice in a bath-type sonicator (2 × 30 s), and extracted nuclear material was recovered as the supernatant after centrifugation (14,000*g* for 10 min) at 4°C. The supernatants were diluted with equal volumes of ice-cold buffer D [20 mM HEPES, 0.1 M KCl, 0.2 mM EDTA, 20% (v/v) glycerol, and 0.5 mM DTT] and immediately frozen in liquid nitrogen. Protein content of the recovered extracts was determined against solutions with increasing concentrations of bovine serum albumin standard using a modified Bradford protein assay (Bio-Rad Laboratories Inc.).

DNA Binding Reaction. The double-stranded blunt-ended NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) consensus oligonucleotide was purchased from Promega (Madison, WI). The double-stranded blunt-ended ICAM-1–specific NF-κB (5′-ATT GCT TTA GCT TGG AAA TTC CGG AGC TGA-3′) oligonucleotide, according to the positions −199 to −170 in the ICAM-1 promotor relative to the transcription start site, was customer-synthesized by VBC-GENOMICS Bioscience Research GmbH (Vienna, Austria) and end-labeled with [γ-^{32}P]ATP (ICN Pharmaceuticals) using T4 polynucleotide kinase (Promega). Unincorporated nucleotides were removed by centrifugation over a Sephadex G-25 spun column (Roche Diagnostics, Mannheim, Germany). Binding reactions containing 7 μg of nuclear extract, 1 μg of poly(dI) poly(dC), and 10^4 cpm of γ-^{32}P-labeled oligonucleotide probe were incubated for 20 min at room temperature. Protein-DNA complexes were resolved by nondenaturing electrophoresis on 4.5% (w/v) polyacrylamide slab gels. Gels were initially prerun in 1× Tris-glycine-EDTA buffer for 30 min at 300V; subsequent to loading of samples, electrophoresis was maintained at 300V for 30 to 40 min. Gels were dried under vacuum, and NF-κB complexes were visualized by autoradiography. Competition experiments were performed as described above except that 100-fold excess competitor DNA was added to the incubations before the addition of probe DNA. The specificity of the DNA binding was confirmed by using a mutant oligo with a G → C substitution in the NF-κB/Rel DNA binding motif (sc-2511; Santa Cruz Biotechnology, Santa Cruz, CA) instead of the NF-κB consensus oligonucleotide.

Antibody EMSA. To 1 μl of nonspecific DNA [1 μg poly(dI) poly(dC)] was added 7 μg nuclear extract, 2 μl of water, 1 μl of polyclonal antisera specific for the NF-κB-subunits p50 (sc-1190 X) and p65 (sc-109 X) (Santa Cruz Biotechnology), and 10^4 cpm of γ-^{32}P-labeled oligonucleotide probe. The reaction was mixed, incubated for 20 min at room temperature, and analyzed as described above.

Binding Reactions with Exogenous Addition of MPA. To determine a possible direct effect of MPA on the binding of NF-κB to DNA, nuclear extracts were incubated with increasing doses (0.1, 0.25, 0.5, 0.75, and 1.0 μM) MPA for 20 min at room temperature, and then EMSA was performed. The concentrations chosen correspond to levels measured in cell lysates after incubation with 5 to 20 μM MPA for 24 h (G. Weigel, unpublished data).

Immunoblotting of Phospho-IκB-α (Ser-32). Aliquots of cytoplasmic extracts (25 μg of protein) were mixed with equal volumes of SDS-polyacrylamide gel electrophoresis sample buffer on ice and then boiled for 3 min. Samples were subjected to electrophoresis on 10% (w/v) SDS-polyacrylamide gel electrophoresis gels overlaid with a 4% (w/v) acrylamide stacking gel. The proteins were electrotransferred to 0.45-μm pore size nitrocellulose membranes (Bio-Rad Laboratories Inc.) using a Trans-Blot cell (Bio-Rad Laboratories Inc.). Transfers were carried out overnight at 4°C with 200 mA of current. Transfer efficiency was checked by cotransfer of both prestained and biotinylated standards (Bio-Rad Laboratories Inc.) as well as staining of the gels with Coomassie Brilliant Blue R-250 (Fluka Chemie AG, Buchs, Switzerland) after transfer. After washing (1× TBS and 0.1% Tween 20 for 10 min at RT), nonspecific binding sites were blocked by immersing the membranes in 1× TBS containing 5% (w/v) nonfat dry milk and 0.1% Tween 20 for 2 h at room temperature on an orbital shaker. After washing (1× TBS and 0.1% Tween 20 for 3 × 10 min at RT), the first antibody [rabbit polyclonal phospho-IκB-α (Ser-32) (New England Biolabs, Beverly, MA) 1:1000 in 1× TBS and 5% bovine serum albumin (BSA)] was added, and the blots were incubated overnight at 4°C. Membranes were washed three times (1× TBS and 0.1% Tween for 10 min at RT) before the second antibody [horseradish peroxidase-conjugated anti-rabbit IgG (New England Biolabs) 1:2000 in 1× TBS and 0.1% Tween] was added, and incubation was continued for 60 min at room temperature. Membranes were washed for 2 × 10 min as above. Immunoreactive bands were visualized using the Phototope-HRP Western detection kit (New England Biolabs). Membranes were exposed to Hyperfilm-ECL autoradiography films (Amersham Biosciences, Piscataway, NJ) for from 1 up to 5 min.

Densitometric Quantification of Immunoblots. The signals obtained in immunoblots were quantitated with a densitometer (PDI). Determination of the signal area to be measured and quantitative evaluation were performed independently by two different investigators.

Immunolocalization of p50 and p65 (Confocal Microscopy). The locations of p50 and p65 were evaluated as additional indicators of the activation of NF-κB. Localization of these subunits in the cytoplasm indicates that the NF-κB heterodimer is still in its "dormant form" and hence located in the cytoplasm. In contrast, localization for p50 and p65 in the nucleus indicates that the NF-κB heterodimer has translocated into the nucleus and is therefore able to activate the transcription of NF-κB–dependent genes. At the end of the incubation period, HUVECs grown on glass culture slides (Falcon; BD Biosciences, San Jose, CA) were fixed in fresh 4% (w/v) paraformaldehyde for 20 min. The cells were permeabilized with 0.3% (v/v) Triton X-100 in PBS for 5 min. Nonspecific adsorption was minimized by incubating the cells with 1% (w/v) BSA in PBS for 30 min. The cells were then incubated with anti–NF-κB p65 antibody (sc-7178) or anti-NF-κB p50 antibody (sc-109x) [both 1:300 in PBS containing 1% BSA (w/v); Santa Cruz Biotechnology]. Controls included buffer alone or nonspecific purified rabbit immunoglobulin G. Specific labeling was detected with a fluorescein-isothiocyanate–conjugated goat anti-rabbit IgG (1:500 in PBS containing 1% BSA) (BD PharMingen, San Jose, CA). Cells were washed with PBS, mounted with ProLong Antifade Kit (Molecular Probes), and examined with a laser scanning confocal fluorescence microscope (LSM 510; Carl Zeiss GmbH, Jena, Germany). An excitation wavelength of 488 nm and a 560-nm long-pass filter were used.

Statistical Analysis. Flow cytometry data are presented as the mean and S.E. For concentration-dependency experiments, blockage experiments, and stimulation experiments in HUVECs, p values were determined by analysis of variance. p values of < 0.05 were considered statistically significant.

Results

Expression of Adhesion Molecules. HUVECs showed constitutive expression of ICAM-1 on a low level (mean fluorescence intensity, 4.1 ± 0.47 arbitrary units). Incubation with MPA dose dependently led to a significant increase of ICAM-1 surface expression, reaching its maximum (6.5 ± 0.11) at a concentration of 20 μM ($p < 0.001$; Fig. 2). To test whether alterations of the endothelial purine metabolism caused by MPA are responsible for this finding, cells were cultivated in the presence of either 1.6 or 0.1 μM guanine. When cells cultivated in the presence of 0.1 μM guanine were preincubated with MPA and then stimulated with IL-1β, a much stronger ICAM-1 expression occurred in these cells compared with those cultivated in the presence of 1.6 μM guanine (Fig. 3). This indicates that a concentration of 0.1 μM guanine, which corresponds to the physiological plasma

level, is not effective for preventing the MPA-induced ICAM-1 expression (Fig. 4). Only when HUVECs were incubated with MPA in the presence of a high dose of guanine (100 μM) was the enhanced ICAM-1 expression abrogated ($p < 0.001$), which proves that only a supraphysiological level of a substrate for the salvage pathway can compensate for the effect of MPA on endothelial cells.

In an attempt to study the mechanism by which MPA-induced changes of endothelial guanine nucleotide metabolism influence ICAM-1 synthesis, several substances that interfere with NF-κB activation were tested, because ICAM-1 mRNA transcription is highly regulated in a NF-κB–dependent fashion. Preincubation of HUVECs with the antioxidant PDTC altered neither the constitutive ICAM-1 expression nor that measured in response to MPA (data not shown). When HUVECs were preincubated with the antioxidant curcumin or the protease inhibitor TPCK, the effect of MPA on ICAM-1 expression could be prevented. The most potent inhibitor was the proteasome inhibitor MG-132, which even reduced the constitutive expression of ICAM-1 (Fig. 5A).

When HUVECs were activated with IL-1β, an increase in ICAM-1 expression of about 2-fold was observed that was further enhanced when the cells had been pretreated with MPA. As was shown for MPA alone, preincubation with curcumin, TPCK, or MG-132 neutralized the stimulating and synergistic effects of MPA and IL-1β (Fig. 5B).

ICAM-1 mRNA Transcription. To determine whether the increased surface expression of ICAM-1 by MPA was mediated by an increased transcription of mRNA encoding ICAM-1, Northern blots were performed with RNA from confluent HUVECs that were left untreated or incubated with MPA for 24 h (Fig. 5C). In untreated HUVECs, the ICAM-1 signal on Northern blots was very weak, but hybridization with 18S rRNA, used as internal reference, confirmed equal RNA loading and transfer. Treatment with MPA led to a significant increase in ICAM-1 mRNA transcription (Fig.

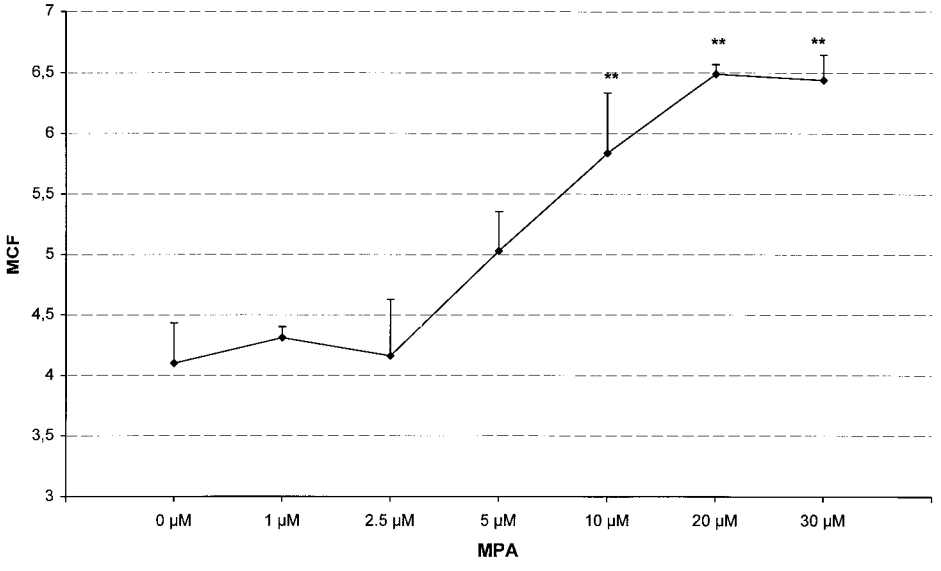

Fig. 2. Surface expression of ICAM-1 on HUVECs after incubation with increasing concentrations of MPA (0, 1, 2.5, 5, 10, 20, and 30 μM) for 48 h as determined by flow cytometry. Data are given as mean channel fluorescence (MCF arbitrary units) and expressed as mean ± S.E. of four independent experiments. **, statistically significant versus control (0 μM MPA) ($p < 0.001$).

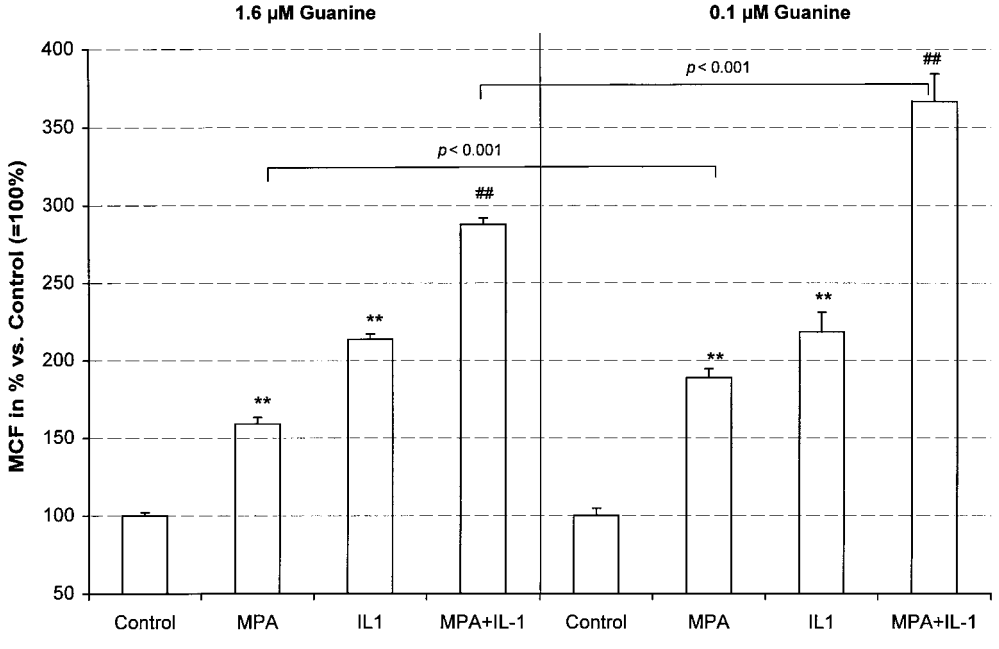

Fig. 3. Surface expression of ICAM-1 on HUVECs cultivated in the presence of 1.6 or 0.1 μM guanine. Cells were incubated with 15 μM MPA for 24 h before stimulation with 100 pg/ml IL-1β for another 24 h and examined by flow cytometry. Data are given as percent of mean channel fluorescence (MCF) compared with control (100%) and expressed as mean ± S.E. of six independent experiments. **, statistically significant versus control ($p < 0.001$). ##, statistically significant versus IL-1β alone ($p < 0.001$).

5C). Upon stimulation with IL-1β, a significant increase in ICAM-1 mRNA was detectable in MPA-untreated cells. In contrast, in cells pretreated with MPA and activated with IL-1β, a sustained increase of ICAM-1 mRNA was detectable compared with IL-1β activation alone (Fig. 5D). The effect of MPA and/or IL-1β on ICAM-1 mRNA transcription and its reduction by curcumin, TPCK, and MG-132 was analogous to that observed for the surface expression of ICAM-1 (Fig. 5, C and D).

ICAM-1 mRNA Stability. HUVECs were left untreated or incubated with MPA (15 μM) for 24 h and activated with IL-1β (100 pg/ml for 4 h) to achieve peak message levels.

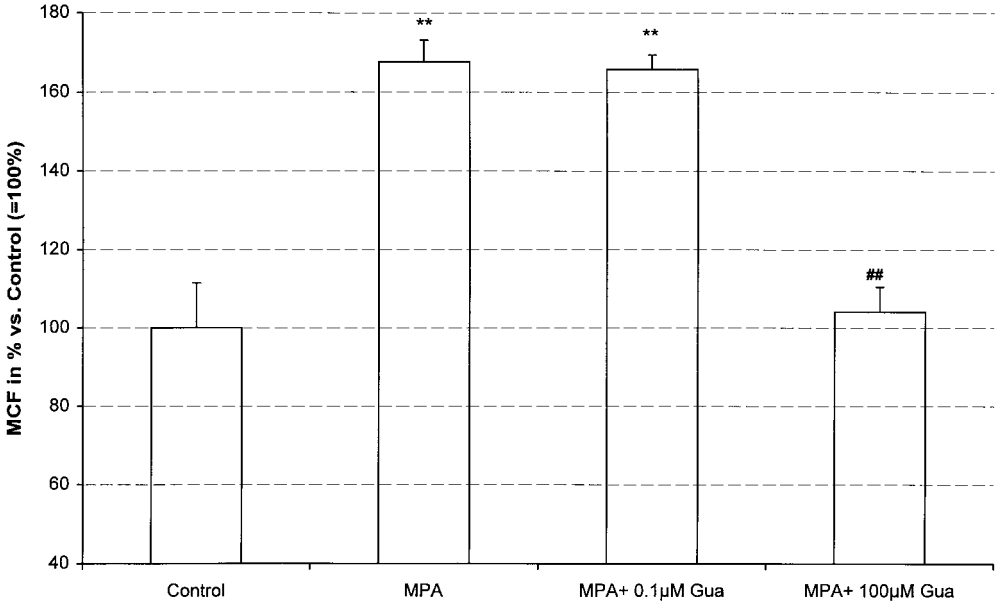

Fig. 4. Surface expression of ICAM-1 on HUVECs. Cells were incubated with 15 μM MPA either alone or in combination with 0.1 or 100 μM guanine (Gua) for 48 h and examined by flow cytometry. Data are given as percent of mean channel fluorescence (MCF) compared with control (100%) and expressed as mean ± S.E. of four independent experiments. ⋆⋆, statistically significant versus control ($p < 0.001$). ##, statistically significant versus MPA alone ($p < 0.001$).

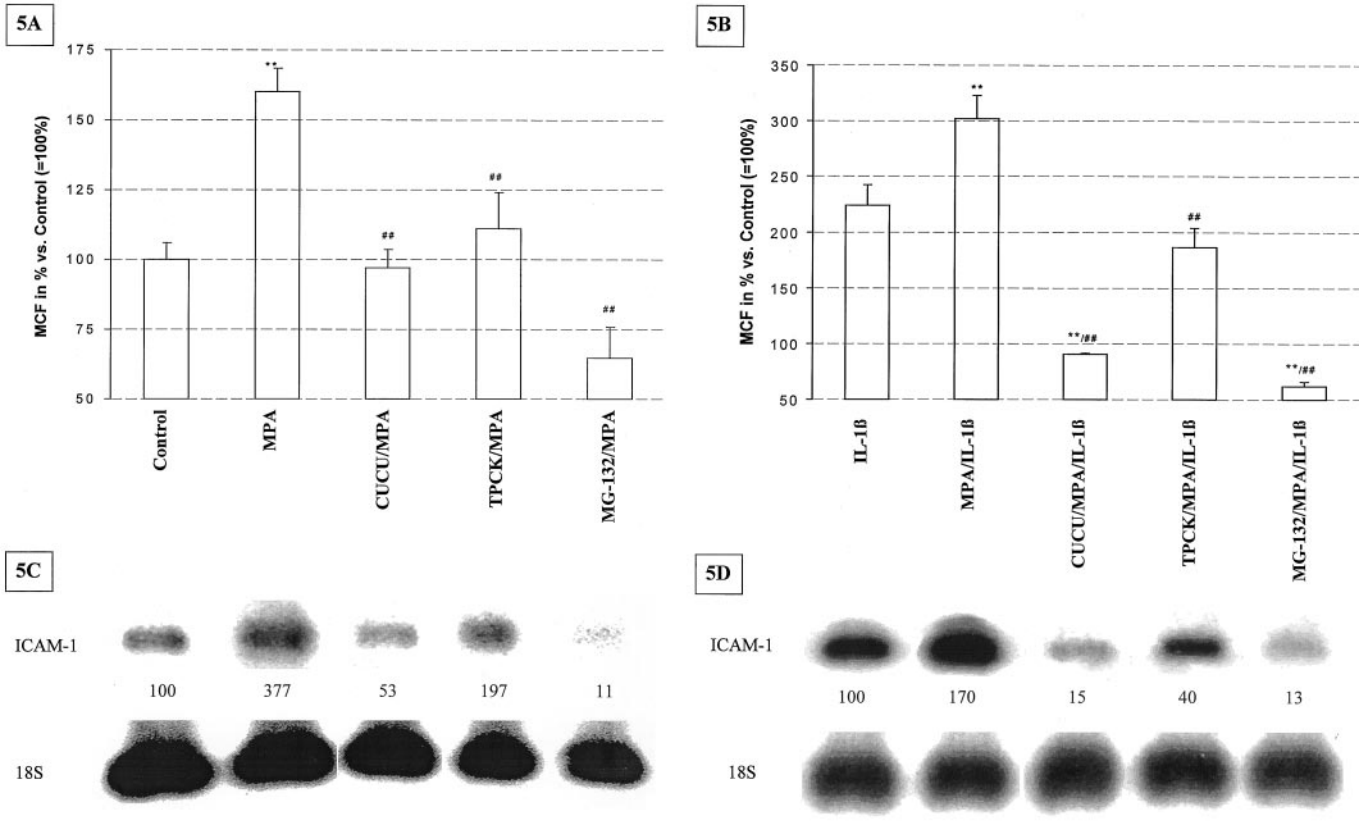

Fig. 5. MPA-induced (15 μM) ICAM-1 surface expression (A and B) and mRNA transcription (C and D) are inhibited by curcumin (20 μM), TPCK (25 μM), and MG-132 (20 μM) as determined by flow cytometry and Northern blot analysis. Flow cytometric data are given as percent of mean channel fluorescence (MCF) compared with control (100%) and expressed as mean ± S.E. of four to six independent experiments. ⋆⋆, statistically significant versus control ($p < 0.001$). ##, statistically significant versus MPA ($p < 0.001$). Total RNA was extracted, and Northern blots for ICAM-1 mRNA were performed. Blots were then stripped and rehybridized for 18S rRNA. Autoradiographic signals for ICAM-1 mRNA were quantitated by densitometry and normalized to 18S rRNA signals. C and D, representative blots. Relative densitometric scores for ICAM-1 mRNA are noted.

Actinomycin D (10 μg/ml) was then added to inhibit transcription, and the decay of ICAM-1 mRNA was examined over a period of 4 h. The half-life of the ICAM-1 mRNA induced by IL-1β alone was estimated to be 3 h. In contrast, the IL-1β–induced ICAM-1 message in MPA-pretreated cells did not decay appreciably after addition of actinomycin D, demonstrating that ICAM-1 mRNA synthesis is not only sustained in the presence of MPA but also that the message is stabilized by MPA compared with IL-1β alone (Fig. 6). The half-life of ICAM-1 message in unstimulated cells could not be determined because of the low basal mRNA levels.

NF-κB (Immunolocalization and EMSA). In HUVECs subjected to MPA, there was staining for p50 and p65 in the nuclei indicating translocation of NF-κB to the nucleus (Fig. 7, A and D). In HUVECs activated with IL-1β, an increase of NF-κB translocation was observed (Fig. 7, B and E). When cells were pretreated with MPA and then stimulated with IL-1β, a most intensive staining for p50 and p65 in the nuclei was seen (Fig. 7, C and F).

EMSA of nuclear extracts isolated from HUVECs after MPA treatment showed an increase in binding of NF-κB to the consensus sequence as well as to the ICAM-1–specific sequence. When the cells were subsequently stimulated with IL-1β, a strong increase in binding of NF-κB to both the consensus and the ICAM-1–specific sequences occurred. Although in cells preincubated with RPMI 1640 medium and

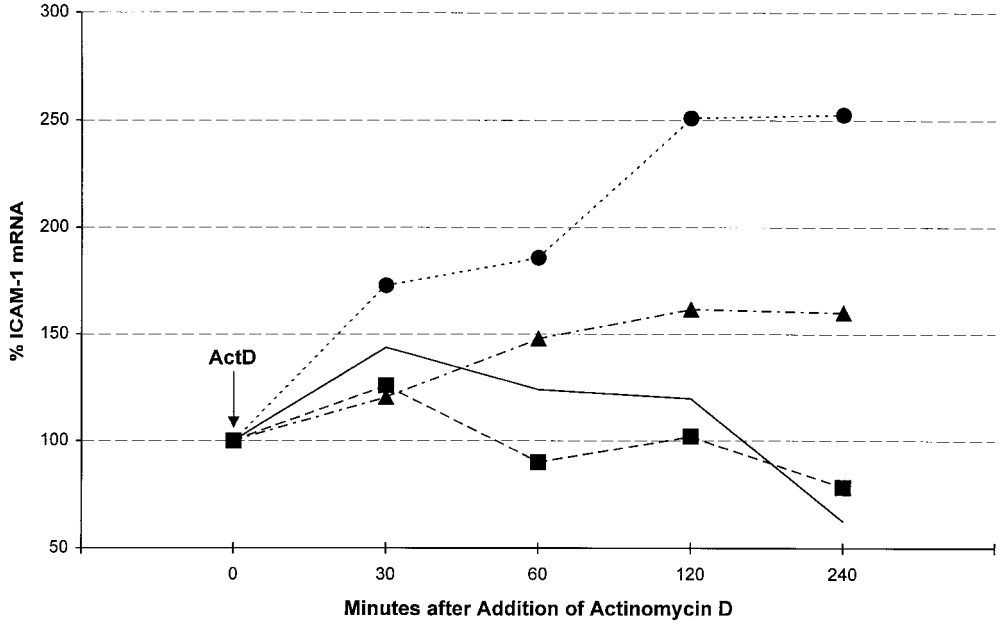

Fig. 6. HUVECs were left untreated or incubated with 15 μM MPA for 24 h and then stimulated with 100 pg/ml IL-1β. Four hours after stimulation with IL-1β, actinomycin D was added at a final concentration of 10 μg/ml. At various time points (0, 30, 60, 120, and 240 min), cells were harvested, total RNA was isolated, and 20 μg of each sample was subjected to Northern blot analysis as described above. Blots were then stripped and rehybridized for 18S rRNA. Autoradiographic signals for ICAM-1 mRNA were quantitated by densitometry and normalized to 18S rRNA signals. Values are given as percentage of ICAM-1 mRNA levels measured before addition of actinomycin D. −, IL-1; ■, IL-1 + ActD; ●, MPA + IL-1; ▲, MPA + IL-1 + ActD.

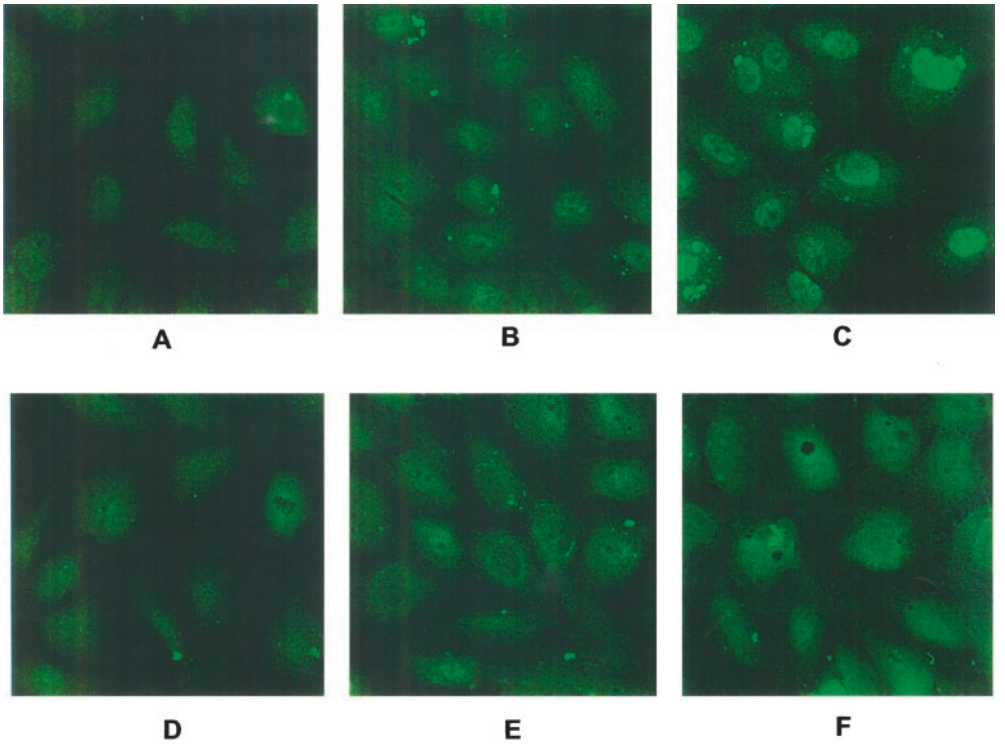

Fig. 7. Immunolocalization of p50 (A-C) and p65 (D-F) as determined by confocal microscopy. Fluorescent images of HUVECs that were treated with 15 μM MPA for 24 h (A and D), treated with 100 pg/ml IL-1β for 3 h (B and E), or incubated with MPA 24 h before stimulation with IL-1β for further 3 h (C and F). Cells were washed, stained, and analyzed by confocal microscopy as described under *Materials and Methods*. Please note the strong positive staining for p50 (C) and p65 (F) in the nuclei of HUVECs pretreated with MPA and stimulated with IL-1β.

activated with IL-1β, the binding of NF-κB to DNA (consensus as well as ICAM-1–specific) started to decrease after 2 h, a sustained binding was seen in cells that had been treated with MPA before the addition of IL-1β (Fig. 8).

Supershift experiments performed with nuclear extracts isolated from HUVECs after MPA treatment showed an involvement of the subunits p50 and p65 (Fig. 9). Stimulation with IL-1β further enhanced the binding of NF-κB to DNA (Fig. 9B). Curcumin, TPCK, or MG-132 inhibited the effects of IL-1β and MPA on NF-κB binding (Fig. 7, A and B). In the presence of curcumin or TPCK, the nuclear binding of NF-κB was strongly reduced, and in the presence of MG-132, it was abolished. These findings correspond to the pattern of ICAM-1 mRNA transcription and ICAM-1 surface expression. To rule out a possible direct influence of MPA on the binding of NF-κB to DNA, increasing doses of MPA were added to the binding reaction of the EMSA, which proved that MPA does not directly interfere with the binding of p50/p65 to DNA (data not shown). From these observations, it was obvious that MPA does not modify the ability of NF-κB to bind to the DNA but influences the NF-κB pathway upstream from interaction with DNA.

pIκB-α Immunoblotting. Cells were preincubated with MPA (15 μM) and stimulated with IL-1β (100 pg/ml) for 0, 10, 20, 30, and 60 min. Cytoplasmic extracts were analyzed for their contents of phosphorylated IκB-α. When endothelial cells were treated with IL-1β, IκB-α phosphorylation peaked after 20 min. Upon incubation with MPA alone, there was a significant increase of pIκB-α in cell lysates compared with cells without MPA treatment (Fig. 10A). When cells were preincubated with MPA and then stimulated with IL-1β, a strongly enhanced formation of pIκB-α was detectable compared with IL-1β alone (Fig. 10B).

Discussion

In the present report, we demonstrate that guanine nucleotide depletion in human endothelial cells enhances constitutive and IL-1β–stimulated ICAM-1 surface expression. The plasma levels obtained with a single dose of the IMPDH inhibitor MPA (Weigel et al., 2001) are comparable with the concentrations used in this series of in vitro experiments. However, because MPA is thought to inhibit leukocyte adhesion to the endothelium (Allison et al., 1993; Blaheta et al., 1998), this in vitro effect is an apparent paradox. The up-regulation of ICAM-1 by MPA was prevented by replenishing intracellular GTP pools by high doses (100 μM) of guanine via the salvage pathway. This finding is in accordance with that reported for MPA-induced reduction of nitric oxide production in endothelial cells (Senda et al., 1995). Because the concentration of guanine in human plasma is reported to be very low (<0.1 μM), cells were also treated with low doses of guanine during incubation with MPA for simulating the in vivo situation (Eells and Spector, 1983). Under these conditions, ICAM-1 up-regulation could not be prevented, proving the clinical relevance of our findings. This is in line with the observations made by Eugui and Allison (1993) showing that at least 50 μM of guanosine are necessary to restore DNA synthesis in MPA-treated and phytohemagglutinin-stimulated peripheral blood cells. In normal lymphocytes, much higher concentrations are needed (Eugui and Allison, 1993).

The MPA-mediated augmentation of ICAM-1 mRNA levels induced by IL-1β could be a consequence of enhanced transcription or RNA stabilization. Indeed, we were able to demonstrate that MPA not only stabilizes ICAM-1 mRNA but also increases magnitude and duration of ICAM-1 transcription. To investigate the signaling events leading to the MPA-induced up-regulation of endothelial ICAM-1 expression, the involvement of NF-κB (which is known to promote ICAM-1 transcription) was studied (Chen et al., 1995a). The increase and prolongation in NF-κB translocation and binding to NF-κB consensus and most importantly, to ICAM-1–specific

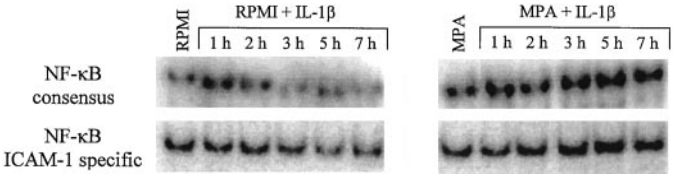

Fig. 8. EMSA of nuclear extracts from HUVECs compares binding of NF-κB consensus sequence with ICAM-1–specific sequence. Cells were incubated with 15 μM MPA or RPMI 1640 medium for 24 h, and then 100 pg/ml IL-1β were added. At different time points (1, 2, 3, 5, or 7 h), cells were harvested, and nuclear extracts were prepared. The binding to NF-κB consensus or ICAM-1–specific sequences (according to the positions −199 to −170 in the ICAM-1 promotor) was determined by EMSA as described under *Materials and Methods*.

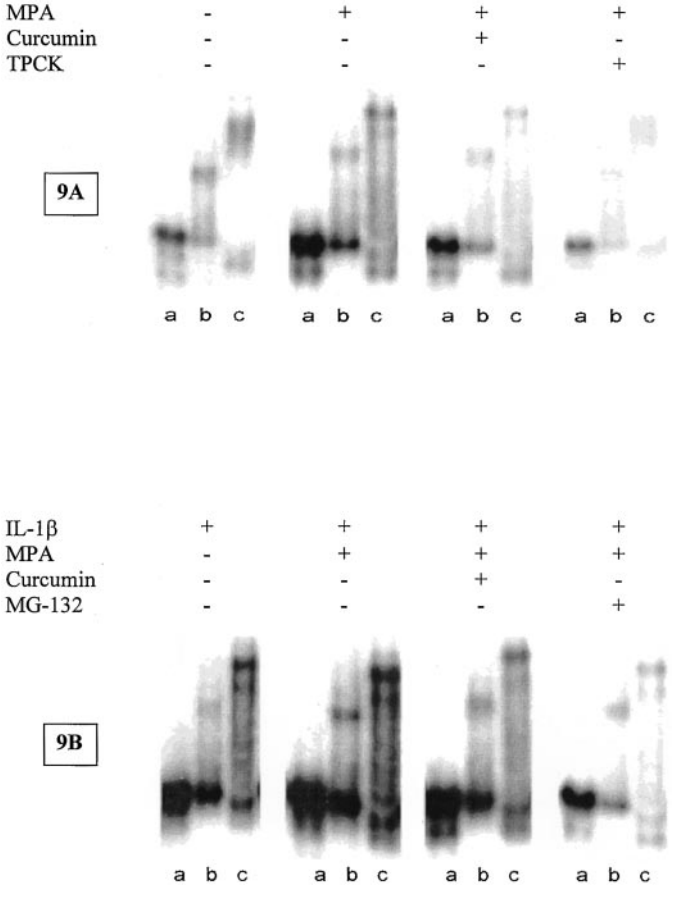

Fig. 9. EMSA of nuclear extracts from HUVECs. Lanes a, binding of NF-κB. Lanes b and c, representative supershifts for the subunits p50 and p65, respectively. Cells were incubated with 15 μM MPA for 24 h and then vehicle (A) or 100 pg/ml IL-1β (B) were added for further 3 h. NF-κB–blocking agents curcumin (20 μM), TPCK (25 μM), and MG-132 (20 μM) were added at time points indicated in Fig. 1. Subsequent to preparation of nuclear extracts, NF-κB DNA binding activity was determined by EMSA as described under *Materials and Methods*.

sequences, strongly indicates that gene activation is involved. Because the dynamics of NF-κB activation after MPA treatment paralleled ICAM-1 mRNA transcription, it seems obvious that the stability of ICAM-1 mRNA results from persistent NF-κB activation and its binding to a sequence in the ICAM-1 promotor.

The effect of MPA on ICAM-1 could be blocked by interfering with the proteasomal degradation of phosphorylated and ubiquitinated IκB using TPCK or MG-132 (Henkel et al., 1993; Mackman, 1994; Rock et al., 1994). Curcumin, an inhibitor of a signal that leads to IκK activity (Singh and Aggarwal, 1995; Bierhaus et al., 1997; Jobin et al., 1999), also prevented the effects of MPA on ICAM-1 synthesis and binding of NF-κB to DNA. To rule out a possible direct influence of MPA on DNA binding, increasing doses of MPA were added to the binding reaction of the EMSA, which proved that MPA does not directly interfere with the binding of p50 or p65 to DNA (data not shown). From these observations, it was obvious that MPA does not modify the ability of NF-κB to bind to DNA but influences the NF-κB pathway upstream from interaction with DNA. In another series of experiments, we studied the pattern of IκB-α in cells that were left untreated or preincubated with MPA and then stimulated with IL-1β. The results proved that MPA pretreatment shifts endothelial cells toward a higher level of phosphorylated IκB-α. Similar to our findings, Sadeghi et al. (2000) observed the up-regulation of IL-1–induced adhesion molecule expression in HUVECs pretreated with simvastatin, a hydroxymethylglutaryl-CoA reductase inhibitor. The authors speculated that there could be a non–IL-1–responsive distinct inhibitory $G_{i\alpha}$-coupled receptor that could modulate IL-1 responses and confer an "inhibitory tone" for endothelial adhesion molecule expression. This inhibitory pathway could be sequentially coupled to a downstream kinase, such as NF-κB–inducing kinase, which is crucial for IL-1–mediated NF-κB translocation and activation of ICAM-1 gene transcription. Thus, it seems possible that through the depletion of intercellular GTP observed in our study, the inhibitory tone mediated via a G_i-protein-coupled receptor is attenuated and ICAM-1 overexpression occurs. In our study, when cells were cultivated in the presence of only 0.1 μM guanine and treated with MPA, which led to a more pronounced GTP depletion, ICAM-1 overexpression was further enhanced. This indicates that guanine nucleotides play an important role in the modulation of ICAM-1 expression and possibly in the activation of NF-κB in endothelial cells in general. Normally, about 0.5 mM GTP is present in resting cells, and the removal of most of it should still leave enough to satisfy the binding affinities and activation of most G-proteins in cell-free systems. Jayaram et al. (1999) proposed that the affinity of G-proteins in vivo for GTP might be much lower, indicating that incomplete depletion of intracellular GTP should result in dramatic down-regulation of G-protein function.

We provide evidence that the enhanced expression of ICAM-1 during MPA treatment occurs through GTP depletion in endothelial cells, which is associated with an enhanced IκB phosphorylation, NF-κB translocation, and binding to a sequence in the ICAM-1 promotor. To additionally demonstrate the clinical relevance of this finding, experiments with the human microvascular endothelial cell line HMEC-1 were performed (data not shown). Similar to the observation made in HUVECs, there was also a significant amplification of IL-1β–induced ICAM-1 expression upon IMPDH inhibition that could only be prevented by addition of high doses (100 μM) of guanine. Because the plasma level of guanine measured in vivo is too low to regenerate endothelial GTP pools via the salvage pathway, pharmacologic manipulation of the de novo GTP synthesis might result in a dysregulation of endothelial ICAM-1 expression and possibly affect other NF-κB–regulated genes.

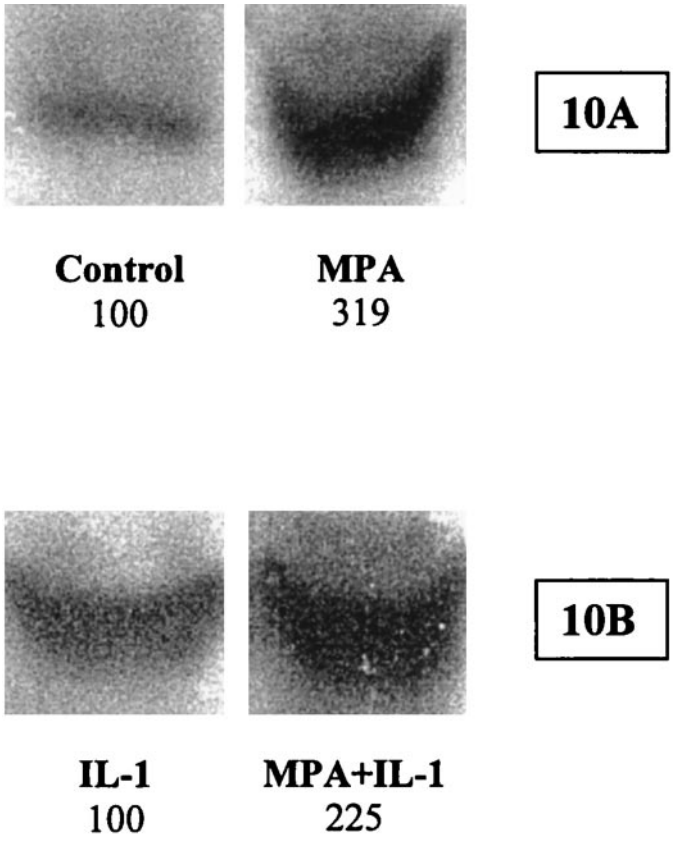

Fig. 10. Immunoblotting for pIκB-α of HUVECs. A, cells were left untreated or pretreated with 15 μM MPA for 24 h, and cytoplasmic extracts were prepared. B, cells were left untreated or pretreated with MPA for 24 h before stimulation with 100 pg/ml IL-1β for 20 min, and cytoplasmic extracts were prepared. pIκB-α content was then determined by Western blotting as described under *Materials and Methods*. Representative blots are presented, and relative densitometric scores are noted.

Acknowledgments

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