Differential effects of NF-κB and p38 MAPK inhibitors and combinations thereof on TNF-α and IL-1β induced pro-inflammatory status of endothelial cells in vitro

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ABSTRACT
Endothelial cells actively participate in inflammatory events by regulating leukocyte recruitment via the expression of inflammatory genes such as E-selectin, VCAM-1, ICAM-1, IL-6, IL-8, and cyclo-oxygenase (COX)-2. In this study we showed by real time RT-PCR that activation of human umbilical vein endothelial cells (HUVEC) by TNF-α and IL-1β differentially affected the expression of these inflammatory genes. Combined treatment with TNF-α/IL-1β resulted in an exaggerated induction of IL-6, IL-8 and COX-2 gene expression. In contrast an attenuated increase in VCAM-1 and ICAM-1 gene expression was observed. Overexpression of dominant negative (dn)IκB protein blocking NF-κB signalling confirmed a major role of this pathway in controlling both TNF-α and IL-1β induced expression of most of the genes studied. While pyrrolidine dithiocarbamate (PDTC) and dexamethasone (DEX), both inhibitors of NF-κB controlled gene expression, exerted limited effects at 1 µM, the thioredoxin inhibitor MOL-294 that regulates the redox state of NF-κB mainly inhibited adhesion molecule expression. Its most pronounced effect was seen on VCAM-1 mRNA levels, especially in IL-1β activated endothelium. One µM RWJ 67657, an inhibitor of p38 MAPK activity, diminished TNF-α and IL-1β induced expression of IL-6, IL-8, and E-selectin, but had little effect on VCAM-1 and ICAM-1. Combined treatment of HUVEC with MOL-294 and RWJ 67657 resulted in significant blocking of the expression of the majority of genes studied. The inhibitory effects were much stronger than those observed by single drug treatment, implying the use of combinations of drugs affecting multiple targets in activated endothelial cells as a potential new therapeutic strategy.

Key words. Endothelial cells, inflammatory gene expression, anti-inflammatory drugs, NF-κB, p38 MAPK
INTRODUCTION

Endothelial cells form the natural barrier between the blood and surrounding tissue. During inflammation they control leukocyte trafficking and actively participate in angiogenesis through differential expression of inflammation- and angiogenesis-associated genes, including cytokines, chemokines, growth factors, and adhesion molecules. Prevention of activation of endothelial cells has been suggested to be beneficial in the treatment of chronic inflammatory diseases like rheumatoid arthritis (RA), and inflammatory bowel disease (IBD). Furthermore, their position in the body makes them an easy accessible target for therapeutic intervention.

The pro-inflammatory cytokines TNF-α and IL-1β, having a similar but not identical effect on gene expression, are often present simultaneously in chronic inflammatory diseases. They exert a prominent effect on the expression of pro-inflammatory genes in endothelial cells. This effect predominantly takes place through activation of intracellular signalling pathways involving NF-κB and p38 MAPK. The transcription factor NF-κB is present in the cytoplasm of unstimulated cells in an inactive form due to its association with the inhibitory protein IκB. Upon cytokine activation degradation of IκB and subsequent nuclear translocation of active NF-κB takes place. The p38 MAPK activation pathway engages diverse upstream kinases responsible for p38 MAPK activation, as well as downstream substrates. In endothelial cells both NF-κB and p38 MAPK are involved in the regulation of the expression of genes encoding E-selectin, VCAM-1, ICAM-1, IL-6, IL-8, and cyclooxygenase (COX)-2, among others. The regulation takes place on transcriptional and posttranscriptional levels.

Both activated NF-κB and p38 MAPK have been shown to be present in RA and IBD lesions, and are therefore interesting targets for pharmacological intervention. However inhibition of NF-κB or p38 MAPK can have the serious drawback of undesired toxic effects on non-diseased cells. Incorporation of these drugs in endothelial cell specific drug delivery systems can theoretically overcome these undesired side-effects. The antioxidant and metal-chelating compound pyrrolidine dithiocarbamate (PDTC), the glucocorticoid dexamethasone (DEX), the thioredoxin inhibitor MOL-294, and the p38 MAPK inhibitor RWJ 67657 are potential candidates for incorporation in drug targeting constructs. Yet, limited data are available on quantitative comparison of the effects of these anti-inflammatory drugs on endothelial cell gene expression under pro-inflammatory conditions.

In the current study we investigated the effects of TNF-α, IL-1β, and a combination of TNF-α/IL-1β activation on the kinetics and levels of expression of the pro-inflammatory genes E-selectin, VCAM-1, ICAM-1, IL-6, IL-8, and COX-2 by human umbilical vein endothelial cells (HUVEC). The importance of NF-κB signalling in TNF-α and IL-1β induced gene expression was investigated by overexpression of an IκB mutant inhibiting NF-κB signal transduction. Furthermore, we analysed the effects of the above-mentioned drugs on the expression levels of the pro-inflammatory genes chosen and their capacity to potentiate gene expression inhibition when added simultaneously. To quantitatively compare the effects of activators and drugs, both real time RT-PCR and, in distinct experiments, ELISA analyses of produced cytokines were performed.
MATERIAL AND METHODS

Endothelial Cells
HUVEC obtained from the Endothelial Cell Facility (UMCG, Groningen, The Netherlands) were isolated from two umbilical cords to circumvent donor bias, and cultured as previously described 22. In short, the cells were cultured on 1% gelatin-precoated tissue culture flasks (Corning, Schiphol, The Netherlands) at 37°C under 5% CO₂/95% air. The endothelial culture medium consisted of RPMI 1640 supplemented with 20% heat-inactivated FCS, 2 mM L-glutamine, 5 U/ml heparin, 100 IE/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml endothelial cell growth factor supplement extracted from bovine brain. Upon confluence, cells were detached from the surface by trypsin/EDTA (0.5/0.2 mg/ml in PBS) and split at a 1:3 ratio into 12- or 6-well tissue culture plates (Corning). In the experiments performed, HUVEC were used up to passage 4. All experiments were performed with confluent HUVEC monolayers, except when using adenovirus encoded dominant negative (dn)IκB, for which confluency was 70%. The experiments were performed with at least two and in most cases with four different HUVEC isolates in independent experiments. Data shown are representative for the data from the different experiments.

Activation of HUVEC
Confluent HUVEC were activated with 1 and 10 ng/ml TNF-α (Boehringer, Ingelheim, Germany), and 1 and 10 ng/ml IL-1β (R&D Systems, Minneapolis MN, USA), added separately or in combination, for 6 hours (early gene expression) and 24 hours (late gene expression). After incubation cells were microscopically analyzed with regard to their morphology and consistently were found to be adherent and viable. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Promega Benelux BV, Leiden, The Netherlands) assays were occasionally executed according to the manufacturers protocol, to corroborate the light microscopy analysis.

Dominant negative IκB Adenovirus
Recombinant, replication-deficient adenovirus Ad5IκBAA, hereafter named dnIκB, was a gift from Prof. C. Trautwein from the Medical School of Hannover, Germany. Adenovirus contained an IκBα sequence, in which serine at positions 32 and 36 were substituted by alanine, and which was fused to influenza A virus hemagglutinin (HA)-tag. The expression was controlled by the cytomegalovirus promoter/enhancer 23. Virus was grown on HEK293 cells and purified from cell lysates by banding twice on CsCl gradients. Virus was desalted using a 10 kD slide-A-lyzer ® (Pierce Chemical Company, Rockford IL, USA) in Hepes/Sucrose buffer, pH 8.0 and stored at -80°C. Viral particles (vp) were determined by UV spectrophotometric analysis at 260 nm. Furthermore a standard limiting dilution assay was performed to determine the vp/plaque forming unit (pfu) ratio. As a control, adenovirus Ad5LacZ, containing the Escherischia coli β-galactosidase gene 24, was grown and purified as described above.

Virus infection protocol
For the transduction of HUVEC with dnIκB or the control virus Ad5LacZ, HUVEC were plated at 12,500 cells/cm² in 6-wells tissue culture plates (Corning), and cultured
overnight before actual transduction. The viral vectors diluted in DMEM (Gibco™, Paisley, Scotland, UK) without FCS, were added at 500 pfu/seeded cell (corresponding to 7.5x10⁵ vp/cell) and incubated for 90 min at 37°C. The incubation medium was then replaced by endothelial culture medium. Cells were subsequently incubated for 24 hours prior to activation to allow transgene expression.

**Western blot analysis of dnIkB expression in HUVEC**

After 24 hours of culturing, cells were detached from the surface by trypsin/EDTA treatment, lysed in the cell culture lysis reagent (Promega Corporation, Madison, WI, USA) and sonicated twice at 4°C for 5 sec. After centrifugation for 10 min at 10 000 g and at 4°C, cleared cell lysates were collected and the protein content was determined using the Bradford protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA), with BSA as the standard. Samples were then mixed with reducing SDS sample buffer, boiled for 5 min, and 30 µg of protein was loaded on SDS-PAGE 10% acrylamide gel. After separation proteins were electrophoretically transferred on a nitrocellulose membrane (Bio-Rad Laboratories). Blots were blocked in blocking buffer containing 5% non-fat dry milk in PBS/0.1% Tween for 2 hours. Next blots were incubated for 1 hr with rabbit anti-HA-probe antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:200 in blocking buffer) for dnIkB detection, and rabbit anti-IkBa antibody (Santa Cruz Biotechnology; dilution 1:20 in blocking buffer) for both endogenous and dnIkB detection. Blots were washed with PBS/0.1% Tween and incubated for 1 hr with horseradish peroxidase-conjugated swine anti-rabbit antibody (DAKO, Glostrup, Denmark) diluted 1:2000 in blocking buffer. After washing as described above, detection was performed using enhanced chemoluminescence detection reagent (Amersham Corp., Arlington Heights, IL, USA) according to the manufacturers protocol.

**Incubation of HUVEC with drugs**

The following drugs were used: PDTC (Sigma-Aldrich, Zwijndrecht, The Netherlands), DEX (9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione; Genfarma B.V., Maarssen, The Netherlands), MOL-294 (methyl (4R/S)-4-hydroxy-4-[(5S,8S)/(5R,8R)]-8-methyl-1,3-dioxo-2-phenyl-2,3,5,8-tetra-hydro-1H-[1,2,4]triazolo [1,2-a]pyridazin-5-yl]-2-butyroate; kindly provided by Dr. M. Kahn from Pacific Northwest Research Institute, Seattle, Washington, USA), and RWJ 67657 (4-(4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1H-imidazol-2-yl)-3-butyln-1-ol; kindly provided by Johnson & Johnson Pharmaceutical R&D, Raritan, New Jersey, USA). Stock solutions (10 mM) of PDTC, DEX, MOL-294, and RWJ 67657 were diluted in DMSO (Merck, Darmstadt, Germany). The stock solutions were diluted in endothelial culture medium to final concentrations as indicated in each experiment.

Anti-inflammatory drugs were added to confluent HUVEC one hour before activation by TNF-α or IL-1β. After 6 and 24 hours of stimulation cells were analyzed microscopically with regard to their morphology and viability after which cells or supernatants were subjected to further analysis. By MTS assay the occurrence of toxic effects of drugs to the cells were excluded.
RNA isolation and real time RT-PCR analysis

Total RNA was isolated using the Absolutely RNA Microprep Kit (Stratagene, Amsterdam, The Netherlands) according to the protocol of the manufacturer. RNA was analyzed qualitatively by gel electrophoresis and quantitatively by RiboGreen RNA Quantitation Kit (Molecular Probes Europe B.V., Leiden, The Netherlands). One µg total cellular RNA was subsequently used for the synthesis of first strand cDNA using SuperScript III RNase H⁻ Reverse Transcriptase (Invitrogen, Breda, The Netherlands) in 20 µl final volume containing 250 ng random hexamers (Promega) and 40 units RNase OUT inhibitor (Invitrogen). After RT-reaction cDNA was diluted with distilled water to 100 µl. Exons overlapping primers and Minor Groove Binder (MGB) probes used for real time RT-PCR were purchased as Assay-on-Demand from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands): housekeeping gene GAPDH (assay ID Hs99999905_m1), endothelial cell marker CD31 (PECAM-1, Platelet Endothelial Cell Adhesion Molecule 1, Hs00169777_m1), E-selectin (Hs00174057_m1), VCAM-1 (Hs00365486_m1), ICAM-1 (Hs00164932_m1), IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1), COX-2 (Hs00153133_m1). The final concentration of primers and MGB probes in TaqMan PCR MasterMix (Applied Biosystems, Foster City, CA, USA) for each gene was 900 nM and 250 nM respectively. As controls, RNA samples not subjected to reverse transcriptase were analyzed to exclude unspecific signals arising from genomic DNA. Those samples consistently showed no amplification signals.

TaqMan real time RT-PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). Amplification was performed using cycling conditions: 2 min 50°C, 10 min 95°C, and 40 to 45 two-step cycles of 15 sec at 95°C and 60 sec at 60°C. Triplicate real time RT-PCR analyses were executed for each sample, and the obtained threshold cycle values (Ct) were averaged. According to the comparative Ct method described in the ABI manual (http://www.appliedbiosystems.com), gene expression was normalized to the expression of the housekeeping gene GAPDH, yielding the ΔCt value. The average ΔCt value obtained from resting, non-treated HUVEC was then subtracted from the average ΔCt value of each sample subjected to the experimental conditions described, yielding the ΔΔCt value. The gene expression level, normalized to the housekeeping gene and relative to the control sample, was calculated by 2⁻ΔΔCt.

IL-6 and IL-8 production measured by ELISA

In designated drug combination treatment experiments, HUVEC medium was harvested, centrifuged and stored at -20°C prior to ELISA based cytokine quantification. Ninety-six-well plates (Corning) were pre-coated with MoAb.anti-IL-6, clone 6/16 (Sanquin, Amsterdam, The Netherlands) 1:1000 diluted in PBS for IL-6, and with MoAb.anti-IL-8 (R&D Systems) for IL-8 analysis. After blocking with 2% BSA/0.05% Tween in PBS, samples were incubated for 2 hours in incubation buffer containing 0.2% gelatine/0.05% Tween in PBS. After washing, bound IL-6 or IL-8 were detected with biotinylated polyclonal swine anti-human IL-6 (Sanquin) or polyclonal swine anti-human IL-8 (R&D Systems), respectively, in combination with streptavidin-E⁺ (Sanquin). Peroxidase activity was determined using tetramethylbenzidine (Roth, Karlsruhe, Germany) as substrate. IL-6 and IL-8 levels...
were calculated in the linear range of the assay from a standard curve (10-1000 pg/ml) using r-hIL-6 and r-hIL-8 (both from R&D Systems).

**STATISTICS**
Statistical significance of differences was studied by means of the two-sided Student’s t-test, assuming equal variances. Differences were considered to be significant when p<0.05.

**RESULTS**
*In endothelial cells different gene expression patterns are induced by TNF-α and IL-1β*
To study gene expression profiles induced by TNF-α and IL-1β, HUVEC were activated with 1 and 10 ng/ml of both cytokines for 6 and 24 hours, and examined for the gene expression of E-selectin, VCAM-1, ICAM-1, IL-6, IL-8, and COX-2. The 6 hours time point reflects early gene expression profiles, while the 24 hours time point reflects late gene expression during prolonged activation, possibly comparable with prolonged exposure of the endothelium to activators in chronic inflammatory disorders.

![Figure 1. Differences in endothelial gene expression profiles induced by TNF-α or IL-1β activation. HUVEC were stimulated with 1 or 10 ng/ml of TNF-α (black bars) or IL-1β (open bars) for 6 (A) or 24 (B) hours. mRNA levels of genes studied were determined with real time RT-PCR and adjusted to the expression of house keeping gene GAPDH. mRNA levels were normalized to untreated, control cells arbitrary set at 1. Note the different values on Y axis. The data present mean values ± s.d. (n=3).](image)

Expression of the endothelial cell marker CD31 remained constant under all conditions studied (figure 1), a result corroborating previously published studies. Patterns of pro-inflammatory gene expression, however, markedly differed with regard to the
activator used and the incubation time studied. TNF-α induced the expression of adhesion molecules VCAM-1 and ICAM-1 to a higher extent than IL-1β, whereas IL-1β more profoundly affected IL-6, IL-8, and COX-2. For E-selectin a similar extent of expression was observed during activation by TNF-α and IL-1β with the exception of activation with 1 ng/ml of TNF-α or IL-1β at 24 hours. This apparent distinction was more pronounced at the early time point. At 6 hours, the level of gene expression induction was independent of the concentration of each activator, the only deviation being IL-1β induced IL-6 and ICAM-1 expression. Especially for TNF-α induced activation at 24 hours higher concentration of activator induced gene expression to the highest level. Stimulation of the expression of cell adhesion molecules was higher at 6 hours of activation compared to 24 hours of activation, while the opposite situation was observed for IL-8 and COX-2. IL-6 exhibited a mixed response, as its level of expression induced by TNF-α was highest at 24 hours, while IL-1β induced expression was higher at 6 hours.

**Adhesion molecule and cytokine gene expression are differently affected by TNF-α/IL-1β co-treatment**

Since the pro-inflammatory cytokines TNF-α and IL-1β can be present simultaneously at sites of inflammation, we investigated the gene expression profile of HUVEC when incubated with both TNF-α and IL-1β. A striking difference in gene expression was observed between cell adhesion molecules on one hand and cytokines and COX-2 on the other hand (figure 2). The observed level of gene expression of adhesion molecules upon co-treatment was lower than the expected level, which was calculated by summation of mRNA levels induced by separate cytokine treatment. This effect was most pronounced for VCAM-1 and ICAM-1 expression at 24 hours at 10 ng/ml concentration of both activators. In contrast, mRNA levels of cytokines and COX-2 were higher than expected from separate TNF-α or IL-1β treatment, with the strongest effects seen on IL-6 mRNA levels. Although the cytokine mediated effects on gene expression differed at the time points (as shown in figure 1), it is noteworthy that the pattern of deviation from the calculated values was similar at both time points. E-selectin was the only exception to this rule.

**Dominant-negative IκB mutant inhibits both TNF-α and IL-1β driven pro-inflammatory gene expression**

TNF-α and IL-1β induced signal transduction in endothelial cell relays mainly through the NF-κB and p38 MAPK routes. Yet the relative contributions of these pathways to the control of the expression of the genes under study are unknown. We investigated the functional relationship between NF-κB activity and pro-inflammatory gene expression in HUVEC using overexpression of dnIκB. HUVEC infected with LacZ adenovirus and HUVEC infected with dnIκB adenovirus were activated with 1 and 10 ng/ml of TNF-α or IL-1β for 6 and 24 hours. Approximately 70-80% of cells were infected by adenoviral infection as established with green fluorescent protein reporter protein (K.I. Ogawara et al, unpublished observations). Expression of dnIκB
Figure 2. Effects of TNF-α and IL-1β co-treatment on HUVEC pro-inflammatory gene expression.

HUVEC were activated for 6 and 24 hours with mixtures of TNF-α and IL-1β in designated concentrations. Gene expression determined by quantitative RT-PCR, was adjusted to GAPDH and normalized to untreated, control HUVEC. The experimentally measured gene expression levels induced by TNF-α/IL-1β combination treatment were compared to the theoretical, expected gene expression levels calculated by summation of gene expression levels observed when cells were treated with TNF-α or IL-1β separately. This later value was arbitrary set at 1. Values above 1 represent situations where experimentally determined mRNA levels were higher than the expected additive value. Values below 1 indicate experimentally determined mRNA levels lower than the expected additive value. The gray area borders the lower through upper limit of the observed level of gene expression that is considered to be within the range of the expected additive level. The data present mean values ± s.d. (n=3).

transgene in HUVEC was confirmed by western blot analysis (figure 3A). In the conditions studied only a minor fraction of cells went into apoptosis upon TNF-α activation. Likely, NF-κB independent cell survival was controlled by serum derived growth factors as the cells were continuously cultured in medium containing FCS.

An almost total inhibition of adhesion molecule expression induced by 10 ng/ml TNF-α or IL-1β was observed in dnIkB-expressing HUVEC compared to uninfected or LacZ infected cells, at both time points studied (figure 3B). A more limited inhibition of gene expression was observed for IL-6 and IL-8 at 6 hours, while at 24 hours the inhibition of these genes was also blocked. The limited COX-2 gene expression observed after 6 hours after start of activation was not NF-κB dependent. Possibly p38 MAPK and PKC control COX-2 expression here\(^{27,28}\). However, after 24 hours a significant inhibition of COX-2 expression was observed in dnIkB expressing cells. Likely, during this longer time period cytokines expressed in an NF-κB independent manner induce, in an autocrine fashion, NF-κB dependent COX-2 expression\(^{29,30}\). A similar pattern of inhibition of gene expression for all genes was seen with 1 ng/ml activator (data not shown).
These data demonstrated in a quantitative manner that the upregulation of pro-inflammatory genes induced by TNF-α and IL-1β are largely (adhesion molecules) or at least partly (interleukins and COX-2) under the control of NF-κB.

Figure 3. Overexpression of dominant-negative IκB inhibits TNF-α and IL-1β driven pro-inflammatory gene expression.

(A). dnIκB-HA was overexpressed in HUVEC as demonstrated by Western Blot. HUVEC were infected with adenovirus and 24 hours later cell lysates were subjected to SDS-PAGE. Immunoblotting with antibody directed against HA-tag showed a significant expression of immuno-detectable dnIκB compared to endogenous protein level.

(B). Effect of adenovirally induced dnIκB expression on TNF-α and IL-1β induced pro-inflammatory gene expression. Uninfected HUVEC (open bars), and HUVEC infected with LacZ as a control (black bars), or dnIκB (grey bars) were stimulated with 10 ng/ml of TNF-α or IL-1β for 6 or 24 hours. mRNA levels were determined by real time RT-PCR, adjusted to the house keeping gene GAPDH, and normalized to the control, non-infected HUVEC cells arbitrary set at 1. The data present mean values ± s.d. (n=3).

Effects of chemical inhibitors of intracellular signalling pathways on inflammatory gene expression

From the experiments performed with dnIκB expressing HUVEC a possible involvement of (an)other cell activation pathway(s) in regulation of cytokine induced inflammatory gene expression became apparent. Therefore we investigated the effects of NF-κB inhibitors with different molecular targets, and of one p38 MAPK inhibitor.
HUVEC were incubated with 1 µM of PDTC, DEX, MOL-294, and RWJ 67657 starting 1 hour before the addition of 10 ng/ml TNF-α or IL-1β. The choice of this fixed concentration was based on the experience that approximately 1 µM of drug can be delivered using drug targeting constructs. As shown in figure 4, the drugs affected inflammatory gene expression differently with respect to the activator used and the time interval studied. RWJ 67657 and MOL-294 were the most potent inhibitors showing downregulation of several inflammatory genes. Specific inhibition of p38 MAPK activity by RWJ 67657 resulted in blocking of gene expression of the interleukins at 6 hours and additionally of the adhesion molecules after 24 hours. MOL-294 treatment resulted in an inhibitory effect on both TNF-α and IL-1β induced adhesion molecule expression, with the most pronounced effect on VCAM-1 expression at both 6 hours and 24 hours. We consistently found significantly higher IL-8 mRNA levels at 6 hours after TNF-α treatment in combination with this drug, which is at present difficult to explain. DEX only downregulated IL-6 mRNA gene expression after 6 hours of TNF-α activation, and VCAM-1 mRNA levels after 24

Figure 4. Effects of inhibitors of signal transduction on pro-inflammatory gene expression by HUVEC.

Drugs were added at 1 µM 1 hour before activation with 10 ng/ml of TNF-α or IL-1β. mRNA levels of designated genes at 6 or 24 hours were determined with real time RT-PCR, adjusted to the house keeping gene GAPDH, and normalized to untreated, non-activated control HUVEC arbitrary set at 1. The 100% value represents activation in the absence of drugs. 0% represents the mRNA level of control cells. Mean values ± s.d. (n=3); *, p < 0.05; **, p < 0.001 between cells activated with TNF-α or IL-1β in the absence of drugs, and cells treated with drugs.
hours of IL-1β activation. Moreover, pretreatment of HUVEC with 1 µM DEX resulted in a significant increase in IL-6 mRNA level 24 hours after IL-1β stimulation compared to untreated cells. The minor modulatory effects of PDTC on gene expression was possibly due to the use of relatively low concentrations of this drug as compared to the concentrations used in other studies

**Combination of drugs enhance inhibitory effects on gene and protein expression in HUVEC**

RWJ 67657 and MOL-294 affect different routes of the main pro-inflammatory activation pathways. Since they both showed pronounced inhibitory effects on TNF-α and IL-1β induced gene expression in HUVEC, we hypothesized that combination treatment employing these two drugs might result in enhanced inhibitory effects. Therefore the effects of simultaneous addition of these drugs in concentrations ranging from 0.1 to 10 µM on the expression levels of studied genes were investigated. These effects were measured at 6 hours after induction of activation by 10 ng/ml TNF-α or IL-1β. In all concentrations and combinations studied the drugs appeared not to be toxic to the HUVEC (data not shown). In the case of expression of the cell adhesion molecules an additive inhibitory effect of RWJ 67657/MOL-294 co-treatment was observed on TNF-α-mediated E-selectin gene expression (figure 5). One µM MOL-294 by itself downregulated E-selectin mRNA levels from 5725 (± 1583) fold increase to 4340 (± 614) fold increase (n=3 ± s.d.). Co-incubation with 0.1, 1, and 10 µM RWJ 67657 resulted in further statistically significant decrease of gene expression to respectively 3532 (± 485; p<0.05 vs. activated cells) fold, 2943 (± 283; p<0.05) fold, and 2117 (± 223; p<0.005) fold. Both TNF-α and IL-1β induced IL-6, IL-8, and COX-2 expression was inhibited in an additive way by drug combination treatment, with different concentration combinations being responsible for the enhanced inhibition of genes expression observed.

To confirm this increase in the inhibitory potential of RWJ 67657 and MOL-294 combination treatment on the expression of IL-6 and IL-8, proteins were measured by ELISA in the supernatants of selected samples harvested at 6 hours (figure 6). The cytokine levels reflected the gene expression data, although the additive effects of drugs co-treatment were less pronounced. This may be due to the limit of detection of the ELISA assays used. A consistently found deviation from this pattern was IL-8 expression in samples treated with 10 µM MOL-294. Total blocking of the protein expression was paralleled by upregulated mRNA levels.
**Figure 5.** Effect of combination treatment of HUVEC with RWJ 67657 and MOL-294 on pro-inflammatory gene expression.

HUVEC were treated with RWJ 67657 and MOL-294, added alone or in combination in concentrations ranging from 0.1 – 10 \( \mu \)M. After 1 hour 10 ng/ml of TNF-\( \alpha \) or IL-1\( \beta \) was added and cells were incubated for another 6 hours. mRNA levels were determined using real time RT-PCR. Data were adjusted to the house keeping gene GAPDH, and normalized to untreated, non-activated control HUVEC arbitrary set at 1. The data present mean values ± s.d. (n=3); *, p < 0.05 between cells treated by the combination of drugs and cells treated with each drug separately.
Figure 6. Comparison of IL-6 and IL-8 gene and protein levels in HUVEC treated by RWJ 67657, MOL-294, or a combination. HUVEC were treated with designated concentrations of RWJ 67657 and MOL-294 for 1 hr, after which either TNF-α or IL-1β was added at 10 ng/ml to activate the cells. Fold induction of mRNA level (left axis) at 6 hours after start of activation was compared with the amount of secreted IL-6 or IL-8 protein determined by ELISA (right axis). Undetectable protein levels (ELISA detection limits are 20 pg/ml) are marked by asterisks. The data present mean values ± s.d. (n=3).

DISCUSSION
In the current study we showed that TNF-α and IL-1β differentially activated inflammatory gene expression in endothelial cells in vitro. Of the four different inhibitors of intracellular signalling cascades studied, p38 MAPK inhibitor RWJ 67657 and the small redox active protein thioredoxin inhibitor MOL-294 were
identified as the most potent drugs showing downregulation of a number of different inflammatory genes. Co-treatment with both drugs resulted in an enhancement of the inhibitory effect on pro-inflammatory gene expression. This observation can have important implications for (targeted) pharmacologic intervention to downregulate endothelial cell activation to reduce leukocyte infiltration in the diseased tissue. It can be envisioned that above mentioned drugs are either administered in a combination treatment protocol or both included in immunoliposomes harnessed with antibodies specifically recognizing activated endothelial cells. Immunoliposome based drug delivery systems can theoretically deliver low micromolar concentration of drugs.

Besides the pharmacologic profile demonstrated here, 1 µM RWJ 67657 was shown to completely inhibit MAPKAPK-2 phosphorylation in HUVEC (Westra et al, Int Immunopharmacol 2005, in press). Low micromolar concentration of MOL-294 was furthermore able to block the DNA binding ability of NF-κB in HUVEC with IC50 value of VCAM-1 expression inhibition of 2.5 µM. Future studies on incorporation of these drugs in the carrier systems developed in our laboratory will allow us to investigate endothelial cell response to these drugs in vitro and in vivo.

We found that the pattern of pro-inflammatory gene expression markedly differed depending on the activator used. IL-1β induced IL-6, IL-8, and COX-2 gene expression by HUVEC to a higher extent than TNF-α, whereas TNF-α more profoundly affected the expression of adhesion molecules. These data are in line with those reported by others, although these latter studies were not performed in a quantitative manner and did not compare all the genes in a direct manner.

Both TNF-α and IL-1β are present simultaneously in pro-inflammatory diseases. Since limited data are available on endothelial cell pro-inflammatory gene expression after simultaneous cytokine treatment, we studied the effects of TNF-α/IL-1β co-treatment on gene expression by HUVEC. The most interesting observation arising from this experiment was the occurrence of diminished upregulation of adhesion molecule mRNA, while an additive or even increased induction of interleukins and COX-2 mRNA was found. A possible explanation is that TNF-α and IL-1β partly utilize the same cell activation pathways. A combinatorial activation might saturate common cofactors or change downstream signaling specificity by utilization of other scaffold proteins leading to differences in response to both activators when compared to either activator alone. Since the molecular mechanism(s) of the observed attenuation in gene expression induced by pro-inflammatory activator co-treatment is/are at present not known, we decided to perform our subsequent pharmacologic studies by using TNF-α and IL-1β separately. Still, this observation justifies further research, as it has important implications for the choice of the experimental conditions to study the pharmacologic potency of new chemical entities in the drug development pipeline.

Both the dnIκB adenovirus and drug treatment experiments confirmed a major role of NF-κB and p38 MAPK pathways in regulation of gene expression in HUVEC as part of the inflammatory response induced by TNF-α or IL-1β. An almost total inhibition of adhesion molecule expression in dnIκB-expressing HUVECs was observed at both early and late time points of activation. The induction of IL-6 and IL-8 expression was also inhibited by dnIκB, but to a lower extent compared to the effects
on the adhesion molecules, indicating considerable involvement of (an)other pathway(s) regulating IL-6 and IL-8. Ridley et al described that the expression of IL-6 and IL-8 induced by IL-1β in HUVEC was p38 MAPK dependent. In another study TNF-α induced IL-8 gene and protein expression was shown to be partly dependent on reactive oxygen species generation and activator protein 1 activation. Also from our pharmacological experiments, the conclusion seems justified that p38 MAPK is, at least partly, controlling expression of these genes.

MOL-294 pretreatment of cells consistently resulted in IL-8 mRNA increases at the early time point compared to untreated controls. Analysis of protein levels, however, revealed a complete block of IL-8 production. This discrepancy may be a result of uncoupling of gene and protein expression, e.g., due to processing of proteins that affect the secretion and/or modifies their recognition sequence directing proteins for ubiquitination. Another explanation may be the fact that thioredoxin is required for efficient proteolysis catalysed by thiol-dependent Cys-proteases such as cathepsin. Cathepsins are known to be essential in processing of mature IL-8 protein at inflammatory sites.

In summary, we performed a quantitative study on pro-inflammatory gene expression in HUVEC, in which we showed that TNF-α and IL-1β differentially induced cell adhesion molecule and cytokine gene expression when added alone. Combination treatment with both cytokines resulted in deviation from the expected induction of mRNA levels of the genes. This observation may be relevant for endothelial cell activation and its complex control mechanisms in inflammatory conditions in vivo. The demonstrated additive effects of combinations of anti-inflammatory drugs that inhibit NF-κB and p38 MAPK signal transduction forms the basis for further research to study whether co-administration can improve the efficacy of the (targeted) drugs to inhibit inflammatory disease activity.

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**REFERENCES**

NF-κB and p38 MAPK inhibition in EC


