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INHIBITION OF NF-κB BY PYRROLIDINE DITHIOCARBAMATE BLOCKS ENDOTHELIAL CELL ACTIVATION+

Christiane Ferran*, Maria T. Millan, Vilmos Csizmadia, Jeffrey T. Cooper, Christine Brostjan, Fritz H. Bach and Hans Winkler

Sandoz Center for Immunobiology, New England Deaconess Hospital, Harvard Medical School, 99 Brookline Avenue, Boston, MA 02215

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SUMMARY: Endothelial cell activation is achieved by the rapid, protein synthesis-independent induction of a characteristic set of genes. Because of the abundance of binding sites for the transcription factor NF-κB in the regulatory region of the aforementioned genes, we hypothesized that this factor might play a key role. Reactive oxygen intermediates act as second messengers in the activation of NF-κB. We have used the antioxidant pyrrolidine dithiocarbamate to analyze the effect of NF-κB inhibition on TNFα-induced EC activation *in vitro*. We show that pyrrolidine dithiocarbamate strongly reduces the TNFα–mediated induction of E-selectin, VCAM-1, ICAM-1, PAI-1, tissue factor, IL-8 and IκB-α. We present evidence identifying NF-κB as a central regulator of EC activation. Therefore, this factor may represent a prime target for therapeutic intervention in pathologic conditions associated with EC activation such as allo- and xenograft rejection, atherosclerosis, ischemic reperfusion injury and vasculitis.

In the healthy vasculature, endothelial cells form a barrier which prevents the egress of proteins and cells into the parenchymal tissue and maintain a physiologic anticoagulatory environment that prevents undesirable intravascular coagulation (1-3). However, under certain inflammatory conditions such as reperfusion injury, bacterial and viral infections, autoimmune diseases as well as allo- and xenograft rejection, EC change their phenotype

+This is manuscript 630 from our laboratories.

*Correspondence should be addressed to Christiane Ferran.

Fax Number: 1-617- 632 08 80. E mail : cferran @ nedhmail. nedh. harvard. edu.

Abbreviations: VCAM-1: Vascular Cell Adhesion Molecule 1, ICAM-1: Intercellular Adhesion Molecule 1, NF-κB: Nuclear Factor kappa B, IκB-α: Inhibitor of NF-κB, TF: Tissue Factor, PAI-1: Plasminogen Activator Inhibitor 1, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, AP-1: Activator Protein 1, ATF: Activating Transcription Factor, CREB: cAMP responsive element binding protein, PDTC: Pyrrolidine Dithiocarbamate, EC: Endothelial Cells.

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and their barrier function is compromised, allowing cell adhesion and transmigration and favor coagulation and thrombosis. These changes have been referred to as EC activation (4). These manifestations of the activated EC are in large measure based on the induction of a wide variety of genes including those encoding adhesion molecules (E-selectin, VCAM-1, ICAM-1, P-selectin), cytokines (IL-1, IL-6, IL-8) and pro-thrombotic molecules (tissue factor (TF), plasminogen activator inhibitor (PAI-1)) as well as the loss of thrombomodulin and heparan sulfate (5,6). *In vitro* such conditions can be generated upon stimulation of EC with TNF, IL-1, PMA or endotoxin (7-9). *In vivo*, especially in the rejection of xenografts, prominent intragraft EC activation has been demonstrated. It is our hypothesis that EC activation is the pathophysiological basis for delayed xenograft rejection which occurs when hyperacute rejection is averted by inhibiting complement activation (9-11). To effectively counteract EC activation in xenografts, it was important to identify a common regulator of EC activation.

It has been noted that the *de novo* expression of genes induced in EC is mainly regulated at the transcriptional level and is independent of protein synthesis. Therefore, the immediate early induction of these genes requires the action of pre-existing transcription factors. Sequence analysis of the regulatory regions of these genes induced upon EC activation reveals that they all share at least one element i.e., a binding site for the preexisting transcription factor NF-κB (12-19). There are only a few reports, dealing with only one or a few genes at a time, one from our laboratories regarding IκB-α up-regulation in porcine aortic endothelial cells (PAEC) (16), from Marui et al. (20) regarding VCAM-1 up-regulation in human umbilical vein endothelial cells (HUVEC), from Read et al. (21) and from Orthner, et al. (22), that suggest that NF-κB is important in the induction of endogenous genes that are up-regulated with EC activation. Several other studies have used reporter constructs, which may or may not reflect the role of NF-κB in the induction of genes in their natural DNA context.

NF-κB is a transcriptional activator associated with immediate early gene expression. The canonical, active DNA binding form is a heterodimer consisting of members of the NF-κB/Rel family of transcription factors, most prominently the NF-κB1 (p50) and RelA (p65) subunits. NF-κB is retained in the cytoplasm of quiescent endothelial cells by association with its inhibitory protein IκB-α (23). Upon activation of the cell, IκB-α is phosphorylated and becomes susceptible to proteolysis, which leads to dissociation from the NF-κB dimer (24,25). The release of NF-κB from IκB-α in turn allows the active p50-p65 dimer to transmigrate immediately into the nucleus, bind to its target DNA sequence element and activate transcription. It has been demonstrated that the activation of NF-κB and the loss of IκB-α can be inhibited by anti-oxidants suggesting a crucial role of reactive oxygen species as mediators of NF-κB activation (26-28).

Because porcine organs represent the most likely source of xenografts, we have specifically chosen human TNF-α to stimulate PAEC and to test the effect of NF-κB inhibition by pyrrolidine dithiocarbamate (PDTC), an anti-oxidant inhibitor of NF-κB activation, on gene up-regulation. In this first extensive study of a xenogeneic setting, we

find that E-selectin, VCAM-1, ICAM-1, TF, PAI-1, IL-8 and ECI-6/IκB-α that are induced upon EC activation are inhibited by PDTC. Jun B, which does not have NF-κB binding sites in its regulatory region, and constitutively expressed genes were not affected by PDTC pre-treatment. Furthermore, there is a very good correlation between inhibition of NF-κB activation and the decrease of expression at the protein level of these genes.

Our finding that NF-κB inhibition is able to notably attenuate activation of porcine aortic endothelial cells suggests NF-κB as a prime target for therapeutic intervention in xenotransplantation.

MATERIALS AND METHODS

PAEC culture and treatment protocols. Fresh endothelial cells (EC) were isolated from porcine aortas by scraping and cultured in Dulbecco's modified Eagles Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, High Clone, Gibco BRL, Grand Island, NY, USA) and 50 U/ml Penicillin/Streptomycin. Confluent cells (between passages 5 and 10) from 175 cm^2 culture dishes (Nunc, Marsh, Rochester, NY, USA) were used for all experiments. Recombinant human TNF was a kind gift from Sandoz Pharmaceuticals (New Jersey, USA). Pyrrolidine dithiocarbamate (PDTC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and diluted in 1 x PBS before adding to cell culture medium. The use of a metal spatula was always avoided. Confluent PAEC monolayers were treated or not with PDTC at 100 μM for 1.5 h prior to stimulation by TNF at 100 U/ml.

Hydrogen peroxide measurements. H_2O_2 production by PAEC was assayed by means of a fluorometric assay as described (29). Briefly, this assay is based on the H_2O_2-dependent oxidation of homovanillic acid (HVA) to a highly fluorescent dimer which is mediated by horse radish peroxidase. The HVA oxidation product was determined fluorometrically using a Perkin-Elmer Model 3000 spectrofluorometer (Perkin-Elmer, Ueberlingen). Excitation was at 321 nm and emission was measured at 421 nm.

Nuclear Extracts. Confluent PAEC monolayers were treated or not with PDTC (100 μM) for 1.5 h prior to stimulation with TNF(100 U/ml). Nuclear proteins were extracted from PAEC before stimulation and 1.5, 6 and 24 h after stimulation with TNF according to the method described elsewhere (30). Protease inhibitors were added to PMSF in all buffers used during nuclear extraction namely leupeptin (0.5μg/ml), antipain (0.5 μg/ml), aprotinin (0.5 μg/ml), pepstatin (1 μg/ml) benzamidine (100 μg/ml), chymostatin (100 μg/ml), TLCK (50 μM) and TPCK (100 μM). Protein concentration of nuclear extracts was determined by a Bradford assay (31) (bovine serum albumin was used as the standard). Nuclear extracts were frozen on dry ice and stored at -80 °C until used.

Electrophoretic Mobility Shift Assay (EMSA). The probes used in all experiments were end-labeled with α-[^{32}P]-dATP (80 μCi at 3000 Ci/ mmol, Amersham, Arlington Heights, IL, USA) using the Klenow fragment of E. coli DNA polymerase I in the presence of unlabeled dTTP, dCTP and dGTP. Binding reactions in 25 μl contained 100,000 cpm of double stranded oligonucleotide, 3 μg of poly (dI-dC), and 5 μg of nuclear extract proteins in 20 mM HEPES pH 7.9, 50 mM NaCl, 1 mM EDTA. 1 mM β-mercaptoethanol and 5% glycerol. Binding reactions were carried out for 30 minutes at ambient temperature. For electrophoresis, high ionic strength 6% - polyacrylamide gels were used as described (32).

Double stranded oligonucleotide containing a porcine E-selectin NF-κB site (pELκB-5'-AATTCATGCTGCTGGGAATTCCTCTGTATGAATTC-3') (H.Winkler, unpublished), the second porcine ECI-6 (IκB-α) oligonucleotide (BS-2-5'-AATTCGGCTTGGAAATTCCCCGAGCG-3'). Nonlabeled probes or nonspecific

oligos such as a mutated κB oligo (mutκB-5'-AGCTTAGATTTTACTTTCCGAGAGGA-3') were sometimes added to the reactions in order to determine specificity of the binding.

RNA Extraction and Northern Blot Analysis. Total RNA was obtained from cultured PAEC according to the method described by Chomczynski and Sacchi (33), which involves a single step of acid guanidinium thiocyanate/ phenol-chloroform extraction. The amount of extracted RNA was calculated from optical density measurements at 260 nm (1 OD_{260} ~ 40 μg/ml). Purity and integrity of RNA samples were confirmed in 1% agarose gels containing ethidium bromide. For Northern Blot analysis, equal amounts of RNA (15 to 20 μg per lane) were loaded and run on a 1.3% agarose/formaldehyde gel. RNA was then transferred to a Nylon membrane and hybridized to cDNA probes encoding for porcine E-selectin, IL-8, and TF derived by Drs. H. Winkler, R. de Martin and E. Hofer, respectively (unpublished data from our laboratories), ECI-6 (the porcine equivalent of the human IκB-α gene) (15), porcine IL-6 (a kind gift from Drs. Molitor and Murtaugh, University of Minnesota, Minneapolis) , human VCAM-1, ICAM-1, junB and thioredoxin (kind gifts from Drs. T. Collins Brigham & Women's Hospital, Boston, J. Pober, Yale University, and E. Wollman, INSERM U 283 Hopital Cochin Paris, France, respectively), mink Plasminogen activator inhibitor (PAI-1) (a kind gift of Dr. B. Kallin, Karolinska Institute, Sweden). In all experiments a cDNA probe for human GAPDH was used to confirm equal loading of RNA in all the wells. All probes were labeled with α–[^{32}P]-dATP (Amersham) using a random primer labeling kit (Stratagene, La Jolla, CA, USA).

E-Selectin ELISA. PAEC were cultured to confluence in 96 well tissue culture plates. They were then treated or not with PDTC (100 μM) 1.5 h prior to stimulation with 100 U/ml of TNF for 4 hours. The cells were then washed 3 times with PBS, fixed in ice-cold 0.02% glutaraldehyde at 4 °C for 5 minutes. Cells were then incubated with a mouse mAb directed against human E-selectin and shown to cross-react with porcine E-selectin (BBA1). The BBA1 mAb was purchased from R&D Systems (Minneapolis, MN, USA) and used at a 1:10,000 dilution of the hybridoma supernatant. A goat anti-mouse peroxidase-coupled polyclonal antibody purchased from Pierce was used as secondary antibody. Optical density was determined at 490 nm on a LKB ELISA reader.

RESULTS

Basal and inducible production of H_2O_2 is inhibited by PDTC. H_2O_2 and its split product, the hydroxyl radical ($HO^{\bullet}$), have been implicated as second messengers in the activation of NF-κB (27). Based on this hypothesis, we tested the thiol compound PDTC, a known anti-oxidant free radical scavenger, for its effect on H_2O_2 levels in supernatants of cultured endothelial cells. Confluent PAEC spontaneously produced low levels of H_2O_2 ranging from 0.005 to 0.009 nmoles/min/1 x 10^6 cells. Upon stimulation with TNF, H_2O_2 production increased by 1.5 to 2 fold ranging from 0.01 to 0.015 nmoles/min/ 1 x 10^6 cells. PDTC treatment (100 μM) of the cells strongly decreased the basal as well as the induced H_2O_2 production by the PAEC ($p < 0.001$) (Figure 1). The concentration of PDTC used in all experiments had no cytotoxic effect during 24 hours of observation, using Trypan Blue uptake as an indicator (data not shown).

NF-κB activation is inhibited by PDTC. Treatment of PAEC with PDTC 1.5 h prior to the addition of 100 U/ml of TNF inhibited NF-κB binding activity at all studied time points i.e. 1.5, 6 and 24 h after TNF addition (Figures 2 A & B). Two NF-κB binding sites were

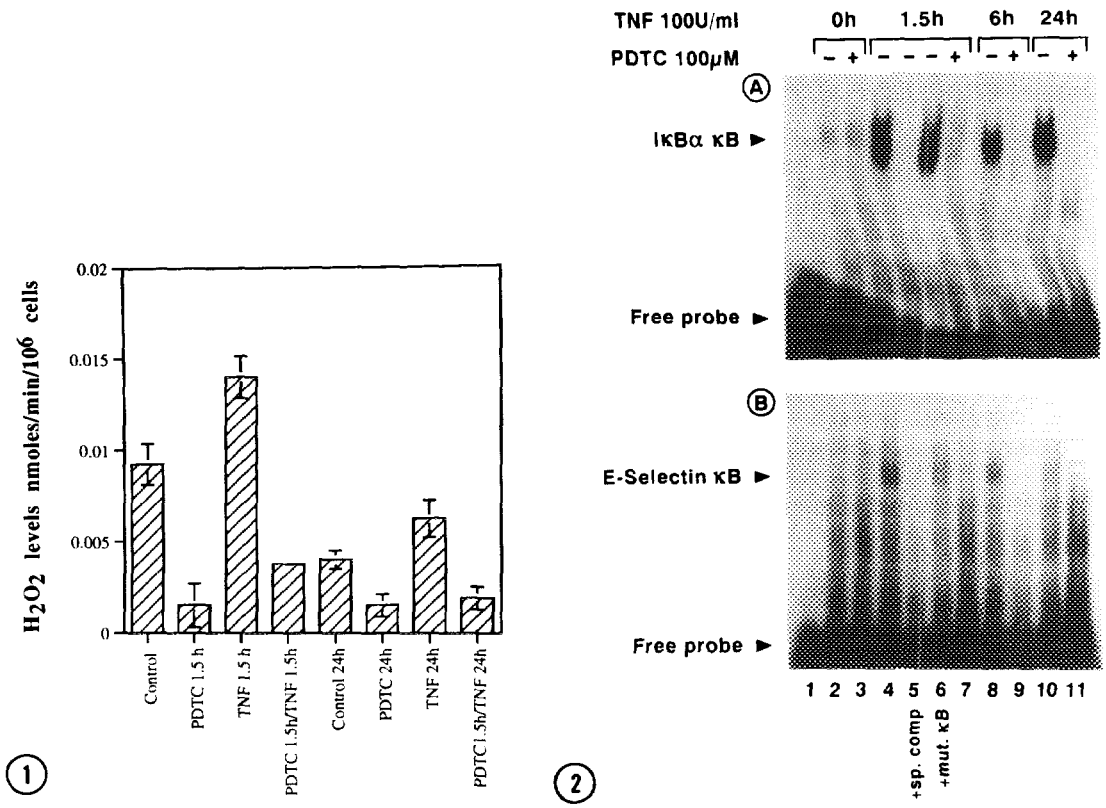

Figure 1. H_2O_2 production by PAEC. Basal levels as well as TNF-induced H_2O_2 levels are significantly decreased when cells are pretreated with PDTC $p > 0.001$. The results shown are representative of at least three independent experiments.

Figure 2. Panel A: Electrophoretic mobility shift assay (EMSA) using a probe corresponding to the second ECI-6 promoter (porcine IκB-α) κB binding site. NF-κB binding is induced upon TNF stimulation of PAEC. This binding is detected up to 24 h (lanes 4, 8 and 10) and is totally abrogated when cells are pretreated with 100 μM of PDTC 1.5 h prior to TNF stimulation (lanes 7, 9 and 11).
Panel B: EMSA using the first porcine E-selectin promoter κB binding site as a probe. Results are similar to that seen in Figure 2 A. mut κB refers to a non-binding, mutated NF-κB oligonucleotide; sp. comp. refers to specific competition oligonucleotide (unlabeled probe). The results shown are representative of at least three independent experiments.

studied: a NF-κB binding site of the porcine ECI-6/IκB-α promoter (15) (BS-2) (Figure 2A) and a NF-κB binding site of the porcine E-selectin promoter (Figure 2B). PDTC alone had no effect on NF-κB binding activity in this system.

Inhibition of NF-κB strongly decreases induction of E-selectin expression. The effect of NF-κB inhibition on E-selectin mRNA expression was evaluated by means of Northern Blot analysis and an E-selectin ELISA was used to assess protein expression. Our results (Figure 3A) show that E-selectin mRNA was not detectable in unstimulated cells. In

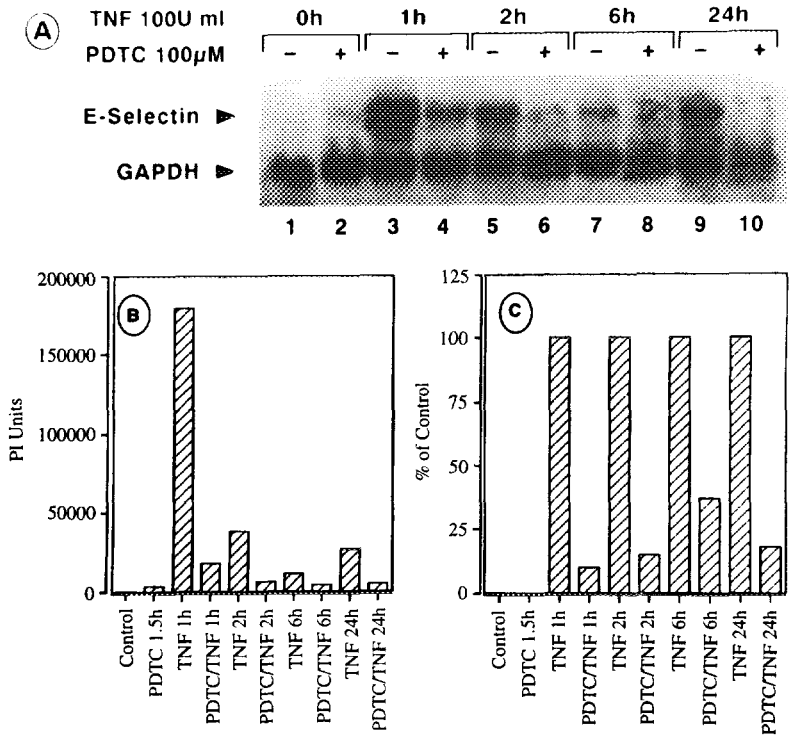

Figure 3. Panel A: E-selectin steady state mRNA levels in PAEC before and after stimulation with TNF alone or in association with PDTC. No mRNA encoding for E-selectin is detectable in quiescent post-confluent endothelial cells. PDTC alone was not able to induce E-selectin expression (lane 2). One hour after TNF stimulation high levels of E-selectin mRNA were detected (lane 3). Bimodal expression was always observed, with 2 peaks of expression at 1 and 24 h after stimulation (lanes 3, 9). PAEC pretreated with PDTC prior to TNF showed a remarkable 80 to 90% decrease of the mRNA levels at all considered time points (lanes 4, 6, 8, 10).

Panels B, C: Phosphorimager quantification of the PDTC-dependent inhibition of E-selectin expression in TNF-activated PAEC. Results are expressed in arbitrary units (generated by the Phosphorimager: PI) in Figure 3B and as percentage of positive controls (cells treated with TNF alone) at each time point in Figure 3C. The results shown are representative of at least three independent experiments.

response to TNF stimulation, there was marked induction of E-selectin mRNA levels in a bi-phasic mode, peaking at 1 and 24 h (lanes 3 and 9). Pretreatment of the PAEC with PDTC reduced the TNF-induced E-selectin mRNA levels by 80% to 90% at all time points tested (Figures 3A, B, and C). Quantitation was performed on a Molecular Dynamics PhosphorImager; the results are shown in Figures 3B and C as phosphorimager (PI) counts and as percentage of control, respectively. The level of inhibition was reproducible. All data shown represent one experiment out of a series of three. In accordance with the observed reduction in E-selectin mRNA levels, the amount of detectable E-selectin protein was equally reduced: inhibition of NF-κB by PDTC decreased TNF induced E-selectin protein expression by 80-90% as compared to untreated cells (Figure 4).

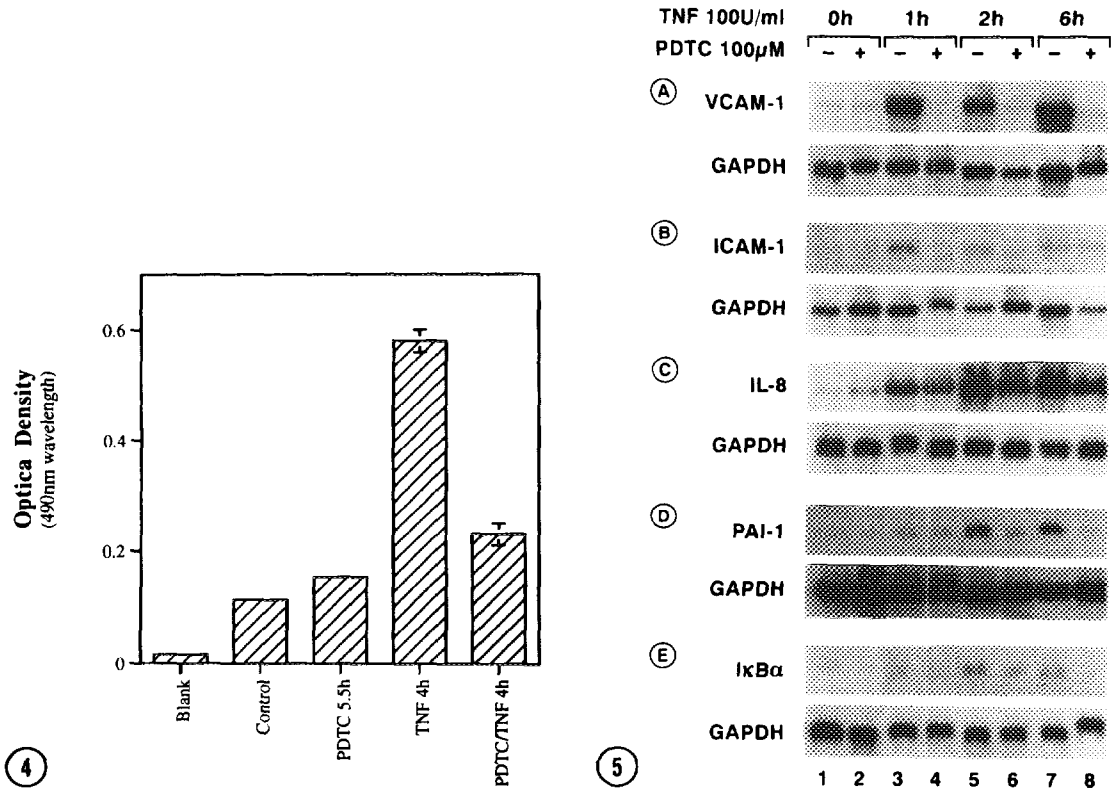

Figure 4. E-selectin protein expression upon TNF stimulation as assessed by means of a specific ELISA. As for the mRNA levels TNF-related E-selectin protein expression is inhibited up to 80% by PDTC pretreatment. The results shown are representative of at least three independent experiments.

Figure 5. Northern blot analysis of gene expression in PAEC treated with TNF as compared to cells pretreated with PDTC prior to TNF stimulation: **Panel A:** VCAM-1 mRNA is not detected in quiescent PAEC. Upon TNF stimulation, peak expression is noted within an hour and lasts up to 24 h. PDTC pretreatment inhibits up to 95% mRNA expression at all tested time points. **Panel B:** ICAM-1 basal mRNA expression is not modified when PDTC alone is added to the culture medium. Upon TNF stimulation, high mRNA levels are detected with a peak expression between 1 and 2 h. PDTC pretreatment also inhibits this expression up to 80% at 1 and 2 h but no longer at later time points starting at 6 h. **Panels C, D, E:** Low levels of basal IL-8, PAI-1 and IκBα mRNA are detected in quiescent untreated PAEC and are not modified by PDTC addition. Upon TNF stimulation, increased expression is noted within an hour, peaking between 1 and 4 h after stimulation and lasting up to 24 h. PDTC pretreatment inhibits up to 80% of all the gene expressions at all tested time points. The results shown are representative of at least three independent experiments.

The induction of a number of genes expressed in activated EC is strongly decreased in the absence of NF-κB. Besides E-selectin, we studied the NF-κB dependence of two additional adhesion molecules induced upon EC activation, namely VCAM-1 (Figure 5A) and ICAM-1 (Figure 5B). No basal VCAM-1 mRNA levels were detected prior to TNF stimulation. High levels of mRNA encoding for VCAM-1 were seen as early as 1 h after

TNF stimulation and up to 24 h. Pretreatment with PDTC led to a very strong (> 90%) reduction of steady state mRNA levels (Figure 5A). While the inhibition of NF-κB by PDTC does not influence basal ICAM-1 mRNA levels, the increase in mRNA levels induced by TNF treatment (seen as early as 1 h after addition of TNF) were strongly reduced up to 6 h following TNF stimulation but not further on (Figure. 5B). The use of a human ICAM-1 probe at high stringency accounts for the weak signal in figure 5 B. Basal IL-8 mRNA levels were not modified by PDTC, TNF-induced expression was reduced by 60-70% by PDTC pretreatment as determined by phoshorimager analysis (Figure 5C). We next analyzed the behavior of PAI-1 and IκB-α, all known to be induced in activated EC. Our results showed that similar to the adhesion molecules, induction of these genes was strongly inhibited by PDTC addition (Figures 5 D and E).

JunB, a gene without NF-κB binding sites in its regulatory region, is not affected by NF-κB inhibition. Jun-B, a proto-oncogene which exhibits cytokine-inducible expression but does not have known NF-κB binding sites in its regulatory region, was tested as a negative control for NF-κB related inhibition of gene expression. Jun-B mRNA was induced by TNF with peak expression at 4 h that was not altered by PDTC pretreatment of the PAEC (Figure 6).

Constitutive mRNA levels of GAPDH and thioredoxin remain unchanged by treatment with PDTC. RNA blots were also probed for thioredoxin, an endogenous protein present in EC which is involved in the oxidant stress response (34). There is a detectable amount of basal expression in quiescent PAEC. Thioredoxin mRNA levels were not affected by TNF stimulation and/or PDTC treatment (Figure 7). The constitutive expression of the housekeeping gene GAPDH was equally unaffected by TNF and PDTC.

DISCUSSION

The data presented in this study support the hypothesis that NF-κB is a major factor that leads to changes of gene expression in endothelial cells upon cytokine-induced

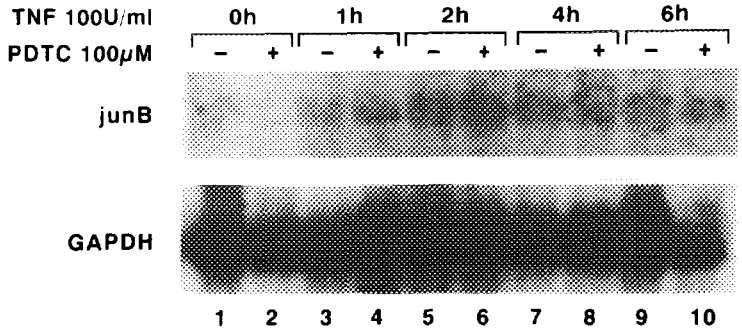

Figure 6. Northern Blot analysis using a cDNA junB probe. jun-B mRNA levels are increased by TNF stimulation with a peak expression seen at 4 h. This TNF-related increase of mRNA levels is not modified by PDTC pretreatment of the cells. The results shown are representative of at least three independent experiments.

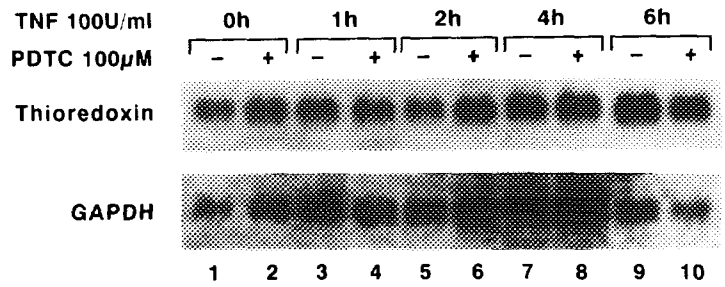

Figure 7. Northern Blot analysis using a cDNA Thioredoxin probe. High constitutive levels of mRNA encoding for Thioredoxin are detected in resting untreated PAEC. These levels are neither modified by TNF stimulation of the PAEC nor by PDTC pretreatment of the cells. The results shown are representative of at least three independent experiments.

activation. We and others have suggested a significant role of NF-κB in EC activation but a broad analysis had not been previously carried out, especially in porcine endothelial cells (3,15,16,21,35,36). We show that inhibition of NF-κB blunts the induction of essentially all the genes we have studied, including those coding for various adhesion molecules (E-selectin, VCAM-1, ICAM-1), a chemokine (IL-8), and prothrombotic molecules (Tissue factor, PAI-1). The induction of junB by TNF is not affected by PDTC. This result is consistent with our hypothesis since junB expression is independent of NF-κB.

With regard to regulation of endogenous gene expression, our results are in accordance with previously published data of Marui et al. on VCAM-1 expression and its sensitivity to antioxidants (20). However, we find different results for E-selectin and ICAM-1. Whereas Marui et al. find that E-selectin and ICAM-1 expression is poorly or not suppressed by anti-oxidants, we , in this manuscript and others for ICAM-1 regulation in fibroblast (37) were able to show strong inhibition of these genes by PDTC. This discrepancy may be explained by at least two differences in the experimental design between the two studies. First, Marui et al. used human umbilical vein endothelial cells (HUVEC) while in this work porcine aortic endothelial cells were studied. Second, in the published study PDTC was used at 50 μM and pretreatment was for 30 min as opposed to 100 μM and 90 min pretreatment in our work. Since NF-κB has been shown to be essential for E-selectin and ICAM-1 expression (13,14), inhibition of this factor is expected to inhibit both genes regardless of the stimulus. Although Marui et al. show inhibition of NF-κB they do so by using a higher concentration of PDTC (100 μM) and a longer pretreatment (1hr) and without showing the influence of this alternative treatment on gene expression. Taken together we believe that the differences relate mostly to the different PDTC concentration used and only indicate a higher sensitivity to anti-oxidants of VCAM-1 as compared to E-selectin and ICAM-1. We reproducibly find a bi-phasic induction of E-selectin with peak mRNA at 1 and 24 hours post-induction. The difference to the reported kinetics of E-selectin induction in HUVEC may relate to the different origin of the cells.

That PDTC effectively blocks inducible NF-κB activity has been previously reported in Jurkat T cells, in B and pre B cell lines, in fibroblasts as well as in monocytic cell lines and monocytes (26,28,37-39). Our group have extended these findings to primary cultured PAEC as confirmed in this work. It is conceivable that PDTC exerts its effects at several levels. It may interfere with superoxide dismutase activity by scavenging superoxide and thus prevent the generation of H_2O_2. Furthermore, PDTC has the potential to chelate Fe^{2+} and therefore prevent the Fenton reaction, which generates hydroxyl radicals ($HO^{\bullet}$) from H_2O_2 (26,40). Our data show that PDTC is mainly acting upstream of the Fenton reaction and is able to decrease *in vitro* EC production of H_2O_2. Although PDTC decreased basal H_2O_2 levels basal gene expression was not affected. This is in accordance with the finding that basal expression of NF-κB inducible genes (i.e. IκB-α, GM-CSF) is NF-κB independent (41).

In addition to NF-κB, at least one other transcription factor, NF-ELAM1, is required for efficient transcription of the E-selectin gene (42,43). We therefore studied the effect of PDTC on the binding of NF-ELAM1. Interestingly, we found increased binding activity of this factor in response to PDTC treatment in activated endothelial cells while E-selectin mRNA levels were markedly decreased (data not shown). Whether increased NF-ELAM1 binding is involved in the PDTC dependent inhibition of E-selectin remains to be determined.

To control for possible non-specific effects of PDTC on gene expression, we have tested the activity of PDTC on a cytokine inducible gene that has no NF-κB site in its 5' regulatory region as well as on constitutively expressed genes whose transcription is apparently independent of NF-κB. Neither the TNF-inducibility of jun-B nor the constitutive expression of thioredoxin and GAPDH were affected by PDTC. These results lead us to conclude that, at least as far as gene regulation is concerned, the effect of PDTC is mainly mediated by NF-κB inhibition.

The data we present in this paper supports our hypothesis that NF-κB is a critical transcription factor in the induction of a large array of genes that are induced with EC activation. Our experiments were designed to study this question at the level of induction of genes in their DNA context rather than with reporter constructs as well as to address this issue in a xenogeneic combination, a major focus of our laboratory. We conclude that NF-κB may be a key regulator of genes induced upon EC activation.

From a clinical standpoint, the ability to interfere with the phenotypic changes of endothelial cells in response to activating cytokines represents a potential therapeutic strategy for the treatment of chronic atherosclerosis, reperfusion injury, septic shock, allograft rejection and especially delayed xenograft rejection (44-46).

On the basis of this extensive work done on PAEC *in vitro*, we are now aiming at specifically targeting NF-κB in endothelial cell by means of genetic manipulation, for instance by temporally controlled IκB-α expression (manuscript in preparation). This alternative approach would offer the advantage of solely inhibiting NF-κB whereas most of the drugs including the anti-oxidant here used have a broad array of effects which will render interpretation of the data obtained *in vivo* difficult.

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