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Evidence Against the Involvement of Multiple Radical Generating Sites in the Expression of the Vascular Cell Adhesion Molecule-1

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The present study was undertaken to investigate the hypothesis that multiple oxygen radical generating systems contribute to the tumor necrosis factor (TNF) α-stimulated transcriptional activation of the vascular cell adhesion molecule (VCAM)-1 in endothelial cells. Experimental evidence has implicated the cytochrome P450 monooxygenase and a phagocyte type NADPH-oxidase as a source of oxygen radicals in these cells. We show here that endothelial cells exhibit cytochrome P450 activity by measuring the O-dealkylation of the exogenous substrate 7-ethoxyresorufin, but components of the phagocyte-type NADPH oxidase could not be demonstrated in endothelial cells. In that latter respect it was surprising that the NADPH oxidase inhibitor apocynin completely prevented the accumulation of VCAM-1 mRNA. However, we found that apocynin also acts as an inhibitor of cytochrome P450 activity in endothelial cells. Therefore the inhibitory effect of apocynin on the induction of VCAM-1 may no longer be used to demonstrate a role for the NADPH oxidase in this process. Furthermore, different cytochrome P450 inhibitors Co^{2+}, metyrapone, SKF525a decreased the endothelial VCAM-1 expression stimulated by TNFα. Also under hypoxic conditions the expression of VCAM-1 was reduced. On this basis we assume that the oxygen dependent step in the intracellular signalling cascade underlying the TNFα stimulated transcriptional activation of VCAM-1 resides in the activity of a cytochrome P450 dependent monooxygenase. The finding that the phospholipase A_2 inhibitor bromophenacylbromide inhibited the expression of VCAM-1 may indicate that arachidonic acid serves as a substrate for the cytochrome P450 monooxygenase reaction, but further research is needed to elucidate the particular cytochrome P450 family member mediating the expression of VCAM-1.

Keywords: Vascular cell adhesion molecule-1, tumor necrosis factor-α, intracellular signalling

INTRODUCTION

Endothelial cell adhesion molecules play an important role in the recruitment of leukocytes into the extravascular space during the inflammatory response and in the repair of tissue injury.[1,2]

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The expression of the adhesion molecules vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1) and endothelial leucocyte adhesion molecule-1 (E-selectin) is induced (VCAM-1 and E-selectin) or increased (ICAM-1) by the cytokine tumor necrosis factor-α (TNFα).[1,2] A pivotal event in the stimulated transcription of the adhesion molecule genes is the activation of the transcription factor nuclear factor-κB (NFκB), a family of dimeric transcription factor complexes. NFκB resides in the cytosol in an inactive form complexed to members of the inhibitory protein family IκB. Upon activation IκB is removed from the complex by degradation (IκB-α) or processing (p105) in the proteasome proteolytic pathway.[3,4,5] NFκB then translocates to the nucleus where it binds to κB regulatory elements in the promoter regions of the VCAM-1, ICAM-1 and E-selectin genes.[6,7,8] Although essential, the activation of NFκB is not sufficient for cytokine-induced transcription. Several other factors are likely involved in the assembly of unique transcriptional activation complexes.[5]

The signal transduction pathways underlying the expression of VCAM-1, ICAM-1 and E-selectin may not be completely overlapping. Marui *et al.* showed that the expression of VCAM-1, but not ICAM-1, is controlled by the redox status of the endothelial cell.[9] The redox status supposedly is modulated through the formation of reactive oxygen species (ROS) on the one hand, and the level of intracellular antioxidants and the activity of the antioxidant enzymes on the other. It has been hypothesized that ROS function as second messenger molecules in the activation of NFκB.[10]

Previously, it has been suggested that in L929 fibroblast cells the mitochondrion is the source of oxygen radicals which would serve as common mediators of the cytotoxic and gene regulatory effects of TNFα in these cells.[11]

The TNFα-induced expression of VCAM-1 seems to involve several radical-generating systems. It has been suggested by Weber *et al.* that in endothelial cells these systems include the flavoenzymes cytochrome P450 monooxygenase and a phagocyte-type NADPH oxidase, since inhibition of these two systems by, respectively, SKF525a and apocynin (4-hydroxy,-3-methoxy-acetophenone) inhibited the TNFα induced expression of VCAM-1 specifically.[12] This effect most likely was due to an effect on the activation of NFκB, as demonstrated for SKF525a.[12] However, in contrast to the flavoprotein dependent cytochrome P450 monooxygenase[13] the expression of a phagocyte-type NADPH-oxidase activity remains to be demonstrated in endothelial cells.[12] Leaving the possibility that the effect of apocynin on the endothelial VCAM-1 induction has to be attributed to a completely different mode of action.

It has been demonstrated before that apocynin can inhibit cytochrome P45011β in sheep adrenal cells.[14] This might imply that apocynin, like SKF525a, inhibits the cytochrome P450 monooxygenase present in endothelial cells. This would shed doubt on the idea that multiple radical generating sites are involved in the TNFα-induced expression of VCAM-1. We therefore questioned whether the flavoenzyme cytochrome P450 monooxygenase may be the sole oxygen dependent step in the intracellular signalling cascade leading to the expression of VCAM-1. To that end we have investigated the presence of NADPH oxidase and questioned whether apocynin could act as an inhibitor of cytochrome P450 monooxygenase in endothelial cells. Finally, we studied the effect of hypoxia and inhibition of the cytochrome P450 electron transport chain on the expression of VCAM-1.

MATERIALS AND METHODS

Monoclonal Antibodies

Monoclonal antibody (mAb) clone MEM112 directed against ICAM-1, and mAb clone 1G11B1 directed against VCAM-1, and clone ENA1 directed against E-selectin were obtained

from Monosan (Uden, The Netherlands). Mouse IgG1κ, was purchased from Sigma (St. Louis, MO) and fluorescein isothiocyanate (FITC)-conjugated rabbit-anti-mouse Fab_2 fragment STAR9 from Serotec (Oxford, UK). Antisera and mAb's directed respectively against p47-*phox*, p67-*phox* and gp91-*phox* were a generous gift of dr. A. J. Verhoeven (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).

Reagents

Reagents were obtained from Merck (Darmstadt, Germany) unless specified otherwise. Diphenylene iodonium obtained from Sigma was dissolved in dimethylsulfoxide by sonication as a 20 mmol/L stock.

Culture and FACS Analysis of Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were cultured as previously described[15] according to the method described by Jaffe *et al.*[16] in M199 (Flow Laboratories) supplemented with 10% newborn calf serum, 10% human serum kindly provided by the Red Cross Bloodbank, Rotterdam, The Netherlands, endothelial cell growth factor, 15 U/ml heparin, 50 U/ml penicillin, and 5 μg/ml streptomycin, under 95% air-5% CO_2. Confluent endothelial monolayers were treated as indicated in the legends. Cells were detached and incubated for 30 min at 4°C with either monoclonal antibody clone 1G11B1 (Monosan, Uden, The Netherlands) directed against VCAM-1, or clone MEM112 (Monosan) directed against ICAM-1, or clone ENA1 directed against E-selectin, or control mouse IgG1κ as described before.[15] The second antibody was a fluorescein isothiocyanate conjugated rabbit anti-mouse Fab_2 fragment. Membrane antigen expression was analysed by fluorescence activated cell sorting (FACS) as previously described,[17] and is expressed as median linear fluorescence intensity (F.I.). Data were corrected for background fluorescence due to binding of IgG1κ, unless specified otherwise.

Assay of Cytochrome P450 Activity in Endothelial Cells

The cytochrome P450 activities were assessed using intact cells according to the method described in detail by Donato *et al.*[18] TNFα-stimulated endothelial cells (25 cm^2) were incubated with 7-ethoxyresorufin (10 μmol/L, Sigma) which can be dealkylated by cytochrome P5450IA1 (CYPIA1)[18,19] and by cytochrome P450-AA.[20] In addition to this cells were also incubated with 7-pentoxyresorufin (20 μmol/L, Sigma) which is specifically dealkylated by P450IIB1.[18,19] After 4 h at 37°C aliquots of supernatant culture media were incubated in the presence of 150 Fishman units of β-glucuronidase/ml and 1200 Roy units of arylsulfatase/ml (Boehringer, Mannheim, Germany) for 2 h at 37°C. The fluorescence of resorufin was measured in a Perkin Elmer fluorimeter with excitation and emission wavelengths set at 530 nm and 590 nm, respectively. A standard curve of resorufin (Sigma) was prepared in culture medium. The results are expressed in pmol resorufin/mg cell protein.

Immunoblot Analysis

Neutrophilic granulocytes (granulocytes) were isolated as previously described[15] and lysed overnight at 4°C in 10 mol/L HEPES, 0.25 mol/L, 5 mmol/L EDTA, 100 μg/ml leupeptin, 2 mmol/L PMSF, and 10 mmol/L β-mercaptoethanol, pH 7.5. Endothelial cells were lysed overnight at 4°C in 4 mmol/L EDTA, 50 mmol/L Tris, 150 mmol/L NaCl, 0.5% (v/v) nonidet P40, and 1 mmol/L PMSF, pH 7.4. Twenty μg of cell homogenate protein was analysed by SDS PAGE. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Boehringer Mannheim, Germany).

Membranes were blocked for 1h with 3% (w/v) albumin in Tris-buffered saline with 0.1% (v/v) TWEEN (TTBS). Blots were washed with TTBS and incubated for 18 h with 1:2000 rabbit antiserum directed against the C-terminus of p67-*phox*, or 1:2000 rabbit antiserum against the C-terminus of p47-*phox*, or 1:1000 mouse mAb 43 directed against gp91-*phox* in TTBS, or in TTBS without antiserum to control for binding of the secondary antibody. Next, immunoblots were incubated with peroxidase conjugated goat-anti-rabbit mAb (1:5000) or with peroxidase conjugated goat-anti-mouse mAb (1:10000). Blots were developed with ECL (Amersham, UK).

Hypoxic Incubations

Hypoxic incubations were performed as previously described at 37°C.[15] In the incubator a continuous flow of 95% N_2-5% CO_2 humidified and warmed to 37°C was maintained during hypoxic incubations. Control incubations were done at 20% O_2-75% N_2-5% CO_2 at 37°C. Buffers and media were equilibrated to ambient conditions before starting the experiment.

Polymerase Chain Reaction

Total RNA was isolated from $2*10^6$ HUVEC stimulated with TNFα (0–100 U/ml in complete M199) according to the method of Chomszynski *et al.*[21] cDNA was produced from 75 ng of each RNA extract by M-MLV reverse transcriptase (Promega) as described before.[21] For VCAM-1 specific oligonucleotide primers were used (forward primer: 5'CGGGATCCATCCACAAAG CTGCAAGAA-3', and reverse primer: 5'-GCGAATTCGCCACCACTCATCTCGATTT-3') which have been previously described.[22] For ICAM-1 specific oligonucleotide primers were used described by Saito *et al.* (forward primer 5'-TGACCATCTACAGCTTTCCGGC-3' and reverse primer 5'-AGCCTGGCACATTGGAGT CTG-3').[23] cDNA was amplified by 30 cycles with Goldstar DNA polymerase (Eurogentec) in a Perkin Elmer thermocycler 480 with the annealing temperature set at 55°C or 60°C, respectively for VCAM-1 and ICAM-1. Homology of the PCR product with the VCAM-1 cDNA or the ICAM-1 cDNA was confirmed by sequence analysis.

Northern Blot Analysis

Total cellular RNA (20 μg) was size-fractioned using 1.2% agarose formaldehyde gel in the presence of 1 μg ethidium bromide. The RNA was transferred onto Hybond and covalently linked by ultraviolet irradiation. Hybridizations were performed at 42°C for 18 h in 50% deionized formamide, 1 mol/L NaCl, 2.5% dextran sulfate, 35 μg/ml denatured fish DNA in 20% P buffer (1% BSA, 1% polyvinylpyrrolidone, 1% Ficoll, 250 mmol/L Tris-HCl, 0.5% Na-pyrophosphate, 5% SDS). The PCR product (approximately 50 ng cDNA) was radiolabeled with [α-^{32}P]dCTP (±50 μCi per hybridization) by the use of a labelling kit (Amersham). After hybridization, filters were washed with a final stringency of 0.2×SSC (1×SSC = 150 mmol/L NaCl, 15 mmol/L Na citrate), 0.1% SDS at room temperature. Autoradiograms were quantified by phosphorimaging (Molecular Imaging System GS-363, Biorad).

Statistical Analysis

Data are expressed as the means ±standard error of mean (SE). The differences between means was assessed by Student's *t*-test. Significance was determined at the 95% confidence level.

RESULTS

Cytochrome P450 Activity in Endothelial Cells

To assess whether apocynin inhibits cytochrome P450 dependent monooxygenase activity in TNFα-stimulated endothelial cells, we set out to determine the rate of dealkylation of the

cytochrome P450 substrates 7-ethoxyresorufin and 7-pentoxyresorufin in unstimulated and TNFα-stimulated endothelial cells. The basal rate of dealkylation of 7-ethoxyresorufin was 74 pmol.mg^{-1} cell protein in 4 h. Stimulating the cells with 100 U/ml TNFα did not significantly increase this rate, i.e. 101 pmol.mg^{-1} in 4 h in the presence of TNFα.

Endothelial cells did not dealkylate 7-pentoxyresorufin within 4 h of incubation. Indicating the absence of the cytochrome P450 family member CYPIIB1. Even with an extended incubation time of 18 h we did not detect any dealkylation of 7-pentoxyresorufin. Stimulating the endothelial cells with TNFα also did not reveal any significant CYPIIB1 activity (data not shown).

Effect of Apocynin and Diphenylene Iodonium on the Cytochrome P450 Enzyme Activity

The effect of apocynin on the cytochrome P450 dependent ethoxyresorufin dealkylation by endothelial cells stimulated with TNFα was investigated next. Cells were preincubated with apocynin or vehicle for 30 min, next 7-ethoxyresorufin dealkylation was assessed in the presence or absence of apocynin. The results presented in Figure 1 show that apocynin significantly decreased the dealkylation of 7-ethoxyresorufin by TNFα-stimulated endothelial cells to about 50%, i.e., 52 pmol.mg^{-1} in 4 h ($P < 0.05$). In unstimulated endothelial cells apocynin decreased

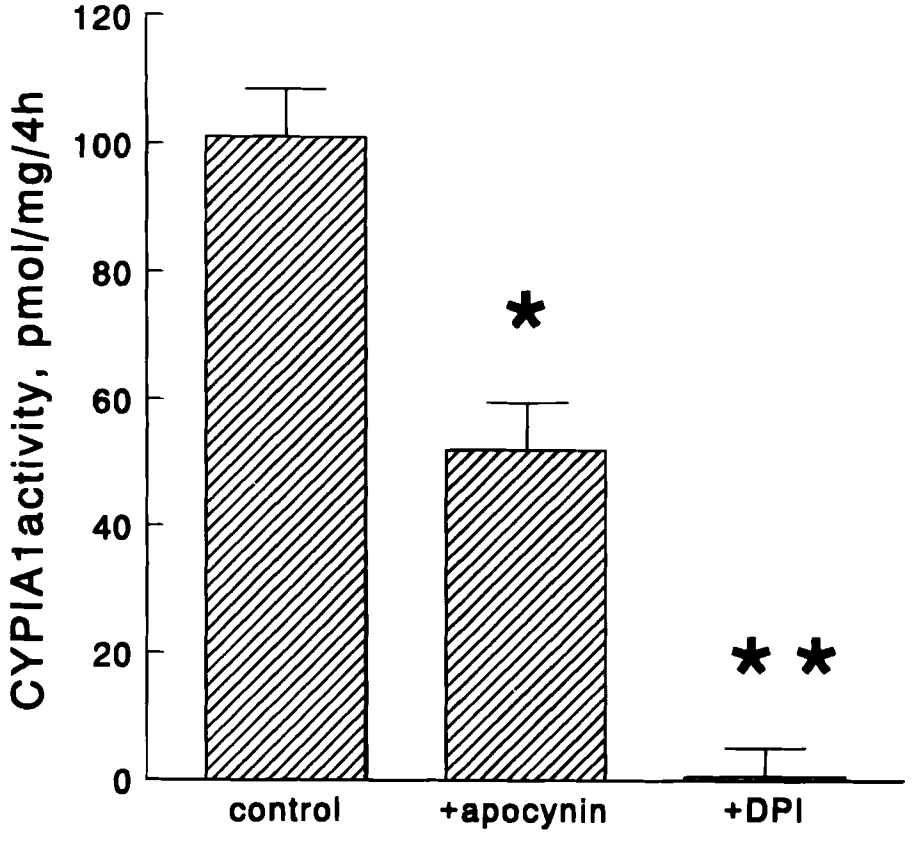

FIGURE 1 Effect of apocynin and diphenylene iodonium (DPI) on the cytochrome P450 activity in endothelial cells. Endothelial cells were preincubated with apocynin (600 μmol/L) or diphenylene iodonium (20 μmol/L) for 30 min, next the cells were stimulated with TNFα (100 U/ml) and 7-ethoxyresorufin dealkylation was assessed in the presence of apocynin or DPI. The results represent at least three experiments. Asterisks denote significant difference from control (*; $P < 0.05$ and **; $P < 0.01$).

the dealkylation rate from 74 pmol.mg^{-1} to 31 pmol.mg^{-1} in 4 h ($P < 0.05$). As a control we also assessed the effect of inhibition of the NADPH cytochrome P450 reductase, essential for cyto-chrome P450 activity, by the well known flavoprotein inhibitor diphenylene iodonium (DPI). The results presented in Figure 1 show that DPI completely inhibited the dealkylation of 7-ethoxyresorufin in endothelial cells.

Assessment of the NADPH Oxidase Components P47-*phox*, P67-*phox* and Gp91-*phox* in Endothelial Cells

To assess whether endothelial cells express the phagocyte-type NADPH oxidase, homogenates of resting or TNFα-stimulated endothelial cells were analysed by immunoblotting, and the expression of the NADPH oxidase components p47-*phox*, p67-*phox* and gp91-*phox* were compared with equal amounts (20 μg) of homogenate protein of resting or phorbol myristate acetate (PMA) stimulated granulocytes. The results presented in Figure 2 show that endothelial cells do not express p47-*phox*, p67-*phox* and gp91-*phox* either in the absence or presence of TNFα. Whereas in granulocytes the p47-*phox* and p67-*phox* are amply present either in resting or PMA-treated cells. Gp91-*phox* could also be detected but to a lesser extent in resting and stimulated granulocytes (Fig. 2).

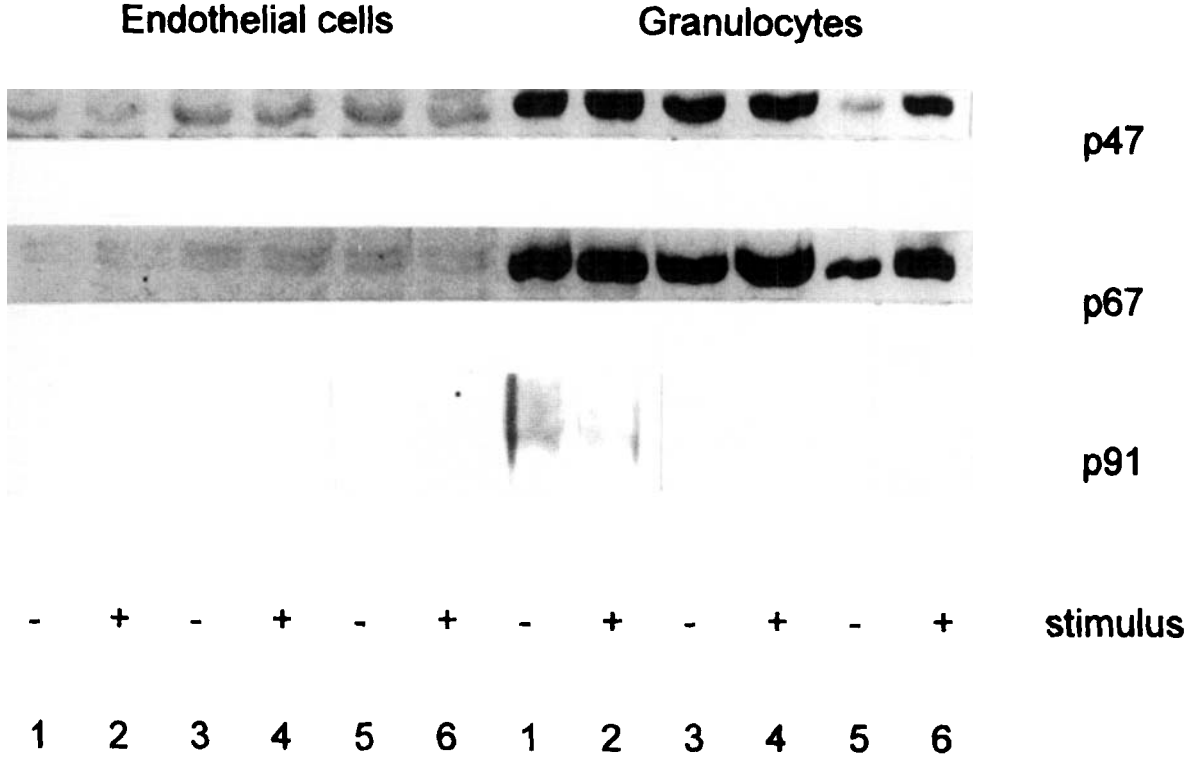

FIGURE 2 Assessment of NADPH oxidase components p47-*phox*, p67-*phox* and gp91-*phox* in endothelial cells and granulocytes. Equal amounts of endothelial cell en granulocyte homogenates (20 μg of protein) were analyzed by SDS-PAGE and immunoblotting as described. Endothelial cells were stimulated in three separate experiments with 0 or 100 U/ml TNFα (1–6) for 4 h. Granulocytes were treated with 0 or 100 ng/ml phorbol myristate acetate in three separate experiments (1–6). The NADPH components p47-*phox* (1–6), p67-*phox* (1–6), and gp91-*phox* (1–2) are respectively denoted as p47, p67 and p91. The density of the bands in the endothelial cell lanes was similar to that obtained when blots were incubated with only the secondary antibody (data not shown).

Effect of Apocynin and DPI on the Expression of VCAM-1

To confirm the inhibitory effect of apocynin on VCAM-1 protein expression demonstrated before[12] endothelial cells were incubated with increasing concentrations of apocynin for 30 min to 1 h. Next, the cells were stimulated with 100 U/ml of TNFα in the presence of absence of apocynin. The expression of VCAM-1 protein (Fig. 3a) was inhibited by apocynin in a dose dependent manner, with a maximum effect at 600 μmol/L. At this concentration apocynin almost completely inhibited the accumulation of VCAM-1 mRNA (Fig. 3b). This effect was specific for VCAM-1 since the accumulation of ICAM-1 protein and mRNA were not significantly affected by apocynin (Fig. 4a and b).

To further substantiate the involvement of flavoenzymes in the expression of VCAM-1 endothelial cells were treated with increasing concentrations of DPI (0, 10 and 20 μmol/L) or vehicle, and next stimulated with 100 U/ml TNFα in the presence or absence of DPI. The results presented in Figure 5 show that 20 μmol/L of DPI significantly inhibited VCAM-1 expression. The equivalent concentration of the vehicle DMSO had no effect on the expression of VCAM-1, which amounted to 94% of the control. The inhibitory effect of DPI was specific for the expression of VCAM-1 since the expression of

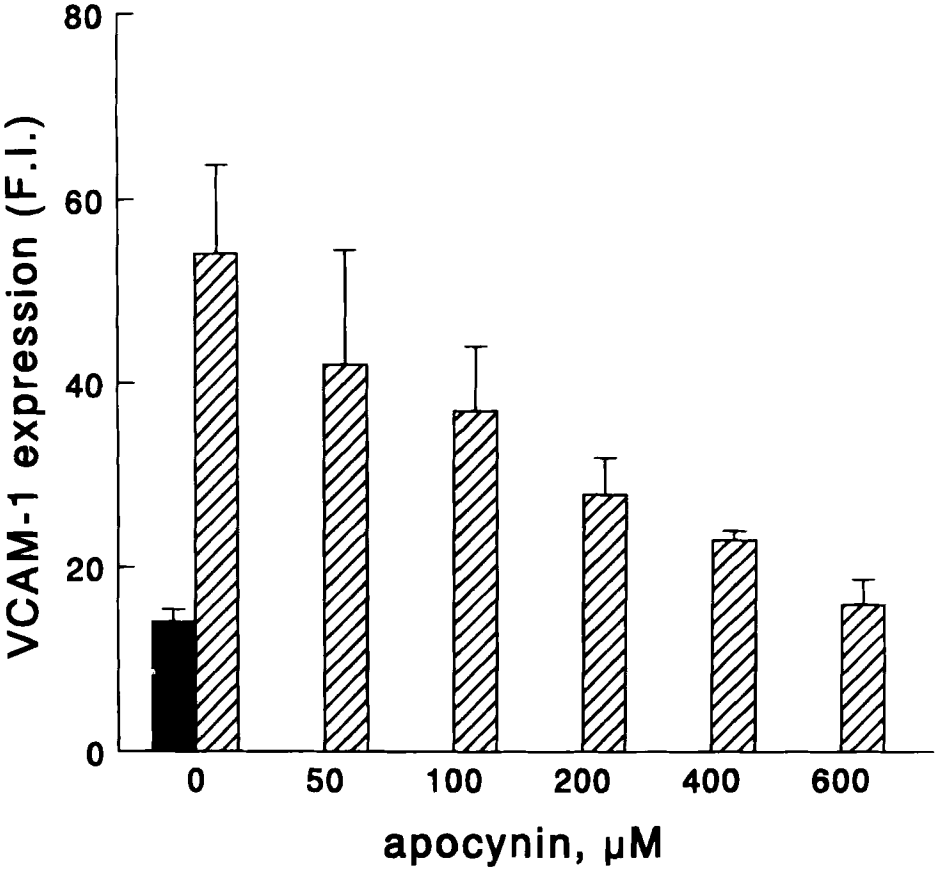

FIGURE 3a

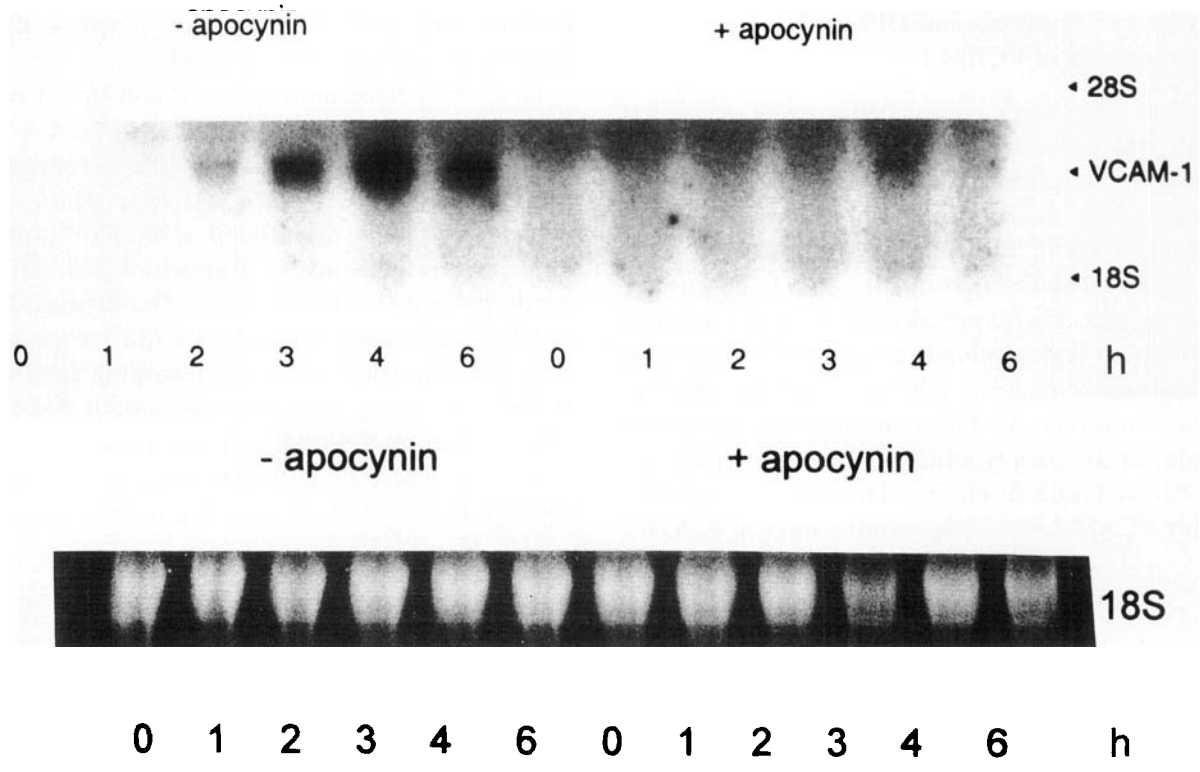

FIGURE 3b and 3c

FIGURE 3 Effect of apocynin on the TNFα induced expression of VCAM-1 protein (**3a**) and mRNA (**3b**). Endothelial cells were preincubated with apocynin (0–600 μmol/L). Next, the cells were stimulated with TNFα (100 U/ml) in the presence of apocynin. After 6 h of stimulation with TNFα the expression of VCAM-1 protein was assessed by FACS analysis (hatched bars, Fig. 3a). The black bar represents binding of anti-VCAM-1 mAb to unstimulated endothelial cells, background fluorescence of cells incubated with IgG1κ, i.e., 5.4 F.I. ±sd = 1.1, remained unchanged under the applied conditions. In Fig. 3b the TNFα stimulated accumulation of VCAM-1 mRNA in time was assessed in the presence or absence of apocynin (600 μmol/L) by Northern blot. In Fig. 3c the ethidium bromide stained 18S RNA band of each lane is shown as a control for equal loading and quality of the RNA samples. Results represent at least three experiments.

ICAM-1 remained unaffected by DPI up to the maximum concentration of 20 μmol/L (Fig. 5).

To exclude the possibility that the effect of DPI could be attributed to an effect on the mitochondrial NADH dehydrogenase (or NADH Q reductase) we tested the effect of rotenone on the endothelial expression of VCAM-1. After treatment of endothelial cells with 5 μmol/L rotenone their oxygen consumption measured by a Clark type electrode was inhibited by 70%. At this concentration rotenone did not inhibit the TNFα stimulated endothelial VCAM-1 expression. In fact it seemed to increase VCAM-1 expression from 22 F.I. ($n = 2$, range 20–23) to 51 F.I. ($n = 2$, range 46–55). This effect was not further investigated since it was beyond the scope of the present study.

Effect of Cytochrome P450- and Phospholipase A_2 Inhibitors on VCAM-1 Expression

To establish the role of the cytochrome P450 monooxygenase in the TNFα-stimulated expression of VCAM-1 we used different inhibitors of the cytochrome P450 electron transport system and tested their effect on the expression of VCAM-1 and ICAM-1 by TNFα-stimulated endothelial cells. The results presented in Table I show that the majority of the cytochrome P450 inhibitors used in this study (significantly)

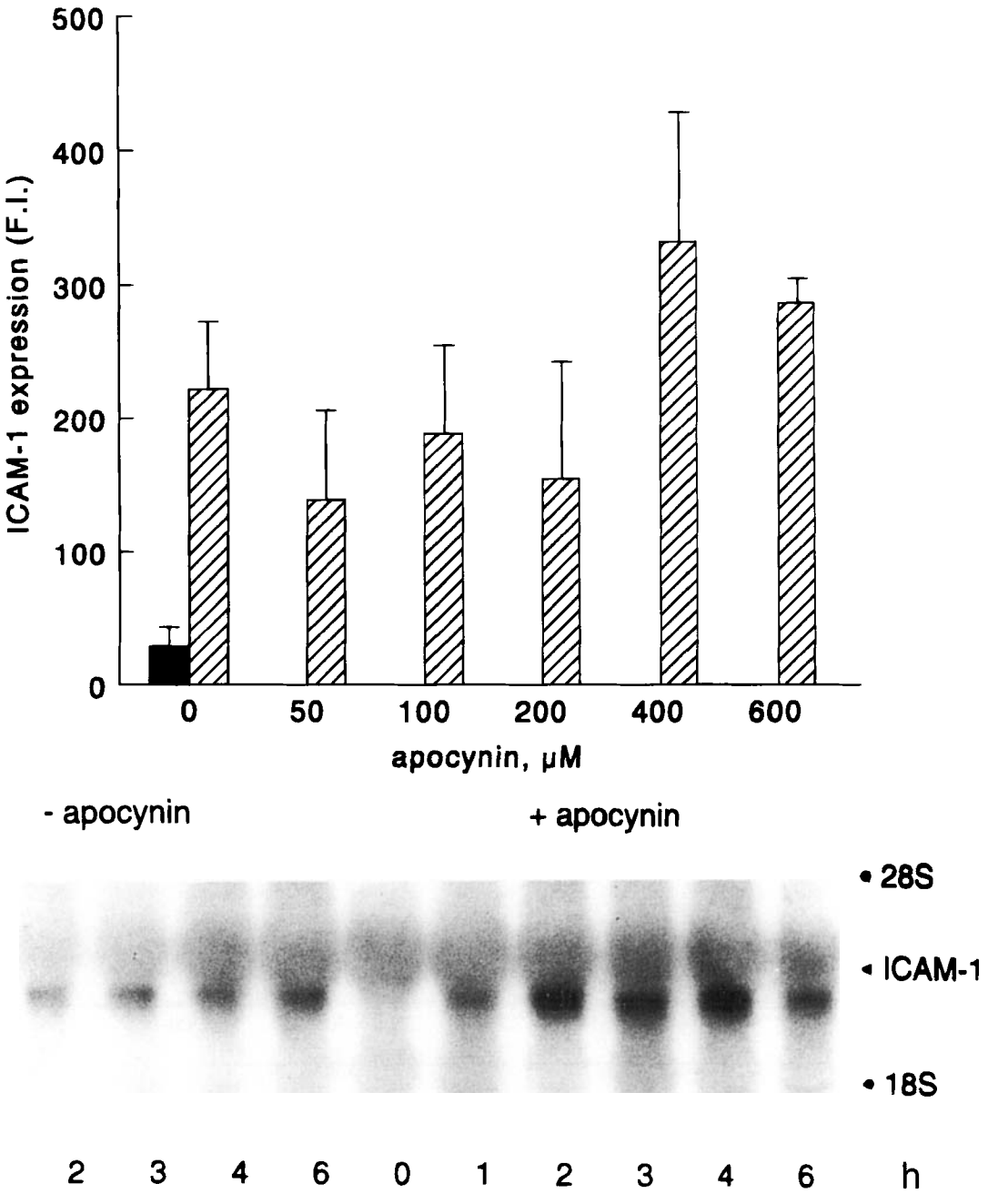

FIGURE 4 Effect of apocynin on the TNFα induced expression of ICAM-1 protein (**4a**) and mRNA (**4b**). Endothelial cells were preincubated with apocynin (0–600 μmol/L). Next, the cells were stimulated with TNFα (100 U/ml) in the presence of apocynin. After 6 h of stimulation with TNFα the expression of ICAM-1 protein was assessed by FACS analysis (hatched bars, Fig 4a). The black bar represents aspecific binding of anti-ICAM-1 mAb to unstimulated endothelial cells, background fluorescence was 5.4 F.I. ±sd = 1.1, and remained unchanged under the applied conditions. In Fig 4b the TNFα stimulated accumulation of ICAM-1 mRNA in time was assessed in the presence or absence of apocynin (600 μmol/L) by Northern blot. In Fig 3c the ethidium bromide stained 18S RNA band of each lane is shown as a control for equal loading and quality of the RNA samples. Results represent at least three experiments.

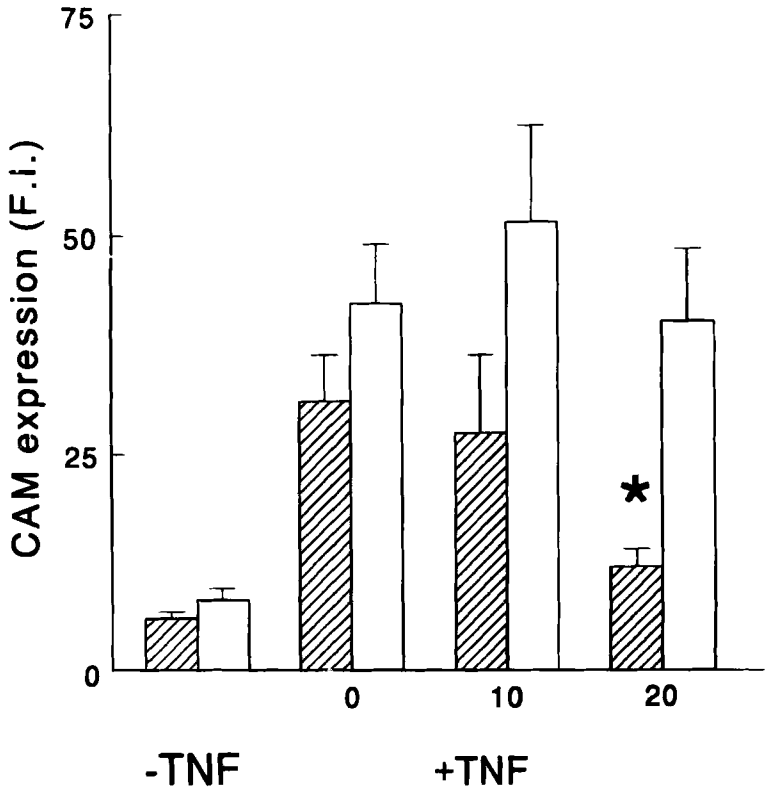

FIGURE 5 Effect of diphenylene iodonium (DPI) on the expression VCAM-1 and ICAM-1. Endothelial cells were preincubated with increasing concentrations of DPI (0–20 μmol/L), and subsequently stimulated with TNFα (100 U/ml) in the presence or absence of DPI. After 6 h of stimulation the expression of VCAM-1 (hatched bars) or ICAM-1 (white bars) was assessed by FACS analysis. Background fluorescence of cells incubated with IgG1κ was 5 F.I. and did not change under the applied conditions. Results represent three experiments.
*Significant difference from control ($P < 0.05$). CAM; Cell Adhesion Molecule.

TABLE I Effect of cytochrome P450 and phospholipase A_2 inhibition on the expression of VCAM-1 by TNFα-stimulated endothelial cells

Inhibitor	Conc.	% VCAM-1 expression	P-value	% ICAM-1 expression	P-value
Cytochrome P450 inhibitor					
Co^{2+}	0.2 mM	17 ($n = 3$)*	0.015	82 ($n = 3$)*	n.s.
AIA	0.2 mM	109 ($n = 3$)*	n.s.	100 ($n = 3$)*	n.s.
metyrapone	1.0 mM	32 ($n = 3$)†	0.017	83 ($n = 3$)†	n.s.
SKF525a	0.1 mM	48 ($n = 3$)†	0.041	89 ($n = 3$)†	n.s.
Phospholipase A_2 inhibitor					
BPB	10 μM	34 ($n = 4$)†	0.048	91 ($n = 2$)†	

Control VCAM-1 and ICAM-1 expression respectively amounted to 39 F.I. and 56 F.I. Cells were pretreated with inhibitor prior to stimulation with TNFα in the presence of inhibitor:
* for 18 h
† for 30 min –1 h
AIA: allylisopropylacetamide
BPB: bromophenacylbromide

decreased the TNFα stimulated expression of VCAM-1. The most dramatic decrease in VCAM-1 expression was observed for Co^{2+}, a metal which is known to decrease the intracellular content of cytochrome P450. The effect of Co^{2+} was only observed with a preincubation time of 18 h and not with a shorter preincubation time of 30 min (data not shown). In contrast to Co^{2+}, metyrapone and SKF525a, another well known cytochrome P450 inhibitor allylisopropylacetamide (AIA), which causes breakdown of the cytochrome P450 haem by a process called 'suicidal inactivation', did not at all affect the endothelial VCAM-1 expression. None of the inhibitors used in this study significantly decreased the TNFα-stimulated expression of ICAM-1.

Inhibition of phospholipase A_2 activity by bromophenacylbromide (BPB) resulted in a 60–70 % decrease ($P < 0.05$) of VCAM-1 expression in TNFα stimulated endothelial cells. Whereas the expression of ICAM-1 remained unaffected (Table I).

Effect of Hypoxia on the Expression of VCAM-1

To investigate whether the TNFα-stimulated induction of VCAM-1 by endothelial cells depends on the presence of oxygen, we studied the effect of hypoxia on the surface expression of VCAM-1. Endothelial cells were preincubated under hypoxic conditions for 30 min, and next stimulated with increasing concentrations of TNFα for 6 h under continuous hypoxia. The results presented in Table II show that hypoxia significantly decreased the TNFα stimulated expression of VCAM-1 to about 60% of the normoxic control ($P < 0.05$). To determine whether this effect was specific for the expression of VCAM-1 we also studied the effect of hypoxia on the expression of ICAM-1 at 6 h after stimulation with TNFα, and of E-selectin at 4 h after stimulation, at which time E-selectin expression is maximal. The results show that the expression of both ICAM-1 and E-selectin under hypoxic conditions did not differ from the normoxic control (Table II).

DISCUSSION

The major conclusion to be drawn from the present study is that the proposed involvement of various flavoenzymes in the TNFα-induced expression of VCAM-1 can be traced back to the cytochrome P450 dependent monooxygenase in endothelial cells. Based upon the evidence obtained with different enzyme inhibitors at least two flavoenzyme dependent systems have been implied that positively affect the endothelial expression of VCAM-1. These are the phagocyte-type NADPH oxidase and the cytochrome P450 monooxygenase electron transport system.[12] It has been hypothesized that both the NADPH-oxidase and the cytochrome P450 monooxygenase, which both can be a source of reactive oxygen species (ROS), mediate the transcriptional activation of VCAM-1 by the generation of these ROS.[12] In our opinion the idea that these two systems contribute to a putative oxygen

TABLE II Effect of hypoxia on the expression of the endothelial leucocyte adhesion molecules

Adhesion molecule	Normoxia	Hypoxia	P-value
VCAM-1	100%	57±14% ($n=4$)	0.029
ICAM-1	100%	100% ($n=2$)	
E-selectin	100%	116% ($n=2$)	

Control VCAM-1 and ICAM-1 expression amounted to 38 F.I. and 72 F.I., respectively. E-selectin expression was determined by ELISA and amounted to 0.137 OD. Endothelial cells were kept under hypoxic or normoxic conditions for 30 min and next stimulated with 100 U/ml TNFα for 6 h (VCAM-1 and ICAM-1) or 4 h (E-selectin), while kept under the same ambient conditions. Data are presented as percentage of the normoxic control. Statistical significance of the difference in fluorescence intensities was assessed by Student's *t*-test for paired data.

radical pool can no longer be sustained. While apocynin inhibits the NADPH oxidase in neutrophilic granulocytes,[24] we now demonstrate that apocynin is an inhibitor of cytochrome P450 activity in endothelial cells. Furthermore we show here that endothelial cells do not express any of the NADPH oxidase components p47-*phox*, p67-*phox*, or gp91-*phox* either under resting or stimulated conditions. Apparently, endothelial cells do not express the phagocyte-type NADPH oxidase. Thus, the inhibitory effect of apocynin on the induction of VCAM-1 and its inhibitory effect on cytochrome P450 activity may now support a role for the cytochrome P450 monooxygenase in the TNFα-induced endothelial expression of VCAM-1. In the present study we also show that the TNFα-stimulated expression of VCAM-1, but not ICAM-1 or E-selectin, is reduced under hypoxic conditions. These findings are in accordance with those of Klein *et al.*,[25] although they reported a slightly larger decrease in the TNFα stimulated VCAM-1 expression (about 60% inhibition) under hypoxic conditions. It remains to be demonstrated whether anoxia can completely abolish TNFα stimulated VCAM-1 expression. If we consider these data together with the fact that various cytochrome P450 inhibitors and the NADPH cytochrome P450 reductase inhibitor DPI can decrease the endothelial VCAM-1 expression stimulated by TNFα we conclude that the oxygen dependent step in the intracellular signalling cascade underlying the TNFα stimulated transcriptional activation of VCAM-1 resides in the activity of a cytochrome P450 dependent monooxygenase.

By the use of the exogenous substrate 7-ethoxyresorufin we were able to demonstrate cytochrome P450 activity in intact endothelial cells. This activity can be attributed to the cytochrome P450 family members cytochrome P450IA1[18,19] and P450-AA.[20] The dealkylation of 7-ethoxyresorufin may therefore confirm the previous observation by Overby *et al.* who showed that CYPIA1 is present in endothelial cells at the protein and mRNA level.[13] However, a physiological substrate for CYPIA1 has not yet been identified. In contrast cytochrome P450-AA oxidizes arachidonic acid into the biologically active epoxyeicosatrienoic acids.[20,26] Our data on the inhibitory activity of the phospholipase A_2 inhibitor BPB on VCAM-1 expression indeed indicate that arachidonic acid may be the substrate for the cytochrome P450 dependent monooxygenase reaction.

An unexpected finding of the present study was that the well known cytochrome P450 inhibitor allylisopropylacetamide (AIA) failed to decrease VCAM-1 expression. Although we expected that AIA like Co^{2+} would drastically reduce VCAM-1 expression this observation may be explained by the selectivity of AIA for distinct cytochrome P450 family members, *i.e.* pentobaribital induced forms of cytochrome P450.[27] For example the cytochrome P450 PB-4 also known as the *CYP2B1* gene product is particularly susceptible to suicidal inactivation by AIA.[28] We therefore conclude that the difference in expression of cytochrome P450 family members between phenobarbital-induced rat hepatocytes and human endothelial cells[18] could make the endothelial cells less susceptible to treatment with AIA. In this respect it may be relevant to note that we already demonstrated no dealkylating activity of 7-pentoxyresorufin in endothelial cells, whereas phenobarbital induced rat hepatocytes can dealkylate this substrate.[18]

During cytochrome P450 dependent monooxygenation electrons are transferred from NADPH to the cytochrome P450 heme. One electron is used to reduce the heme iron, and the second to subsequently bind and cleave the oxygen molecule to generate the active species for insertion into the substrate.[29] The flavoenzyme NADPH-cytochrome P450 reductase temporarily stores electrons during this transfer. Iodonium compounds like DPI typically inhibit flavoenzymes that function as one-electron donors, accepting two electrons but passing them one singly.[30] Whereas all known DPI *insensitive* flavoenzymes simultaneously transfer two electrons during

catalysis.[30] Therefore theoretically the inhibition of VCAM-1 induction could also have involved the iodonium sensitive flavoenzymes mitochondrial NADH dehydrogenase, xanthine oxidase, and nitric oxide (NO) synthase. An important role for the latter flavoenzyme has been suggested recently, when Khan *et al.* demonstrated that the increased delivery of NO suppresses the TNFα-induced transcriptional activation of VCAM-1.[31] However, this negative regulatory role for the nitric oxide synthase can not explain the present observation that DPI inhibits the expression of VCAM-1, because then an increased expression of VCAM-1 would be anticipated. A role for the xanthine oxidase has been excluded previously by Weber *et al.* who demonstrated that allopurinol did not inhibit VCAM-1 expression.[12] In the present study we also excluded the possible contribution of the mitochondrial NADH dehydrogenase.

Taken together we postulate the cytochrome P450 monooxygenase as the sole oxygen dependent step in the TNFα-stimulated induction of VCAM-1 by endothelial cells. A wide variety of structurally diverse compounds, e.g., steroids, fatty acids (including prostaglandins and leukotrienes) are metabolized by the cytochrome P450 monooxygenase system. Also the formation of the ROS hydrogen peroxide as a byproduct of the monooxygenation reaction has been described.[32] Future research is needed to elucidate the type of cytochrome P450 isoenzyme and the nature of its product as the second messenger molecule in the expression of VCAM-1.

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