Expression of inflammation-related genes in endothelial cells is not directly affected by microparticles from preeclamptic patients

CHRISTIANNE ANNE RACHEL LOK, ANITA N. BÖING, PIETER H. REITSMA, JORIS A. M. VAN DER POST, ED VAN BAVEL, KEES BOER, AUGUESTE STURK, and RIENK NIEUWLAND

AMSTERDAM, THE NETHERLANDS

Background: Inflammation and endothelial dysfunction are prominent in preeclampsia. Microparticles (MPs) may link these processes, as MPs induce the production of pro-inflammatory cytokines by endothelial cells and cause endothelial dysfunction. Aim: To study changes in expression of inflammation-related genes in human endothelial cells in response to MPs from preeclamptic patients. Methods: Human umbilical vein endothelial cells (HUVECs) were incubated for various time intervals in the absence or presence of isolated MP fractions from preeclamptic patients (n = 3), normotensive pregnant women (n = 3), non-pregnant controls (n = 3), and interleukin (IL)-1α as a positive control. Total RNA was isolated and used for multiplex ligation-dependent probe amplification (MLPA) and real-time polymerase chain reaction (PCR). Results: IL-1α enhanced the expression of IL-1α, IL-2, IL-6, and IL-8; nuclear factor of kappa light chain enhancer in B-cells (NFκB)-1, NFκB-2, and NFκB-inhibitor; cyclin-dependent kinase inhibitor and monocyte chemotactic protein-1; and transiently increased tissue factor expression. RNA expression of inflammation-related genes and genes encoding adhesion receptors, however, were unaffected by any of the MP fractions tested. Conclusion: MLPA is a suitable assay to test the inflammatory status of endothelial cells, because incubation with IL-1α triggered substantial changes in RNA expression in endothelial cells. Taken together, it seems unlikely that MPs from preeclamptic patients induce endothelial dysfunction by directly affecting the expression of inflammation-related genes in these cells. (J Lab Clin Med 2006;147:310–320)

Abbreviations: ANOVA = analysis of variance; B2M = β-2-microglobulin; BMI = B lymphoma murine leukemia viral oncogen homolog insertion region; bp = base pairs; CDKN-1A = cyclin-dependent kinase inhibitor; cDNA = copy deoxyribonucleic acid; DNA = deoxyribonucleic acid; dNTP = deoxyribonucleotide triphosphate; EDTA = ethylenediaminetetraacetic; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GST-P1 = glutathione s-transferase p1; HUVEC = human umbilical vein endothelial cell; ICAM = intracellular adhesion molecule; IL = interleukin; ISSHP = International Society for the Studies of Hypertension in Pregnancy; KCl = kalium chloride; MCP = monocyte chemotactic protein; $MgCl_2$ = magnesium chloride; MIF = macrophage migration inhibitory factor; MIP = macrophage inflammatory protein; MLPA = multiplex ligation-dependent probe amplification; MP = microparticle; mRNA = messenger ribonucleic acid; NFκB = nuclear factor of kappa light chain enhancer in B-cells; NFκB-IA =

From the Department of Obstetrics, the Department of Experimental Clinical Chemistry, the Department of Experimental Internal Medicine, and the Department of Medical Physics, Academic Medical Center, Amsterdam, The Netherlands.

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Reprint requests: Christianne Anne Rachel Lok, Department of Obstetrics and Gynecology, H4-Zuid, Academic Medical Center, Meibergdreef 9, PO Box 22660, 1100 DD Amsterdam, The Netherlands; e-mail: c.a.lok@amc.uva.nl.

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NFκB inhibitor alpha (IκB); PARN = poly-A specific ribonuclease; PCR = polymerase chain reaction; PDE = phosphodiesterase; PDGF = platelet-derived growth factor; PMP = platelet-derived microparticle; PTP = protein-tyrosine phosphatase; RA = receptor agonist; RNA = ribonucleic acid; RT = reverse transcription; SERP = serine proteinase inhibitor; Taq = Thermus aquaticus polymerase; TF = tissue factor; THBS = thrombospondin-1; TNF-R = tumor-necrosis factor receptor; VCAM = vascular cell adhesion molecule

Inflammation and endothelial dysfunction are both prominent in the development of preeclampsia.[1] The activated endothelium plays a pivotal role in the production of inflammatory mediators such as cell-adhesion receptors, growth factors, cytokines, and mediators that influence the vascular tone. However, the factors that initiate this inflammatory response are still unknown.

MPs, small membrane vesicles released from activated or apoptotic blood cells or endothelial cells, modulate endothelial cell function because MPs from preeclamptic patients impair endothelium-mediated relaxation in isolated myometrial arteries.[2] In addition, *in vitro*-generated PMPs trigger the production of IL-1β, IL-6, and IL-8 in endothelial cells, as reflected by elevated mRNA and protein levels.[3] Leukocyte-derived MPs induced the production of the pro-inflammatory cytokines IL-6 and MCP-1 by endothelial cells,[4,5] and leukocyte MPs from synovial fluid of arthritic joints triggered the production of IL-8 and MCP-1 by fibroblast-like synoviocytes.[6] Elevated levels of MPs from leucocytes have been reported in preeclampsia.[7] Whether isolated MP fractions from preeclamptic patients affect the expression of inflammation-related genes or genes encoding adhesion receptors in endothelial cells is the focus of this study.

To study possible MP-induced changes in RNA expression of endothelial cells, a novel method, MLPA, was used.[8] In parallel experiments, the suitability of this method in endothelial cells was investigated by measuring time-dependent changes in (m)RNA-expression of 40 different inflammation-related genes induced by the pro-inflammatory cytokine IL-1α. Real-time PCR was used to determine RNA expression levels of various adhesion receptors produced by endothelial cells after incubation with MP or IL-1α.

MATERIALS AND METHODS

Patients. The study was approved by the medical ethical committee of the Academic Medical Center and was carried out according to the principles of the Declaration of Helsinki. After obtaining written informed consent, blood samples were obtained from preeclamptic patients (n = 3), normotensive pregnant women (n = 3), and non-pregnant controls (n = 3). The women were matched for maternal age (±5 years) and parity. The preeclamptic patients and normotensive pregnant women were also matched for gestational age (±2 weeks). The non-pregnant controls were healthy women not using any medication, including oral contraceptives. Preeclampsia was defined according to the definitions of the ISSHP: (1) diastolic blood pressure of 110 mm Hg or more on any occasion or 90 mm Hg or more on two separate occasions at least 4 hours apart, (2) proteinuria of 0.3 g protein in 24 hours developing after 20 weeks gestational age, and (3) values returning to normal within 3 months after delivery.

In each group, 2 patients were primiparous and 1 patient was multiparous. As expected, the systolic and diastolic blood pressures were significantly higher in the preeclamptic group compared with the normotensive pregnant women and the non-pregnant controls. The birth weight was lower in the preeclamptic group compared with the normotensive pregnant women. No other differences existed (Table I).

Collection of blood samples. Samples were taken from the antecubital vein without a tourniquet through a 20-gauge needle with a vacutainer system. The samples were collected into a 4.5-mL tube containing 0.105-M (3.2%) buffered sodium citrate (Becton Dickinson; San Jose, Calif). Within 30

Table I. Patient characteristics

	Preeclampsia (n = 3)	Normotensive pregnancy (n = 3)	Non-pregnant controls (n = 3)	*P*	*P**
Age (years)	29.1 ± 5.5	29.6 ± 5.0	25.7 ± 4.6	NS	NS
Gestational age (weeks)	30.2 ± 2.7	31.2 ± 1.2		NS	
Blood pressure					
Systolic (mmHg)	163 ± 7.6	107 ± 5.8	122 ± 14.9	0.002	0.008
Diastolic (mmHg)	112 ± 10.4	65 ± 5.0	69 ± 5.3	0.001	0.001
Body mass index (kg/m^2)	25.0 ± 7.5	25.0 ± 8.2		NS	
Birth weight	1460 ± 370	3295 ± 572		0.02	

Abbreviations: NS, not significant; *P*, *P*-value of the difference in means between preeclampsia and normotensive pregnancy; *P**, *P*-value of the difference in means between preeclampsia and non-pregnant controls.
Note: Data are presented as mean ± SD.

minutes after collection, cells were removed by centrifugation for 20 minutes at 1560 g and 20°C. Plasma samples were then divided into 250-μL aliquots, immediately snap frozen in liquid nitrogen to preserve MP structure, and then stored at −80°C until further analysis.

Isolation of microparticles. Plasma (8 aliquots) was thawed on melting ice, pooled in 2 aliquots of 1 mL, and centrifuged for 60 minutes at 17,570 g and 20°C to pellet the MPs. After centrifugation, 975 μL of the supernatant was removed from each aliquot. The MP pellets (25 μL) were then resuspended in 500-μL culture medium and 25 μL of the MP-free plasma was also diluted in 500-μL culture medium.

Endothelial cell isolation. Umbilical cords were collected at the delivery ward of the Academic Medical Center. Only umbilical cords from healthy pregnant women with uncomplicated singleton pregnancies were used. Our procedures for endothelial cell isolation were described by Abid Hussein et al.[9] As the addition of serum is essential for the survival of endothelial cells, MP were removed from human serum by centrifugation. In the third passage, HUVECs were transferred to a 24-well plate coated with gelatin. After confluence, the cells were maintained for 2–3 days until steady state was achieved before the experiments were started.

Incubation and RNA isolation. For the initial experiments with IL-1α to study the time-dependent changes in mRNA expression in activated HUVEC and the suitability of the MLPA assay, culture medium of the confluent cells was replaced by culture medium containing IL-1α (5 ng/mL; Sigma-Alderich Chemie, Zwijndrecht, The Netherlands) or by culture medium without IL-1α (control). Endothelial cells were incubated at 37°C with IL-1α or culture medium alone for 0, 30, 60, 90, 120, or 240 minutes. To analyze the possible effect of endothelial cell subcultures on RNA expression, endothelial cells from 2 different umbilical cords were cultured and RNA was isolated from the first and third passage cells. All measurements were performed in triplicate. Cells were incubated without or with IL-1α for 1 hour and 4 hours. RNA expression was analyzed by MLPA.

For the MP experiments, endothelial cells were incubated with 500-μL culture medium with either isolated MPs or MP-free plasma. The cellular origin of circulating MPs in preeclamptic patients was previously determined and it was shown that >95% of these MPs are derived from platelets.[7] IL-1α and culture medium alone were used as positive and negative controls. After 1 hour and 4 hours of incubation, culture medium was removed and cells were detached by incubation with 500-μL trypsin. Subsequently, the cell suspensions were mixed with 500-μL culture medium and centrifuged for 10 minutes at 20°C and 300 g. The supernatant was removed, and total RNA was isolated from the endothelial cells using RNeasy columns (Qiagen, Hilden, Germany) according to the protocol of the manufacturer. Total RNA was dissolved in 30-μL RNase-free water (Qiagen, Hilden, Germany) and stored at −20°C until further analysis.

MLPA. MLPA (kit P009; MRC-Holland, Amsterdam, The Netherlands) was performed with RNA in a concentration of 40–60-ng RNA/μL. With the MLPA assay, 40 different genes can be determined simultaneously using 40 different probes.[8] The length of a MLPA probe varies between 130 bp and 500 bp. Each probe consists of a short oligonucleotide that contains a target-specific sequence at the 3′ end and a longer oligonucleotide that contains a target-specific sequence at the 5′ phosphorylated end, and a stuffer sequence of a variable length to enable the separation of the different PCR products during electrophoresis.

The MLPA assay consists of 6 steps, of which the first 5 steps are schematically presented in Fig 1: (1) isolation of RNA, (2) production of single-strand cDNA, (3) hybridization and (4) ligation of different probes, (5) PCR of the ligated probes, and finally (6) capillary electrophoresis. The RNA samples were thawed, and 2.5 μL of each RNA sample was used. For the RT reaction (2), a RT-primer mixture, a RT-buffer, and dNTPs were added to the 200-μL PCR tubes containing the RNA samples. Samples were heated at 80°C for 1 minute in a high-speed thermal cycler with a heated lid (Biometra Uno II, Göttingen, Germany) and incubated for 5 minutes at 45°C. Reverse transcriptase was diluted to 20 U/μL, and 1.5 μL was added per sample. After 15 minutes of incubation at 37°C, the samples were heated at 98°C for 2 minutes. In the hybridization phase (3), 1.5-μL buffer (1.5-M KCl, 1-mM EDTA, 300-mM Tris-HCl, pH 8.5) and 1.5-μL probe mixture (1–4 fmol of each short-probe oligonucleotide and each long-probe oligonucleotide in Tris EDTA) were added to the samples. The samples were then heated for 1 minute at 95°C, followed by incubation for 16 hours at 60°C to allow complete hybridization. The procedure was continued with the addition of a ligase enzyme at 54°C for 15 minutes to enable ligation (4). The samples were heated for 5 minutes at 98°C. Then the PCR reaction was started with 5 μL of the MLPA product (5) and the addition of an enzyme dilution buffer, PCR primers, water, and a polymerase mixture. The amplification cycle (95°C—60°C—72°C) was repeated 32 times. ROX 500, a fluorescence marker, was added to the PCR product. Samples were purified with Sephadex G-50 (Sigma-Alderich Chemie) in filter plates (mahvn4550; Millipore, Billerica, Mass) and analyzed by capillary electrophoresis (6) on a capillary sequencer (ABI 3100; Applied Biosystems, Warrington, UK). The intensity and size of the different probes were calculated with Genescan and Genotyper software packages (Applied Biosystems). In line with the manufacturer's instruction for the application of the MLPA kit in blood cells, B2M was chosen as a reference (household) gene for this study. In preliminary experiments (data not shown), it was confirmed that changes in B2M expression were minimal in control cells and in response to IL-1α. In each experiment, the expression of the B2M gene was arbitrarily set at 1.0 (ie, 100%), to which changes in gene expression were compared (Figs 2 and 3).

Real-time LightCycler PCR. Real-time PCRs were performed on a LightCycler System according to the protocol of the manufacturer (Roche Diagnostics, Mannheim, Germany). RT was carried out at 42°C for 60 minutes (first strand cDNA synthesis kit; Roche Molecular Biochemicals; Mannheim, Germany). For each PCR reaction, 2 μL of cDNA and an 18-μL reaction mixture were used. The reaction mixture contained 2.0-μL DNA Master Mix SYBR Green I (*Taq*

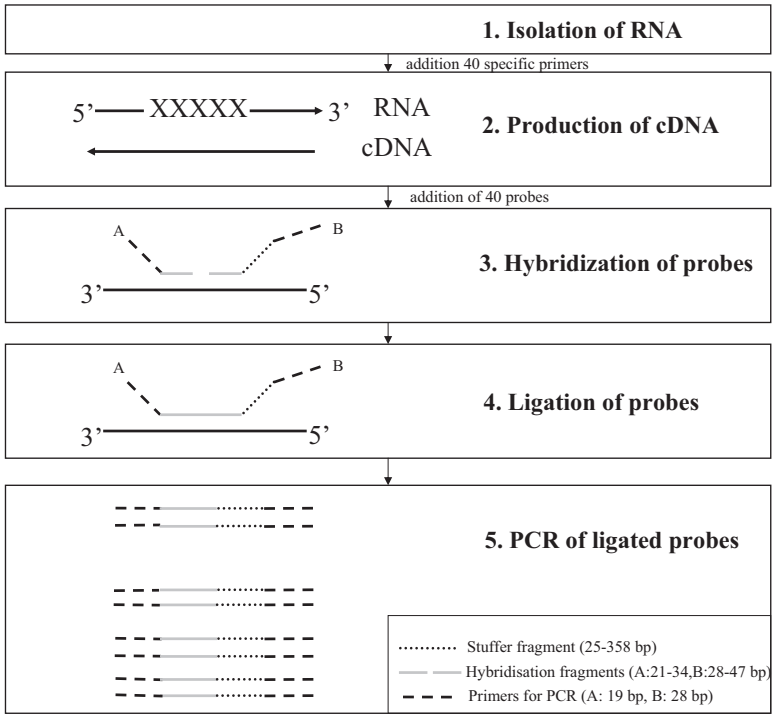

Fig 1. Schematic overview of the MLPA procedure. The MLPA assay consists of 6 steps, of which the first 5 steps are shown: (1) isolation of RNA, (2) production of single-strand cDNA by a reverse transcriptase reaction, (3) overnight hybridization of 40 pairs of a short-probe oligonucleotide (**A**) and a long-probe oligonucleotide (**B**) with the cDNA, (4) ligation of different probes by a ligase enzyme, and (5) PCR of the ligated probes 32 cycles. The last step (6), capillary electrophoresis, is not shown in this figure.

DNA polymerase, SYBR Green I dye, 10-mmol/L $MgCl_2$, and deoxynucleoside triphosphate mixture), 0.5 μL (2.5 ng) of both the forward primer and the reverse primer, 2.4-μL 4.0-mmol/L $MgCl_2$, and 12.6-μL aqua dest. Primers for E-selectin, ICAM-1, VCAM-1, GAPDH, and TF were obtained from Biolegio (Nijmegen, The Netherlands and are summarized in Table II). For DNA amplification, 40 cycles of denaturation (95°C, 30 s), annealing (10 s, primer-dependent; see Table II) and extension (72°C, 10 s) were performed. Water was used as a negative control. The melting curve analysis started at 95°C was then decreased to 5°C below the annealing temperature of each primer and then increased again to 95°C at the rate of 0.2°C/s. Quantification analysis was performed as described by Ramakers et al.[10] In each experiment, the expression of GAPDH was set at 1.0 (ie, 100%), to which changes in E-selectin, ICAM-1, VCAM-1, and TF expression were compared.

Statistics. Data were analyzed with Statistical Package of the Social Science software for Windows, release 11.5 (SPSS Benelux BV, Gorinchem, The Netherlands). The demographic characteristics of patients were normally distributed and therefore analyzed with a one-way ANOVA test for differences among 3 groups and Bonferroni *post hoc* tests. RNA expression of each gene measured with MLPA or real-time PCR was determined as a function of time and compared with the RNA expression of the endothelial cells incubated with culture medium alone (control). A mixed model was used to analyze expression of individual genes as a function of incubation time (repeat measures) and incubation condition. Data were paired because endothelial cells from the same umbilical cord were used for incubations with MP, MP-free plasma, or controls. If the *P*-value of the interaction between incubation condition and time was <0.05, the difference was considered significant.

RESULTS

MLPA ***Effect of IL-1α on mRNA expression in HUVEC.*** To validate the use of MLPA in endothelial cells and the effect of the pro-inflammatory IL-1α, experiments were performed using HUVECs from 7 umbilical cords. Incubation with IL-1α for different time intervals up to 24 hours showed maximal expression of all tested genes within the first 4 hours after addition of IL-1α (data not shown). Therefore, all further experiments were performed within this time interval.

Figure 2 shows changes in expression of interleukins (*A*, *B*), oncogenes and transcription factors/inhibitors (*C, D*), various intracellular enzymes (*E, F*), and chemokines, PDGF-B, TF, THBS-1, and TNF-R (*G*, *H*). Compared with control, especially the expression of genes coding for IL-8 (*A*), NFκB-1, NFκB-1A, and

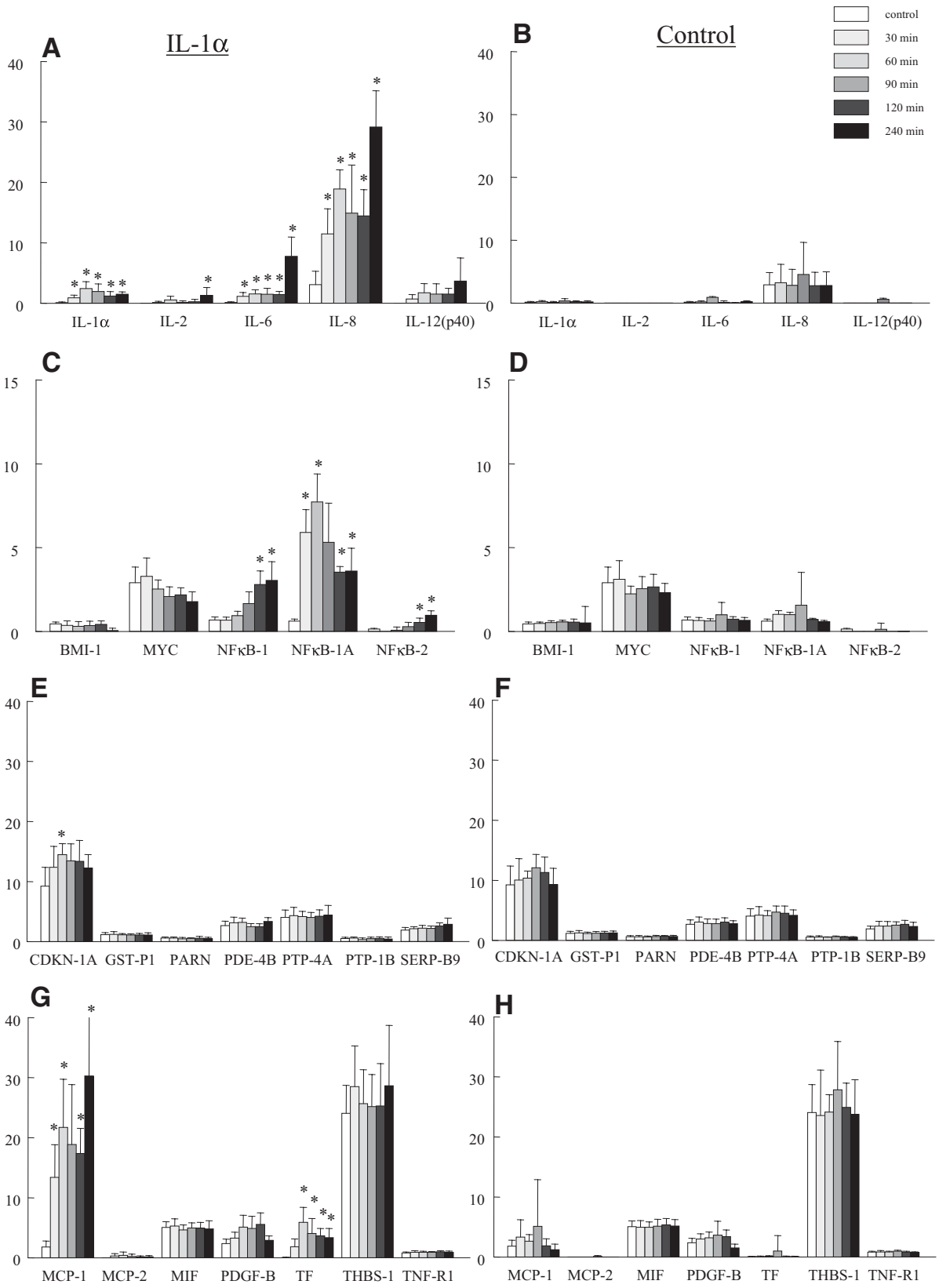

Fig 2. RNA expression in HUVECs after incubation with IL-1α. RNA expression induced by IL-1α (**A, C, E, G**) and control (**B, D, F, H**) of interleukins (**A, B**), transcription factors and oncogenes (**C, D**), enzymes or enzyme inhibitors (**E, F**), and other cellular factors (**G, H**). Incubation times were 0 (white), 30, 60, 90, 120, and 240 minutes (black). Statistically significant changes in expression are marked with *. The Y-axis displays the relative expression of each probe compared with B2M.

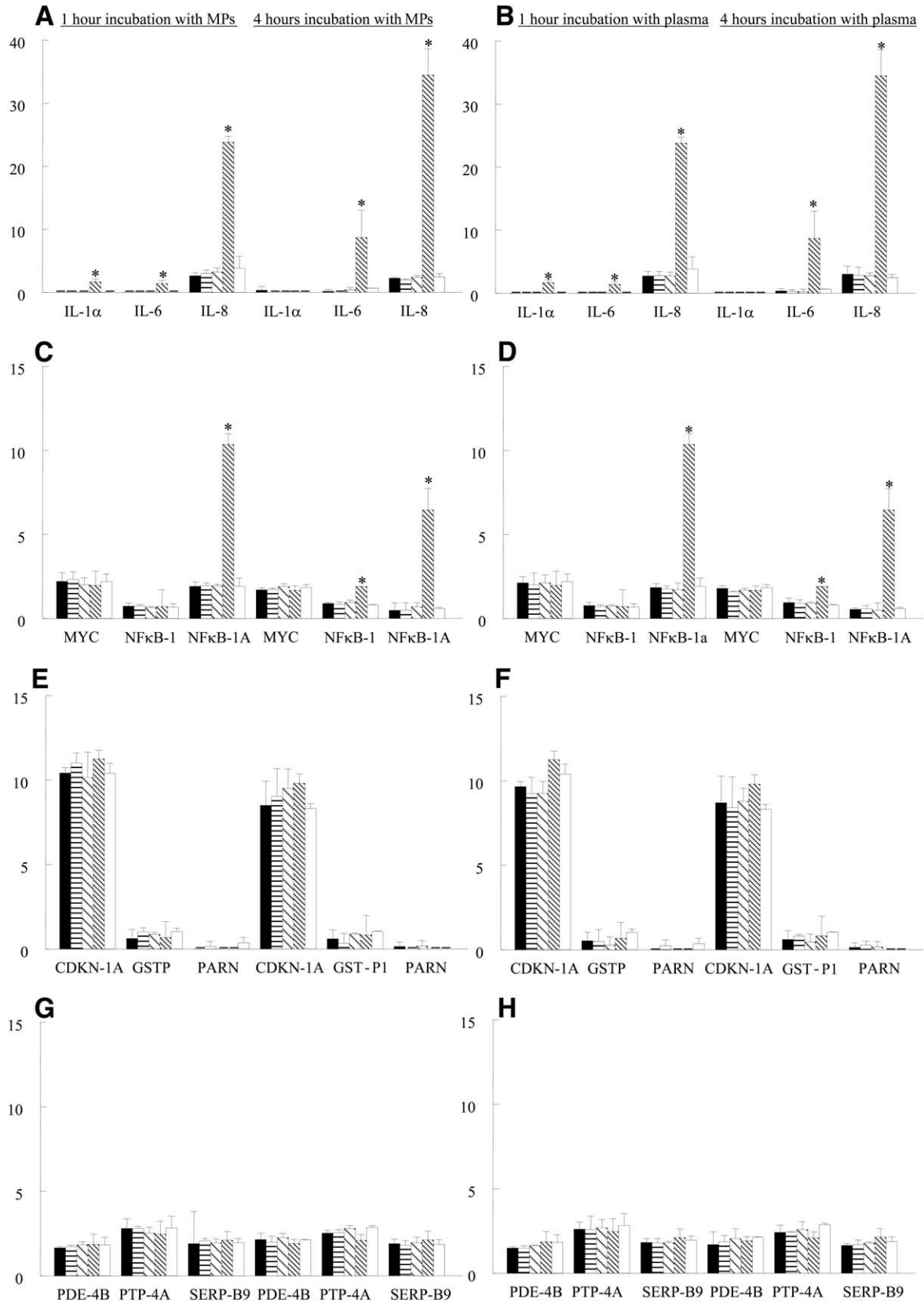

Fig 3. RNA expression in HUVECs after incubation with MPs. This figure shows the RNA expression in endothelial cells after incubation with MPs (**A, C, E, G, I**) or MP-free plasma (**B, D, F, H, J**). The bars represent from the left to the right: preeclampsia, normotensive pregnancy, non-pregnant controls, IL-1α (positive control), and culture medium alone (negative control). The left side of each graph shows the results of 1 hour of incubation, and the right side of each graph shows the results of 4 hours of incubation. The Y-axis displays the relative expression of each probe compared with B2M. Significant changes in expression are marked with *.

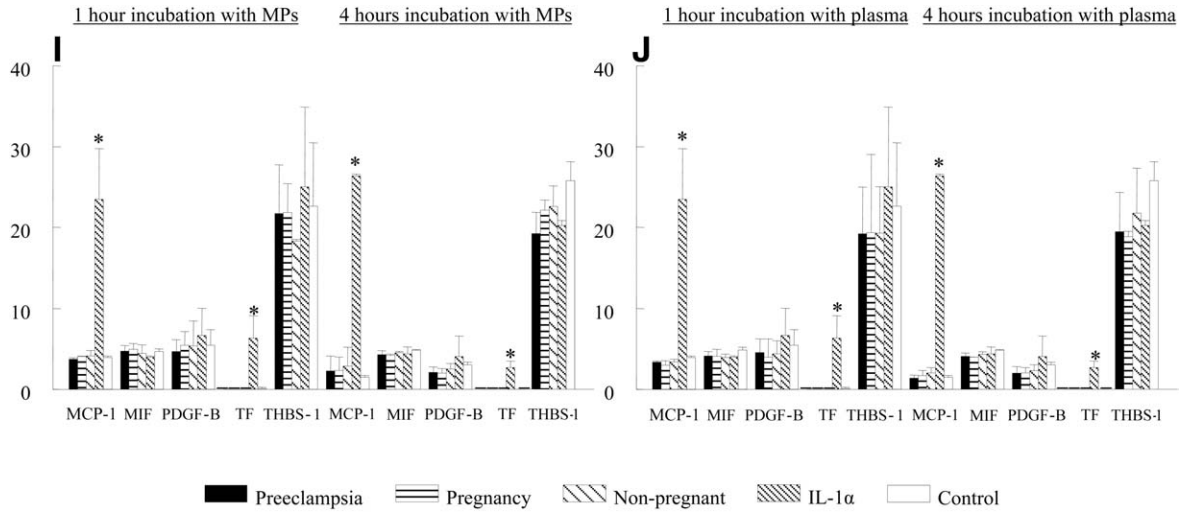

Fig 3. Continued

Table II. Primers used for real-time LightCycler PCR

		Sequence (5′ → 3′)	bp	Annealing Temperature
ICAM-1	forward	TTCCTCACCGTGTACTGGACT	228 bp	60°C
	reverse	TCCATGGTGATCTCTCCTCA		
E-selectin	forward	TGAGCATGGAAGCCTGGTTT	227 bp	60°C
	reverse	AGCTTCCAGGGTTTTGGAAA		
TF	forward	TGAAGGATGTGAAGCAGACGT	237 bp	58°C
	reverse	GGCTTAGGAAAGTGTTGTTCC		
VCAM-1	forward	GGAATTTCTGGAGGATGCAGA	226 bp	58°C
	reverse	TTGCAGCTTTGTGGATGGAT		
GAPDH	forward	GAAGGTGAAGGTCGGAGTC	225 bp	55°C
	reverse	GAAGATGGTGATGGGATTTC		

NFκB-2 (*C*) and MCP-1 (*G*) increased. TF expression was transient and maximal after 1 hour.

Results of duplicate samples of the same umbilical cord before incubation were almost identical (ie, variation in expression was less than 1%) (data not shown). When gene expression was normalized to the mean expression of a series of relatively stable genes (B2M, BMI, and PARN plus TNF-R) rather than B2M alone, similar expression patterns were observed (data not shown).

RNA expression of 37 genes was comparable between the first and third passages. In the absence of IL-1α and compared with the first passage cells, only the expression of BMI (at 1 hour) and CDKN-1A (at 1 hour and 4 hours) were increased. Endothelial cells stimulated with IL-1α showed an increased expression of CDKN-1A after 1 hour but not after 4 hours, and expression of MYC was elevated (at 1 hour). BMI, CDKN-1A, and MYC all play a role in the cell-division-cycle control system, which seems to be affected by cell passage.[10–12] As the expression of all other genes studied was hardly affected, it was decided to perform all experiments with the third passage endothelial cells to circumvent the complication of combining endothelial cells from various umbilical cords in order to obtain sufficient cells for the experimental setup.

In Table III, an overview of the MLPA data of multiple experiments is summarized.[13–40] In this table, the frequencies of detectable quantities of RNA for individual genes in either resting or (IL-1α) activated endothelial cells are indicated. Of the 40 genes studied, 27 were expressed in HUVECs, of which 20 genes had been demonstrated previously. The expression of BMI, PARN, PTP-4A, and MCP-2 has not been described in HUVECs before. BMI is an oncogene, expressed in lymphomas and other malignancies.[10,11] PARN deadenylates mRNAs, and PTP-4A is a tyrosine phosphatase, important for cell development, growth, and differentiation. Finally, MCP-2 is a monocyte chemotactic

Table III. Overview of presence of inflammatory genes in HUVEC

	Probes	MLPA	Literature	Ref
Interleukins	IL-1α	+	+	13, 14
	IL-1β	+	+	13, 15
	IL-1RA	−	+	14, 16
	IL-2	+	−	10, 11
	IL-4(R1)	−	−	13, 14
	IL-4(R2)	−	−	15, 14
	IL-6	+	+	13, 14
	IL-8	+	+	13–15
	IL-10	−	−	13
	IL-12(p35)	−	−	15
	IL-12(p40)	+	−	13
	IL-13	−	−	13, 14
	IL-15(R1)	+	+	13
	IL-15(R2)	−	+	15
	IL-18	−	+	17*
Transcription factors/ oncogenes	BMI	+		
	MYC	+	+	18, 19
	NFκB-1	+	+	20†
	NFκB-1A	+	+	21
	NFκB-2	+	−	22
Enzymes/Enzyme-Inhibitors	CDKN-1A	+	+	23
	GST-P1	+	+	24*
	PARN	+		
	PDE-4B	+	+	25, 26
	PTP-1B	+	+	27
	PTP-4A	+		
	SERP-B9	+	+	28, 29
Other cytokines	B2M	+	+	30‡
	IFNγ	−	−	13, 14
	MIF	+	+	31
	MCP-1	+	+	14, 32
	MCP-2	+		
	MIP-1A	−	+	33*
	MIP-1B	−	−	34
	PDGF-B	+	+	35, 36
	TF	+	+	37, 38
	THBS-1	+	+	39*
	TNF-α	−	+	13
	TNF-R1	+	+	40
	TNF-β	−		

Notes: All probes of the MLPA assay are listed. "+" reflects detection of the RNA of interest in at least two samples in the MLPA (third column) or reported in literature (fourth column), "−" means not detectable in any sample studied (n = 84) in the MLPA (third column) and not detected by other authors (fourth column), not even at protein level.
*Protein level.
†Activity measurement.
‡Flowcytometry.

protein. Current findings also implicate that human endothelial cells may produce IL-2 and IL-12 and express the transcription factor NFκB-2, which is a member of the NFκB/Rel gene family that regulates acute phase and immune responses. Although others reported the expression of IL-1 RA, IL-18, MIP-1A, and TNF-α in endothelial cells, the authors could not detect their expression, which may be due to the use of different agonists.

RNA expression after incubation with MP. Endothelial cells were incubated with isolated MP fractions or MP-free plasma from patients with preeclampsia, normotensive pregnant women, or healthy controls. IL-1α was used as a positive control, and culture medium was used as a negative control. Figure 3 shows the RNA expression of individual cytokines after incubation with these MP fractions (*A, C, E, G, I*) and MP-free plasma (*B, D, F, H, J*) and the controls. The incubation period was 1 hour (left side of each graph) or 4 hours (right side of each graph). The pro-inflammatory interleukins (IL-1α, IL-6, and IL-8) were not upregulated after incubation with MPs or MP-free plasma in any of the 3 groups studied (*A, B*). Also, genes encoding oncogenes, transcription factors (*C, D*), enzymes and enzyme-inhibitors (*E, F, G, H*), MCP-1, macrophage MIF, PDGF-B, TF, and THBS-1 (*I, J*) were unaffected by incubation with MPs or MP-free plasma. In these experiments, the results for IL-1α and culture medium alone were comparable with the initial experiments, which illustrates that HUVECs were viable and sensitive to activation.

Real-time PCR. To validate the sensitivity of MLPA in endothelial cells and to determine RNA expression of well-known endothelial adhesion molecules not included in the MLPA assay, real-time PCR was performed. Incubation with IL-1α resulted in a significant increase in expression of ICAM, VCAM, E-selectin, and TF (Fig 4). Expression of these adhesion molecules and TF was unaffected by incubation with MPs or MP-free plasma from patients with preeclampsia, normotensive pregnant women, or healthy controls.

DISCUSSION

In preeclampsia, the number of leukocyte-derived MPs is elevated compared with normotensive pregnant women and non-pregnant controls.[7] *In vitro*-prepared leukocytic MPs trigger the release of IL-6 and MCP-1 from endothelial cells[4,5] and leukocytic MPs isolated from synovial fluid of arthritic patients induce the release of cytokines from synoviocytes.[6] This process shows that leukocytic MPs promote inflammation. Therefore, we hypothesized that MPs from preeclamptic patients may directly affect expression of inflammation-related genes in endothelial cells.

Inflammation and endothelial dysfunction are closely associated in preeclampsia, and isolated MPs from preeclamptic patients impair endothelial-dependent vasodilatation in resistance arteries.[2] Dilation of blood vessels results from complex interactions between the

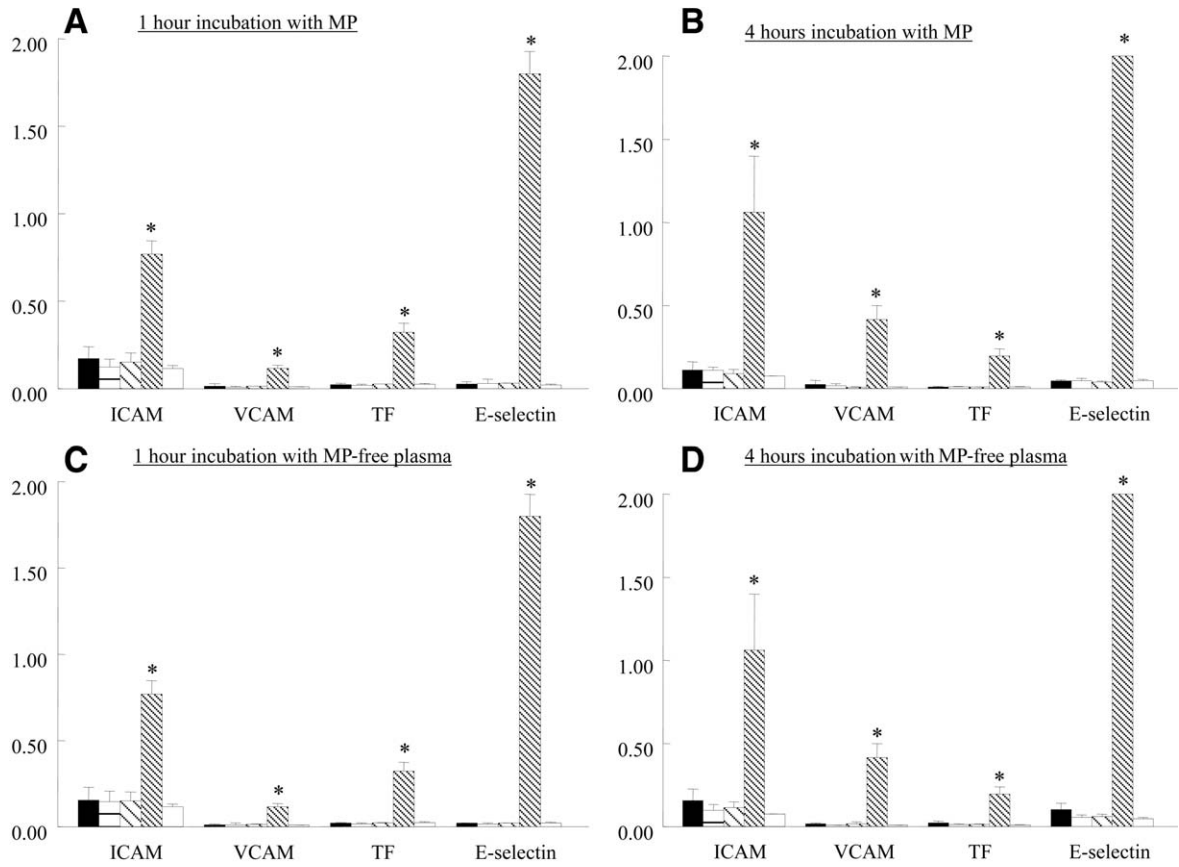

Fig 4. RNA expression in HUVECs measured with real-time PCR. Real-time PCR shows RNA expression in endothelial cells of adhesion molecules and TF. The bars represent from the left to the right: preeclampsia, normotensive pregnancy, non-pregnant controls, Il-1α (positive control), and culture medium alone (negative control). (**A**) Results of 1 hour of incubation with MP. (**B**) Results of 4 hours of incubation with MPs. (**C**) Results of 1 hour of incubation with plasma. (**D**) Results of 4 hours of incubation with plasma. The Y-axis displays the relative expression of each probe compared with GAPDH. Significant changes in expression are marked with *.

endothelium and the underlying smooth muscle cells. MPs may impair dilation by directly affecting endothelial cells, smooth muscle cells, or their interaction. In this study, the first possibility was assessed (ie, whether MPs exert their action directly on endothelial cells). It was demonstrated that MPs from preeclamptic patients did not affect the RNA expression of the studied genes in endothelial cells, not even with higher numbers than *in vivo*. The authors cannot exclude that the expression of other genes in endothelial cells (ie, genes not included in the MLPA) may be affected by MPs.

The second option (ie, the direct binding of circulating MPs to vascular smooth muscle cells) seems unlikely, because an intact endothelium prevents binding of MPs to these cells. However, in preeclampsia, the endothelium is damaged, possibly leading to exposure of the vascular smooth muscle cells to circulating MPs. Finally, MPs may affect the interaction between endothelial cells and vascular smooth muscle cells because MPs modulated isolated arteries.

The MLPA assay monitors changes in RNA expression of inflammation-related genes in human endothelial cells. This study is the first to use the MLPA for analysis of endothelial RNA expression. IL-1α induced a significant inflammatory response in endothelial cells. An effect of MPs could not be monitored. However, it cannot be excluded that subtle changes in gene expression induced by MPs were not detected. Additional studies will be necessary to determine whether minimal changes in RNA expression in endothelial cells can be accurately determined with MLPA. Also, it cannot be excluded that MPs target the endothelium of small arterioles. However, isolating sufficient RNA from such vessels is difficult and carries the risk of contamination by RNA from other cell types (eg, vascular smooth muscle cells), which complicates the interpre-

tation of the results. The effect of MPs on first passage endothelial cells was not investigated because preliminary experiments showed only minimal differences in gene expression between the first and the third passage endothelial cells. Therefore, it is unlikely that MPs would affect RNA expression of first passage cells differently.

In conclusion, the MLPA assay can be used to monitor changes in inflammation-related genes in human endothelial cells *in vitro*, but a direct effect of isolated MPs from preeclamptic patients on the expression of either inflammation-related genes or genes encoding adhesion receptors in endothelial cells could not be demonstrated.

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