CHAPTER 4

A MOLECULAR MAP
OF MICROVASCULAR DIVERSITY
IN RESPONSE TO ACUTE INFLAMMATION
AND ANTI-INFLAMMATORY DRUG TREATMENT

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Abstract

The microvasculature is functionally heterogenic based on its localization in the body. Knowledge on the control of endothelial cell behavior is, however, mainly based on in vitro studies. Our primary aim was to identify the molecular meaning of inter-organ and intra-organ endothelial heterogeneity in mice with regard to basal gene expression, and in response to proinflammatory cytokines and drug treatment. We investigated gene and protein expression levels of endothelial marker genes CD31, VE-cadherin, Endomucin, plasmalemmal vesicle protein (PV)-1, and von Willebrand factor (vWF), and of inflammation related adhesion molecules and chemokines and cytokines in major organs. A highly heterogenic basal gene expression status and response to tumor necrosis factor (TNF)α and interleukin (IL)-1β were observed. The microvascular endothelium furthermore showed organ specific responses to pretreatment with p38 MAPK inhibitor RWJ67657, NF-κB/AP-1 inhibitor MOL-294, corticosteroid dexamethasone, and NF-κB inhibitor BAY11-7082. Interestingly, the in vitro effects of the drugs on inflammatory gene expression were not predictive for their in vivo effects. Analysis of gene expression in endothelium laser microdissected from different microvascular beds in the kidney furthermore revealed local pharmacological effects that were masked by whole tissue analysis. The here described molecular heterogeneity of microvascular endothelial cells in (patho)physiological conditions and in response to drug treatment emphasizes the essence of in vivo studies in microvascular biomedicine research.
INTRODUCTION

Vascular endothelium represents a dynamic organ involved in many physiological and pathological processes of the body, with uniquely differentiated and highly specialized functions (1). Their role in pathology of e.g., inflammation and cancer makes the endothelial cells (ECs) an important target cell for therapeutic interference (2). An additional advantage of ECs as pharmacological target is related to ease of access for blood borne therapeutics. EC biology has been extensively studied, with the majority of studies performed using cultured EC. Although these studies have been valuable for a better understanding of the molecular control of EC behavior, extrapolation of this knowledge to the in vivo situation is difficult. Yet, for the design of effective pharmacological treatment regimens, a better understanding of EC behavior in physiological and pathophysiological conditions is instrumental.

ECs interact with their local microenvironment that consists of surrounding tissue, blood and blood borne cells (3). In inflammation, a quick and sequenced activation of intracellular signaling via nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) leads to changes in the expression patterns of endothelial adhesion molecules, and cytokines and chemokines. As a result, leukocytes adhere to ECs and subsequently migrate into the tissue. According to our current view, leukocyte migration into inflamed tissues takes place in post-capillary venules, the vascular bed that is not committed to essential organ functions. In these venules, blood flow is low, and their small diameter facilitates an intricate contact between leukocyte and endothelium. This paradigm however, is not observed in lungs where the principal site of leukocyte migration is the capillary bed (4). Moreover, this model of leukocyte adhesion differs significantly between organs. For example, in skeletal muscle, heart, kidney, and skin, selectins were shown to be key mediators of leukocyte rolling (5), while leukocyte recruitment to the liver and lungs was largely selectin-independent (6; 7). These findings demonstrate an important heterogeneity in processes underlying microvascular endothelium facilitated leukocyte recruitment into organs, the molecular basis of which is not well understood.

From a pharmacological point of view, it is highly likely that also in response to drug treatment endothelial heterogeneity exists. In recent years numerous drugs have been developed to interfere with inflammatory EC activation (2). Although evidence for strong inhibitory effects has been provided in vitro, little is known on how these drugs affect EC behavior in vivo. Understanding the molecular response of microvascular bed-specific endothelium to drug treatment is of critical importance in the development of effective therapeutic regimens for the treatment of inflammatory diseases.

In the current study we aimed to investigate the molecular meaning of microvascular heterogeneity in mice. Besides analyzing organ related expression profiles of genes that control endothelial homeostasis, i.e., CD31 (PECAM-1), vascular endothelium (VE)-cadherin (CD144), Endomucin, plasmalemmal vesicle protein-1 (PV-1, gp68), and von Willebrand factor (vWF), responses to acute inflammatory activation inferred by systemic tumor necrosis factor (TNF)α and interleukin (IL)-1β administration
were investigated. Furthermore, the effects of p38 MAPK inhibitor RWJ67657, NF-κB/activator protein (AP)-1 inhibitor MOL-294, corticosteroid dexamethasone (DEX), and NF-κB inhibitor BAY11-7082, on TNFα induced gene expression were studied in the major organs. Within the kidney, we furthermore unveiled vascular bed specific responses to the different experimental conditions by studying gene expression in microdissected glomeruli, and EC microdissected from arteriolar and postcapillary venule beds. We determined mRNA, and where possible, protein levels of P- and E-selectin, vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, IL-6, mouse IL-8 homologue KC (keratinocyte-derived chemokine, or CXCL1), and monocyte chemoattractant protein-1 (MCP-1). By this means, this study provides a detailed view into the molecular diversity of the microvasculature in the body, and its distinct adaptation to pathological changes and anti-inflammatory drug treatment.

MATERIALS AND METHODS

Drugs
IκB inhibitor BAY11-7082 ((E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile, Sigma) (8), glucocorticoid dexamethasone (DEX, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione, Genfarma B.V., Maarssen, The Netherlands), NF-κB and activator protein (AP)-1 transcription factor inhibitor MOL-294 (methyl (4R/S)-4-hydroxy-4-[(5S,8S)/((5R,8R))-8-methyl-1,3-dioxo-2-phenyl-2,3,5,8-tetrahydro-1H-[1,2,4]triazolo[1,2a]pyrazin-5-yl]-2-butyanoate) (9), and p38 MAPK inhibitor RWJ67657 (4-(4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1H-imidazol-2-yl)-3-butyn-1-ol, kindly provided by Johnson&Johnson Pharmaceutical R&D, Raritan, New Jersey, USA) (10) were prepared as 10 mM stock solutions in dimethyl sulfoxide (MERCK, Darmstadt, Germany). Further dilutions were made in saline (0.9% NaCl) or endothelial cell medium for the experiments with animal or with cells, respectively, to final concentrations as indicated in each experiment.

Animals
Male C57bl/6 mice (15-20 g: Harlan, Zeist, The Netherlands) were randomly divided into experimental groups. All intravenous (i.v.) injections were performed in the orbital plexus, during which mice were kept under anesthesia by inhalation of isoflurane/O2. To induce systemic activation, 200 ng TNFα (Biosource Netherlands, Etten-Leur, The Netherlands) or IL-1β (Biosource Netherlands) in 20% bovine serum albumin/saline was injected per mouse. Animals and vehicle treated control groups were sacrificed 2h later, and organs were immediately snap frozen in liquid N2, and stored at -80°C prior to use.
To investigate the pharmacological effects of the drugs, mice were i.v. injected with NF-κB inhibitor BAY11-7082 (8) (400 µg), the corticosteroid DEX (25 µg), the NF-κB/AP-1 transcription factors inhibitor MOL-294 (9) (250 µg), or the p38 MAPK inhibitor RWJ67657 (10) (160 µg), 5 – 35min prior to TNFα administration. Two hours after TNFα administration mice were sacrificed, organs harvested and snap frozen.
All animal experiments were approved by the local Animal Care and Use Committee of University of Groningen.
**Drug effects on TNFα mediated activation of HUVEC**

Confluent human umbilical vein endothelial cells (HUVEC) obtained from the Endothelial Cell Facility UMCG, were pretreated with 10 \(\mu\)M of either BAY11-7082, DEX, MOL-294, or RWJ67657. One hour later, cells were activated with 10 ng/ml of human recombinant TNFα (Boehringer, Ingelheim, Germany). After 6h of incubation at 37°C, 5% CO₂/95% air, cells were harvested for RNA extraction as described previously (11). (Nuclear) protein extraction and further processing for phospho-MAP kinase-activated protein kinase (MAPKAPK)-2 Western Blotting and NF-κB Electrophoretic Mobility Shift Assay were performed as described below.

**Detection of the phosphorylated substrate of p38 MAPK by Western Blotting**

HUVEC were incubated with 10 \(\mu\)M drugs for 1h and stimulated with 10 ng/ml TNFα for 30min, washed with ice-cold PBS and lysed in 100 \(\mu\)l of freshly prepared 1 x SDS sample buffer consisting of 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 62.5 mM Tris-HCl (pH 6.8) and processed as described previously (12). After SDS-PAGE and electroblotting, blots were incubated in blocking buffer (TBS/0.1% Tween20/5% nonfat milk), washed extensively in wash buffer (TBS/0.1% Tween20), incubated overnight at 4°C with anti-phospho-MAPKAPK-2 (Thr334) antibody (#3041, Cell Signaling Technology, Danvers, MA, US; dilution 1:1000 in TBS/0.1% Tween20/5% BSA), next incubated with horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (#7074, Cell Signaling Technologies, dilution 1:2000 in blocking buffer) for 1h at room temperature while shaking. Detection was carried out by chemiluminescence using Lumi-Light plus Western Blotting Substrate (Roche, Penzberg, Germany) and captured on X-ray film. Equal loading was confirmed by staining with mouse anti-human actin antibody (C4, IgG_κ, #69100, MP Biomedicals, Solon, OH, US; dilution 1:4000 in PBS/0.1% Tween20/2% BSA)/HRP-conjugated secondary goat anti-mouse antibody (Dako, dilution 1:2000 in blocking buffer) on blots which were earlier stripped with Restore™ Western Blot Stripping Buffer (Pierce, Perbio Science Nederland B.V., Etten-Leur, The Netherlands).

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)**

HUVEC were incubated with 10 \(\mu\)M drugs for 1h and stimulated with 10 ng/ml TNFα for 30min. For preparation of nuclear extracts, cells were placed on ice, the medium was removed, and cells were washed once with cold PBS. Cells were scraped and collected by centrifugation at 1,500 \(\times\) g for 5min. Nuclear extracts were prepared according to manufacturers protocol, using NE-PEC Nuclear and Cytoplasmic Extraction Reagent (Pierce). Protein concentration was determined using Bio-Rad Protein Assay reagent (BioRad Laboratories B.V., Veenendaal, The Netherlands) with bovine serum albumin as the standard. For EMSA 3 \(\mu\)g protein were incubated in a total volume of 20 \(\mu\)l for 20min at room temperature in binding reaction buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 2.5% glycerol, 5 mM MgCl₂, 1 \(\mu\)g of poly dI-dC, 1% NP-40 with 0.2 pmol of infrared dye end-labeled double-stranded NF-κB oligonucleotide (5'-IR dy681-ACTCAGTGGATATTCCCAGAAAAC; Isogen Life Science, IJsselstein, The Netherlands). Protein-DNA complexes were separated electrophoretically in the dark on a 4% polyacrylamide gel. After electrophoresis, the gel was imaged using Odyssey Infrared Imaging System (Li-Cor Biotechnology Lincoln, Ne, USA).

Competition experiments were carried out in the presence of 10 pmol unlabeled NF-κB oligonucleotides and supershift experiments with 1 \(\mu\)g anti-p65 antibody (Santa Cruz Biotechnology...
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Inc., Tebu-Bio, Heerhugowaard, The Netherlands), demonstrating NF-κB specificity of the signal and p65 being the DNA binding protein in the extracts (not shown).

Gene expression analysis by quantitative RT-PCR
Extraction of total RNA from cryostat sections of kidney, brain, heart, liver, and lungs were carried out according to the protocol of RNeasy Mini kit (Qiagen, Leusden, The Netherlands). One µg of total RNA was subsequently reverse transcribed and real-time PCR performed as described previously (11). Exons overlapping primers and MGB probes used for quantitative RT-PCR were purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands): housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Mm99999915_g1), endothelial genes: CD31 (Mm00476702_m1), VE-cadherin (Mm00486938_m1), PV-1 (Mm00453379_m1), vWF (Mm00550376_m1), Endomucin (Mm00497495_m1), inflammatory genes: P-selectin (Mm00441295_m1), IL-6 (Mm00446190_m1), MCP-1 (Mm00441242_m1), KC (Mm00433859_m1), cytokine receptors: TNFR1 (Mm00441875_m1), TNFR2 (Mm00441889_m1), IL-1R1 (Mm00434235_m1), IL-1R2 (Mm00439623_m1). Apart from these genes, the expression of Krüppel-like factor (KLF)2 (Mm00500486_g1) was studied in the microdissected material. Primers & probes for E-selectin (forward primer: CAACGTCATGTTCAAAACAATCAG, probe: CACAAATGCATCGTGGGA, reverse primer: TTAAGCAGGCAAGGAACCA), VCAM-1 (forward primer: TGAAGTTGGCTCACAATTAAGATGT, probe: AACACTTGATGTAAAAGGA, reverse primer: TGGCCAGTAGGTGCAAGAGGA) and ICAM-1 (forward primer: ATGGGAATGTCACCAGGAATG, probe: CAGTACTGTCACACCTTC, reverse primer: GCACAGAATGATTATGTCAGCAGT) were designed in house according to the same quality criteria as Assays-on-Demand primer & probes from Applied Biosystems. The primers and MGB probes used for HUVEC were described previously (11), in addition we used MCP-1 primers (SCYA2, Hs00234140_m1). TaqMan quantitative PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). Duplicate analyses were executed for each sample, the obtained Cₚ values were averaged and data calculated as described previously (11).

Immunohistochemical staining of mouse tissues
Primary antibodies (table 1) diluted in 5% fetal calf serum (FCS) in PBS, or Hank’s balanced salt solution HBSS (GIBCO Invitrogen Corp.) supplemented with 5% FCS for VE-cadherin detection, were applied to acetone fixed tissues according to standard protocols. Detection was performed with appropriate HRP-conjugated secondary antibodies (all from DAKO Cytomation, Glostrup, Denmark) diluted 1:40 in PBS/5% FCS supplemented with 2% normal mouse serum (NMS, DAKO Cytomation). Peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich) and sections were counterstained with Mayer’s hematoxylin (Merck, Darmstadt, Germany).

Staining of P-selectin, E-selectin, and VE-cadherin was performed using primary antibodies described in table 1, unlabeled rabbit anti-rat antibody (Vector Laboratories Inc, Burlingame, CA, USA; dilution 1:500 in PBS/5% FCS/5% NMS/5% NSS), and anti-rabbit polymer labeled with HRP (Dako Cytomation Envision+ System-HRP (AEC) according to manufacturers protocol. Washing buffer used was PBS for P- and E-selectin, and HBSS for VE-cadherin.

All pictures were taken at 200 x magnification. Enlarged version of all pictures is available on: http://www.rug.nl/umcg/faculteit/disciplinegroepen/plg/medbiol/kuldo/index.
**Laser microdissection of renal microvasculature**

Five \( \mu \text{m} \) cryosections mounted on 1.35 \( \mu \text{m} \) polyethylene-naphtalene membranes attached to normal 1 mm slides (P.A.L.M. Microlaser Technology AG, Bernried, Germany) were fixed in acetone and stained with Mayer’s hematoxylin, washed with diethyl pyrocarbonate treated water, and air-dried. ECs from small arterioles (6 x 10^5 \( \mu \text{m}^2 \)) and postcapillary venules (1.3 x 10^6 \( \mu \text{m}^2 \)), as well as glomeruli (3 x 10^6 \( \mu \text{m}^2 \)), were dissected using the Laser Robot Microbeam System (P.A.L.M. Microlaser Technology). For each kidney 700 glomeruli with an area of \(~3 \times 10^6 \mu \text{m}^2\) were dissected. Glomeruli were histologically identified, selected and dissected at 200 x magnification. The actual laser dissection of the tissue was carried out along the Bowman’s capsule. The laser was calibrated for a focus point between 64 and 66 with an energy level between 68 and 73. The speed during dissection was set at the maximum (100 units). For small arterioles an area of \(~6 \times 10^5 \mu \text{m}^2\) (consisting of lumen and cells) was dissected. The EC in the postcapillary venules were identified at 200 x magnification. Their position was marked and the actual selection and dissection of the EC layer was performed at 400 x magnification. The laser was calibrated for a focus point between 64 and 66 with an energy level between 70 and 75, depending on the thickness of the epithelial cell layer neighboring the venules. The speed during dissection was set between 50 and 60. For venules an area of \(~1.3 \times 10^6 \mu \text{m}^2\) (consisting of lumen and cells) was dissected. The EC in the postcapillary venules were identified at 200 x magnification. Their position was marked and the actual selection and dissection of the EC layer was performed at 400 x magnification. The laser was calibrated for a focus point between 47 and 49 with an energy level between 58 and 62, depending on the thickness of the epithelial cell layer neighboring the venules. The speed during dissection was set between 80 and 100. For catapulting glomeruli and arterioles versus postcapillary venules, the laser was focused 2 respectively 3 units lower than during dissection to make sure no tissue was damaged, and the energy level was increased with 20 respectively 17 units. The laser energy-pulse was given at a distance of \(~10\) respectively \(3 \mu \text{m}\) at the side of the selection line. Extraction of total RNA from

### Table 1. List of primary antibodies used for immunohistochemical analysis of protein expression in tissues

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Isotype</th>
<th>Concentration ( \mu \text{g/ml} )</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-selectin</td>
<td>Rat IgG1</td>
<td>1</td>
<td>BD Biosciences PharMingen (Ca, USA)</td>
</tr>
<tr>
<td>E-selectin</td>
<td>Rat IgG2a</td>
<td>10</td>
<td>MES-1, kind gift from Dr. D. Brown, Celltech Group (Slough, UK)</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Rat IgG1</td>
<td>Hybridoma supernatant</td>
<td>M/K-1.9, American Type Culture Collection (Virginia, USA)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Rat IgG2b</td>
<td>Hybridoma supernatant</td>
<td>YN1/1.7.4, American Type Culture Collection</td>
</tr>
<tr>
<td>CD45</td>
<td>Rat IgG2b</td>
<td>2.5</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>CD31</td>
<td>Rat IgG2a</td>
<td>1</td>
<td>MEC13.3, BD Biosciences PharMingen</td>
</tr>
<tr>
<td>Endomucin</td>
<td>Rat IgG1</td>
<td>5</td>
<td>V5c7.8, kindly provided by Dr. D. Vestweber (Munster, Germany)</td>
</tr>
<tr>
<td>PV-1</td>
<td>Rat IgG2a</td>
<td>Hybridoma supernatant</td>
<td>MECA32, Dev. Studies Hybridoma Bank (Iowa, USA)</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Rat</td>
<td>5</td>
<td>A kind gift from Dr. E. Dejana (Milan, Italy)</td>
</tr>
<tr>
<td>vWF</td>
<td>Rabbit</td>
<td>20</td>
<td>DAKO Cytomation</td>
</tr>
<tr>
<td>-</td>
<td>Rat monoclonal control</td>
<td>2</td>
<td>Antigenix America, USA</td>
</tr>
</tbody>
</table>
the dissected materials and subsequent DNase treatment were carried out according to the protocol of StrataPrep total RNA microprep kit (Stratagene).

Statistics
Linear Mixed effects Models and Analysis (LME) to account for duplicate PCR analysis values were used for data analysis to address the significance of observed effects (13). Multiple testing was controlled by the False Discovery Rate-controlling procedure of Storey and Tibshirani (14) for the different research questions at level of q < 0.05. All analyses were performed using R (15).

RESULTS

Expression of Endothelial marker and inflammation-related genes differed between vascular beds

Since literature provided evidence of organ differences in gene expression (5-7; 16), we first systematically investigated basal gene and protein expression patterns of endothelial markers and inflammatory molecules in the kidney, brain, heart, liver, and lungs. The constitutive expression of EC marker genes varied strongly between organs (Fig. 1). Lungs showed the highest expression of all marker genes compared to the other organs, which can be explained by its high degree of vascularization. Of note is the exceptionally low gene expression of PV-1 in the brain, and of vWF in the kidney. Phenotypic differences between microvascular endothelial subsets in the organs were visualized by immunohistochemical analysis: while CD31 and VE-cadherin stained in a pan-endothelial manner, Endomucin, PV-1, and vWF were restricted to specific subsets (Fig. 1). Both PV-1 and Endomucin were absent from arterioles in all organs, significantly expressed by endocardial endothelium in the heart, and heterogeneously expressed in the smaller vascular branches. While in kidney, liver, and lungs, the glomerular and peritubular endothelium, sinusoidal endothelium, respectively alveolar endothelium expressed both PV-1 and Endomucin, brain and heart capillaries were only positive for Endomucin. vWF was focally produced in endothelium in all vascular branches studied, except in sinusoidal endothelium in the liver and in the majority of brain capillaries.

In basal conditions, the highest respectively lowest gene expression of inflammatory cell adhesion molecules was observed in lungs and brain (Fig. 2). Selectins showed low constitutive expression, while high basal mRNA levels of VCAM-1 and ICAM-1 were found in all organs, and especially in the lungs. Using immunohistochemistry, P- and E-selectin proteins were undetectable in all microvascular beds (not shown). VCAM-1 and ICAM-1, on the other hand, clearly stained subsets of endothelium (Fig. 2). VCAM-1 was expressed by arteriolar endothelium throughout the organs, and exerted scattered expression in the venules and endocard, while capillaries were negative. Of interest is the finding that in brain only venular endothelium was modestly ICAM-1 positive, while its expression in arterioles and in capillaries in the parenchyma was undetectable. In contrast, in all other organs all vascular subsets expressed this cell adhesion molecule. These data substantiated the existence of microvascular endothelial heterogeneity at the level of inflammatory adhesion molecule expression.
Relative gene expression levels of endothelial markers genes (mRNA levels versus GAPDH x 1000)

<table>
<thead>
<tr>
<th></th>
<th>CD31</th>
<th>VE-cadherin</th>
<th>PV-1</th>
<th>Endomucin</th>
<th>vWF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>8.8 ± 1.0</td>
<td>10.6 ± 0.6</td>
<td>21.7 ± 2.2</td>
<td>12.9 ± 1.1</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>Brain</td>
<td>3.6 ± 1.9</td>
<td>5.9 ± 1.4</td>
<td>0.02 ± 0.01</td>
<td>1.5 ± 0.2</td>
<td>3.4 ± 1.3</td>
</tr>
<tr>
<td>Heart</td>
<td>24.5 ± 18.8</td>
<td>53.0 ± 13.4</td>
<td>1.1 ± 0.4</td>
<td>7.8 ± 0.4</td>
<td>4.8 ± 2.7</td>
</tr>
<tr>
<td>Liver</td>
<td>4.0 ± 0.7</td>
<td>14.7 ± 2.6</td>
<td>13.0 ± 2.7</td>
<td>0.9 ± 0.2</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Lungs</td>
<td>571.5 ± 69.0</td>
<td>1462.3 ± 234.5</td>
<td>589.5 ± 32.2</td>
<td>86.1 ± 11.2</td>
<td>336.5 ± 56.7</td>
</tr>
</tbody>
</table>

Figure 1. Microvascular heterogeneity presented at the level of endothelial marker gene mRNA expression (quantitative RT-PCR; table, mean of 3 mice per experimental group, ± s.d.) and the location of protein expression (immunohistochemistry; figure). The highly vascularized lungs showed the highest mRNA expression of all markers. CD31 and VE-cadherin proteins were expressed to different extents by all vascular endothelial cells while all other proteins exhibited vascular bed specific expression patterns. Black arrowhead: arteriolar EC; white arrowhead: venular EC; black arrows: capillary EC; white arrow: E = endocardium, G = glomerulus. Enlarged pictures used in the figures are available at: http://www.rug.nl/umcg/faculteit/disciplinegroepen/plg/medbiol/kuldo/index.
Relative gene expression levels of adhesion molecule genes (mRNA levels versus GAPDH \( \times 1000 \))

<table>
<thead>
<tr>
<th>Tissue</th>
<th>P-selectin</th>
<th>E-selectin</th>
<th>VCAM-1</th>
<th>ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>0.02 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Brain</td>
<td>0.009 ± 0.002</td>
<td>0.005 ± 0.002</td>
<td>1.5 ± 0.3</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Heart</td>
<td>0.04 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>0.16 ± 0.04</td>
<td>0.02 ± 0.02</td>
<td>2.6 ± 1.4</td>
<td>4.6 ± 1.8</td>
</tr>
<tr>
<td>Lungs</td>
<td>3.5 ± 0.4</td>
<td>0.2 ± 0.2</td>
<td>26.2 ± 15.9</td>
<td>285.5 ± 140.2</td>
</tr>
</tbody>
</table>

**Figure 2.** Differences in the level (mRNA analysed by quantitative RT-PCR; table, mean of 3 mice per experimental group, ± s.d.) and in the vascular location (protein analysed by immunohistochemistry; figure) of adhesion molecule expression in organs. Similar to the EC marker genes, lungs showed the highest expression of adhesion molecules compared to the other organs. P- and E-selectin mRNAs were expressed at a relatively low level, with proteins being undetectable by immunohistochemistry (not shown). Enlarged pictures used in the figures are available at: http://www.rug.nl/umcg/faculteit/disciplinegroepen/plg/medbiol/kuldo/index.

**Systemic proinflammatory cytokine administration caused organ specific endothelial cell activation**

Previously, we showed that TNF\(\alpha\) induced the expression of adhesion molecules in HUVEC to a higher extent than IL-1\(\beta\), while IL-1\(\beta\) more avidly affected cytokine and chemokine production (11). To study whether a similar differential effect prevailed in vivo, activation of EC in distinct organs in reaction to TNF\(\alpha\) and IL-1\(\beta\) administration was investigated. Generally, TNF\(\alpha\) induced higher expression of inflammatory genes compared to the same dose of IL-1\(\beta\), the microvascular response to either cytokine being organ specific (Fig. 3). The increase in VCAM-1 and ICAM-1 mRNA transcripts were lowest of all genes, and for all organs in the same range, while P- and E-selectin showed an organ defined differentiated response. Notably was also the striking difference in cytokine and chemokine expression in response to TNF\(\alpha\) and IL-1\(\beta\), with especially high induction in the kidney.

The differentiated response of the microvasculature with regard to inflammatory adhesion molecule expression was immunohistochemically substantiated for their reaction to TNF\(\alpha\) (Fig. 3). P-selectin was again undetectable (not shown), while ICAM-1 expression changes could only be visualized in the brain due to its presence in other organs at already high levels under basal conditions. Significant vascular bed differences were observed at the level of E-selectin and VCAM-1 expression. For example, both in brain and lungs subsets of arterioles and venules expressed
E-selectin in response to TNFα, while in the liver and kidney the specialized capillary sinusoidal respectively glomerular endothelium became E-selectin positive. In the heart, especially the endothelium covering the endocard and the larger venular endothelium expressed E-selectin. VCAM-1 followed the E-selectin expression pattern.

Figure 3. Cytokine-induced expression of inflammatory genes showed significant inter-organ vascular heterogeneity. The responses of kidney, brain, heart, liver, and lungs to i.v. administered TNFα (open bars) or IL-1β (filled bars) were assessed at 2h after injection. Using quantitative RT-PCR, gene expression was compared to untreated controls as described in Materials and Methods. Asterisks represent significant difference in gene expression induction between TNFα and IL-1β for the particular organ. *, q < 0.05. The table presents relative expression of cyto/chemokine genes in basal conditions in the organs, with accompanying changes in expression levels upon cytokine administration in the figure. Mean of 3 mice per experimental group, ± SEM. Immunohistochemistry visualized the microvascular localization of the expression of the cell adhesion molecules upon TNFα stimulation. P-selectin was undetectable at the protein level (not shown). Enlarged pictures used in the figures are available at: http://www.rug.nl/umcg/faculteit/disciplinegroepen/plg/medbiol/kuldo/index.
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In heart, liver, and lungs. In the kidney, however, VCAM-1 protein was infrequently expressed in glomeruli where E-selectin was present, while an increased number of VCAM-1 positive peritubular endothelium was detected where at the same time E-selectin was absent.

Both TNF receptors and IL-1R1, which mediate signaling upon binding of their respective cytokine, were abundantly expressed in lungs and liver, whereas heart, kidney, and brain manifested lower constitutive expression (table 2). Overall, these expression levels could not directly relate to the observed pattern and extent of cytokine responsiveness of the vascular beds.

**Table 2. Basal expression levels of TNFα and IL-1β receptors in mouse organs. Values represent mRNA levels versus GAPDH x 1,000. N=3 ± s.d..**

<table>
<thead>
<tr>
<th></th>
<th>TNFR1</th>
<th>TNFR2</th>
<th>IL-1R1</th>
<th>IL-1R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>8.6 ± 0.7</td>
<td>0.2 ± 0.06</td>
<td>0.4 ± 0.2</td>
<td>0.007 ± 0.005</td>
</tr>
<tr>
<td>Brain</td>
<td>2.6 ± 0.2</td>
<td>0.2 ± 0.02</td>
<td>0.2 ± 0.04</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Hart</td>
<td>7.2 ± 1.9</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.08</td>
<td>0.005 ± 0.003</td>
</tr>
<tr>
<td>Liver</td>
<td>24.3 ± 5.4</td>
<td>2.0 ± 0.7</td>
<td>3.0 ± 1.3</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Lungs</td>
<td>185.4 ± 40.2</td>
<td>14.0 ± 1.6</td>
<td>8.8 ± 3.7</td>
<td>1.0 ± 1.0</td>
</tr>
</tbody>
</table>

Inhibitors of inflammatory signal transduction differentially affected TNFα-induced microvascular activation

Next, we investigated the effects of drugs on endothelial cell activation. In HUVEC, both BAY11-7082 and MOL-294 significantly suppressed TNFα induced adhesion molecule and MCP-1 expression, whereas RWJ67657 most strongly, and DEX to a lesser extent, affected the expression of interleukins (Fig. 4). P-selectin was not induced by TNFα activation in HUVEC (not shown), which corroborated previous findings (17). BAY11-7082 selectively interfered in the NF-κB pathway, RWJ67657 affected TNFα mediated p38 MAPK signaling via inhibition of phosphorylation of its substrate MAPKAPK-2 (Fig. 4), while both molecular targets were unaffected by DEX (Fig. 4A/B, (18)) and MOL-294 (not assessed; (9)). Of note is that inhibition of NF-κB by BAY11-7082 was accompanied by an increase in p38 MAPK activity, implying a shift in kinase pathway under pharmacological pressure.

In vivo, the drugs affected gene expression to various extents, but the patterns of genes affected by the drugs did not reflect their effects observed in vitro (Fig. 4C). Among the striking differences was the absence of DEX effects on TNFα-induced adhesion molecule expression in HUVEC, while in vivo the drug significantly inhibited the expression of E-selectin in all organs, and of VCAM-1 in heart, liver, and lungs. These data unambiguously demonstrate that the in vivo pharmacological activity of the anti-inflammatory drugs considerably deviated from the effects observed in an in vitro EC system that is frequently used to study pharmacological effects of new anti-inflammatory drugs in development.
Figure 4. Inhibition of TNFα–induced inflammatory gene expression by anti-inflammatory drugs. (A-B) Inhibitory effect of drugs on nuclear translocation of NF-κB as determined by EMSA (A), and on p38 MAPK signal transduction as determined by MAPKAPK-2 substrate phosphorylation by Western Blotting, with actin as loading control (B). HUVEC were pre-incubated with 10 µM drugs for 1h and stimulated with 10 ng/ml TNFα for 30min. 1: no stimulation, 2: TNFα, 3: BAY11-7082/TNFα, 4: DEX/TNFα, 5: RWJ67657/TNFα. (C) In vitro and in vivo effect of drugs on gene expression as assessed by quantitative RT-PCR. mRNA levels were adjusted to GAPDH and normalized to nonactivated, nontreated controls. Mean values (n=3) ± SEM. Drug effects in cells and organs were related to TNFα treated samples (0% - no effect of drug pretreatment) and quiescent, untreated controls (-100% - drug pretreatment fully inhibited upregulation of the gene in response to TNFα). Asterisks represent significant difference between gene expression levels induced by TNFα and the levels obtained in cells/mice treated with drugs prior to TNFα administration. *, q<0.05.
Endothelial heterogeneity in inflammation

Renal microvascular beds can be molecularly mapped by gene expression analysis in microdissected samples

We recently showed that pharmacological effects of a drug selectively delivered in a subset of endothelial cells can be masked when analyzing whole tissue RNA isolates (19). To be able to identify the vascular localization of (additional) in vivo effects of DEX in the kidney, we separated the microvasculature by laser microdissection of arteriolar and venular endothelium, and glomeruli. The experimental procedure of microdissection combined with real-time RT-PCR allowed isolation of intact RNA from dissected cells (Fig. 5). The vascular subsets expressed the endothelial marker genes to a different extent, which corroborated the protein expression profiles shown in Figure 1. Exceptions were Endomucin and PV-1 in arterioles, where we did detect mRNA but failed to detect the protein, the latter observation corroborating previous findings (20; 21).

Renal microvascular subsets were differentially responsive to TNFα

The microdissection strategy now enabled us to study the molecular basis of microvascular heterogeneity in the kidney under basal conditions, and in response to TNFα and drug treatment. Besides the intra-renal microvascular differences in EC marker gene expression, a remarkable heterogeneity in basal inflammatory gene expression was observed (Fig. 6). Upon TNFα administration another quality of microvascular heterogeneity became visible – each microvascular bed demonstrated its own TNFα driven adhesion molecule expression profile. P- and E-selectin were most strongly induced in glomeruli, while VCAM-1 and ICAM-1 were expressed by all vascular beds to a similar extent. The mRNA levels of the adhesion molecules in the different vascular beds were in accordance with their protein expression profiles as shown in Figure 2. The added value of our strategy of gene expression analysis in microdissected samples was demonstrated by the unmasking of basal expression of P-selectin in arterioles, and its strong induction by TNFα in glomeruli and venules, which was undetectable by immunohistochemistry. In addition, while immunohistochemistry did not allow assessment of changes in ICAM-1 expression due to high protein expression at basal conditions, gene expression analysis demonstrated that it did respond in all vascular beds.

Cytokine and chemokine expression levels were also spatially controlled (Fig. 6C), with arteriolar mRNA levels being below the threshold cycle values (Ct) of 40 cycles and therefore assigned as nondetectable. TNFα strongly induced IL-6, KC, and MCP-1 mRNA levels in all vascular beds, with the most pronounced expression of KC in glomeruli (Fig. 6D).

An important issue in looking at the microvasculature by gene expression analysis of microdissected cells is the choice of the housekeeping gene. We used GAPDH as a reference based on the fact that vascular endothelium in different locations expresses endothelial marker genes at different levels. To correct for endothelial content and hence for impurities by other cells which are inadvertently part of the microdissected samples, we also calculated gene expression levels of endothelial-restricted genes using CD31 as a reference (Fig. 7). This did not change the overall pattern of gene expression.
Figure 5. (A) Laser microdissection of renal microvasculature. a) Representative picture of renal microvascular beds: black arrowhead points at an arteriole, black arrow at a glomerulus, and white arrowhead at a venule. b) Laser cutting of the glomeruli was carried out along the Bowman’s capsule. c) Dissection of the venular EC was carried out across the epithelial cell layer in close proximity to the endothelial layer. Since the laser has a particular width, a small space between the laser and the EC layer was kept to circumvent laser induced damaging of the EC. d) Dissection of arteriolar EC was carried out across the smooth muscle cell layer in close proximity to the arteriolar endothelium. Similar to above described selection of venular endothelium, a small space between the selection-line and the EC layer was kept. (B) The integrity of RNA isolated from microdissected material was verified by quantitative RT-PCR using two CD31 primer sets. The 5’ primer set hybridized to exons 2-3 and the 3’ primer set to exons 11-12. The threshold cycle (Ct) data exemplify a kidney RNA isolate and RNA isolate of microdissected glomeruli, and are representative for similar analyses on other microdissected samples. (C) Expression levels of CD31, Endomucin, PV-1, VE-cadherin, and vWF in whole kidney RNA isolates, and in RNA isolated from microdissected arterioles [A] and venules [V], and from glomeruli [G]. Mean values of 3 mice ± SEM (unadjusted for duplicate PCR values). Asterisks represent significant differences in expression of a marker in the particular vascular bed versus its expression in the other two vascular beds. *, q < 0.05.
Dexamethasone strongly inhibited adhesion molecule expression in vascular subsets. We investigated whether an acute pharmacological effect of DEX on microvascular EC could be visualized by isolating the EC from the rest of the organ prior to gene expression analysis. Indeed prominent inhibition of TNFα-induced adhesion molecule expression was observed in renal microvascular beds of mice pretreated with DEX (Fig. 8). In arteriolar EC, DEX significantly inhibited the expression of all endothelial adhesion molecules, while P-selectin respectively VCAM-1 were inhibited in venular and glomerular EC. In contrast, DEX barely affected the expression of IL-6, KC and MCP-1 in any of these vascular beds (not shown). The inhibitory effects of DEX on adhesion molecule protein expression heterogeneity in the different microvasculatures, implying that the patterns reported reflect a factual heterogeneity between vascular beds.
were semi-quantitatively substantiated by immunohistochemistry for E-selectin and VCAM-1 (Fig. 8), while effects on P-selectin (undetectable) and ICAM-1 (high protein expression) could not be visualized.

**DISCUSSION**

In the present study we investigated the molecular meaning of microvascular heterogeneity in mice. We showed that ECs display a high degree of variation in basal gene expression depending on their location in the body and within the organ. Moreover, activation with TNFα or IL-1β affected the expression of vascular restricted and vascular-associated inflammatory genes in patterns that differed between organs and between microvascular beds within an organ. Interference with endothelial inflammatory intracellular signaling by pretreatment with drugs diminished TNFα-induced upregulation of inflammatory gene expression in an organ specific manner which did not per se correspond to their *in vitro* effects.

This study employed a new experimental strategy that enables a detailed investigation of the molecular basis of microvascular behavior *in vivo*. Using laser microdissection of subsets of cells from morphologically distinct vascular beds, we unmasked in a quantitative manner the meaning of microvascular heterogeneity in the kidney. Since real-time RT-PCR analysis is more sensitive than immunohistochemistry,
Figure 8. *In vivo* inhibitory effect of DEX on TNFα-induced microvascular gene (mRNA analysed by quantitative RT-PCR; graph) and protein (analysed by immunohistochemistry; figure) expression in renal microvascular beds. DEX effects are related to TNFα responses set at 100% and healthy controls set at 0. Mean values of 3 mice ± SEM. Asterisks represent significant difference in gene expression between mice only administered with TNFα and mice treated with DEX prior to TNFα administration. *, q < 0.05. While DEX effects on protein expression of VCAM-1 in glomeruli (white arrow: glomeruli) and E-selectin in arterioles (black arrowhead) corroborated the gene expression data, its local effects on the expression of P-selectin (protein not-detectable, data not shown) and ICAM-1 (protein overexpressed) could only be assessed by microdissection/quantitative RT-PCR.
we identified (cytokine induced) differences in endothelial adhesion molecule (e.g., P-selectin) and cytokine and chemokine (e.g., KC) expression which would have stayed hidden otherwise. The research strategy developed was validated by comparing endothelial specific and endothelial restricted genes with localization of their respective proteins. It should be noted, though, that the microdissection procedure for endothelial cells will not yield a pure endothelial isolate. The thickness of the endothelial layer is more than one order of magnitude smaller than the 1-2 µm span of the laser, as a consequence of which co-dissection of smooth muscle cells, pericytes, and epithelial cells with arteriolar, capillary, respectively venular endothelium is inevitable. Similarly, in case of glomeruli, the RNA of the dissected structures will always contain RNA of podocytes and mesangial cells. Determination of the level of impurity is no sine cure, as RNA of smooth muscle cell, pericyte and epithelial marker genes are readily detectable in endothelial cells (22). Moreover, likely heterogeneity in microenvironment controlled gene expression exists in other cell types as well, thereby complicating quantitation of impurities. Irrespective of this yet unsolved issue, we showed here that the experimental strategy developed allowed the reproducible dissection and gene expression profiling of endothelial enriched microstructures of the vascular branch that until now were difficult to approach.

In the kidney, the microvascular endothelium was highly responsive to acute inflammation induced by TNFα, and exhibited its response in a vascular bed specific manner. Especially cells in the glomeruli responded with a massive upregulation of both EC-restricted and more general inflammatory genes. Previously, high expression of signaling receptor TNFR1 in glomeruli was reported (23), where besides EC also mesangial cells and podocytes may contribute to responsiveness to TNFα (24). Interestingly, however, TNFR expression was quite similar in the three microvascular beds studied (Fig. 9), which cannot explain e.g., the minor upregulation of E-selectin

![Figure 9. Constitutive expression of TNFR1, TNFR2, and KLF2 in renal microvascular beds.](image)

Results of quantitative RT-PCR (gene expression adjusted to GAPDH) of RNA isolated from arterioles, glomeruli, and venules microdissected from kidneys of control mice. Mean values of 3 mice ± SEM. Asterisks represent significant differences in expressions of a gene in a particular vascular bed versus its expression in the other two vascular beds. *, q < 0.05.

in arterioles and venules. We also investigated whether differential expression of Krüppel-like factor (KLF)2 transcription factor as a controller of inhibition of cytokine-induced adhesion molecule expression could be an underlying mechanism for the different vascular reactions (25). We found that KLF2 was highly expressed in arteriolar endothelium and in glomeruli, both vascular beds belonging to the arteriolar branch
of the vascular tree, and to a lesser extent in venular endothelium (Fig. 9). Since all microvascular endothelium expressed their own proinflammatory adhesion molecule repertoire in reaction to TNFα, the differential expression of this transcription factor per se could also not explain the observed vascular responses. Additional differences in basal signaling scaffolds, kinase expression and activity levels, as well as differences in location inferred epigenetic control of gene expression may all contribute to the observed heterogeneity. Studies aiming to reveal insight into these matters should employ in vivo approaches to prevent loss of organ-imprinted control mechanisms. One attractive strategy to this end might be functional investigations in conditional gene knockouts created by targeted delivery of small interfering RNA into subsets of ECs, for which identification of EC subsets specific target epitopes is an essential prerequisite (26; 27).

The divergent pharmacology of DEX in EC in vitro and in subsets of EC in vivo cannot be explained by pharmacokinetic parameters such as plasma half-life, or organ distribution differences, as DEX exerted in all organs an effect on at least one EC restricted gene. It is possible that the effects observed in vivo are not a direct effect of DEX on the microvascular endothelium, but mediated by leukocytes known to be affected by the drug. Irrespective of the underlying cause, our data clearly indicate that in drug development a thorough in vivo study should be executed to molecularly map the effects of a drug on the microvasculature, irrespective of the outcome of the initial in vitro screening. Moreover, when the molecular target of a drug is localized in a designated cellular compartment of an organ, whole organ analysis may hamper proper interpretation of the data. Whether MOL-294 and RWJ67657 also exerted a pharmacological effect that was masked in the whole tissue analysis as performed here, is now under investigation in our laboratory.

In summary, the presented results indicate that ECs are highly heterogeneous depending on their location in the body with location-controlled microenvironmental conditions to which they can adapt. Further studies need to be undertaken to determine the molecular mechanisms underlying the observed microvascular heterogeneity. Unraveling these mechanisms will be instrumental in the development of therapeutic regimens that can effectively interfere with microvascular dysfunction in disease. Application of innovative tools to in vivo molecular pharmacology as described here, using preclinical or clinical tissue biopsies, taken for diagnostic purposes or before and after drug treatment, will be of key importance in this development.

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