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Vasospastic persons exhibit differential expression of ABC-transport proteins

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Purpose: To quantify the gene expression levels of the ABC-proteins MDR1 (P-glycoprotein) and MRP (multidrug resistance-associated protein) isoforms in isolated mononuclear cells of vasospastic persons with increased Endothelin-1 plasma levels.
Methods: Quantitative real-time RT-PCR was performed to determine the expression levels of the MDR1 (P-glycoprotein) gene and MRP1 to MRP5 genes as well as the expression of the ET_A and ET_B receptor in mononuclear cells derived from 11 vasospastic subjects compared to 10 healthy controls.
Results: Mononuclear cells of vasospastic subjects showed a significant decrease in the expression of MDR1 (P-glycoprotein) gene (p=0.029), MRP2 gene (p=0.003), and MRP5 gene (p=0.013) when compared to healthy controls. These effects were poorly correlated with ET-1 plasma levels. No significant ET_A and ET_B receptor expression was observed in both groups.
Conclusions: Vasospastic persons differ in their expression pattern of MDR proteins from healthy controls. This might be an indirect effect of elevated ET-1 levels.

ATP binding cassette (ABC) transporter proteins belong to a large superfamily of transport proteins that are highly conserved across evolution [1]. These transport proteins mediate the translocation of different structurally unrelated molecules across various membranes and are expressed in different tissues. They are located in the plasma membrane or in the membrane of different cellular organelles [2]. Therefore, they control the distribution of endogenous metabolic products and exogenous xenobiotics on a subcellular level as well as in the organism as a whole. Some of these proteins form specific membrane channels [3]. Others facilitate the transport of inorganic ions, or pump various organic compounds [4]. For this transport activity, ABC proteins utilize the energy of ATP hydrolysis [5].

Numerous clinical data, mainly derived from cancer research, have revealed that the multidrug resistance phenotype is often associated with the over-expression of certain ABC transporters, termed multidrug resistance (MDR) proteins. The P-glycoprotein (Pgp, MDR1, ABCB1) mediated multidrug resistance was the first discovered [6-9] and probably still is the most widely observed mechanism in clinical multidrug resistance [10].

Beside Pgp, other efflux-pumps belonging to the group of multidrug resistance-associated proteins with 7 homologues (MRP1-MRP7) were characterized. Over-expression of some of these transport proteins lead to MDR phenotype [11,12]. MDR proteins possess a broad substrate specifity [2]. Therefore, acute inhibition or decreased expression of such MDR proteins may result in an enhanced uptake and systemic accumulation of drugs, which may lead to an increased sensitivity or toxicity.

Self-reported observations of vasospastic subjects revealed an enhanced sensitivity to different drugs such as beta blockers and calcium channel blockers (many of them are substrates of MDR transport proteins). All these subjects showed characteristic symptoms for the vasospastic syndrome like an inborn tendency towards cold hands and sometimes cold feet, a low body mass index, and low blood pressure that fluctuates markedly [13]. They also often show a slower sleep onset [14], significantly less feelings of thirst coupled with less daily fluid intake (unpublished data), and a higher plasma level of endothelin [13].

Recently it was shown that Endothelin-1 (ET-1) in subnanomolar to nanomolar concentrations was able to rapidly reduce the activity of MRP2 mediated drug transport in shark rectal gland [15]. This effect of MRP2 function could be confirmed in killifish renal proximal tubules [16] and a similar inhibitory effect was seen for P-glycoprotein. Both effects could be abolished when an ET_B receptor antagonist was given but not when an ET_A receptor antagonist was given. This prompted us to investigate the expression levels of P-glycoprotein and MRP1 to MRP5 in subjects with vasospastic syndrome and elevated ET-1 plasma levels, and compare it with the expression of these transport proteins in healthy controls.

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METHODS

Blood samples: Blood samples were collected from 11 vasospastic subjects and 10 healthy controls. Vasospastic subjects and controls were recruited from the study "Pathophysiology of vascular dysregulation" (Swiss national protocol number 65/00; this study was performed at the University Eye Clinic of Basel, Switzerland). All participants gave written informed consent for all procedures before inclusion in the study. The protocol was approved by the Ethical Committee of the Department of Internal Medicine, University Hospital Basel Switzerland, and adhered to the guidelines laid down in the Declaration of Helsinki.

A detailed medical history excluded individuals with a history of alcohol or drug abuse, systemic diseases (e.g., diabetes, high concentration of blood lipids, major arterial hypertension or other systemic circulatory diseases other than vasospasm) or who had been taking any medication at least 4 weeks prior to the study. Subjects were included in the study after an ophthalmological examination without pathological findings and a screening for indicators of vasospasm. After local cooling of the fingers, vasospastic subjects exhibited a stop in blood flow for more than 20 s, which was detected by nailfold capillaromicroscopy [17]. In addition, ET-1 plasma levels were determined by a specific radioimmunoassay, as described by Goerre et al. [18]. Therefore, blood samples were taken by venopuncture after 30 min of a rest in a supine position. All vasospastic subjects tested here exhibited an increased plasma level of ET-1, ranging from 2.13 to 4.13 pg/ml (reference value for females: 1.42±0.28 pg/ml; for males: 1.67±0.34 pg/ml) [19]. Examination of healthy controls showed no vasospastic response and low ET-1 plasma levels. Individual ET-1 plasma concentrations are shown in Figure 1.

Isolation of mononuclear cells: Mononuclear cells were isolated from 10 ml heparinized whole blood by density centrifugation as described by Maurer and colleagues [20] using a lymphocyte separating medium (Lymphodex; Innotrain, Kronberg, FRG). After extensive washing (three times) with phosphate buffered saline (PBS) cells were centrifuged at 440x g for 5 min and the supernatant was aspirated. Dry pellets were frozen immediately and stored at -75 °C until use.

RNA extraction and reserve transcription: Total RNA was isolated using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) following the instructions provided by the manufacturer. RNA was quantified with a GeneQuant photometer (Pharmacia, Uppsala, Sweden). After DNase I digestion (Gibco, Life Technologies, Basel Switzerland) 2 μg of total RNA was reverse-transcribed by Superscript (Gibco, Life Technologies, Basel Switzerland) according to the manufacturer's protocol using random hexamers as primers.

Standards for Quantitative Real-Time PCR (TaqMan).: To generate the standard curves, we used gene-specific cDNA amplicons as standards. These standards cover the TaqMan primer/probe areas and were obtained by PCR amplification. Since all the genes of interest are expressed in Caco-2 cells we used reverse transcribed RNA of Caco-2 cells as a template. Total RNA was extracted from confluent monolayers at passage 52 using the RNeasy mini kit (Qiagen GmbH). For each gene-specific PCR, we used 25 ng of reverse-transcribed Caco-2 RNA per 25 μL reaction. The final concentration of each primer was 300 nM. The primers (Table 1) were designed using the primer express software 2.0 (Applied Biosystems, Rotkreuz, Switzerland) and were manufactured by Invitrogen (Basel, Switzerland). The components of the PCR reaction (AmpliTaq Gold, 10x PCR buffer, dNTPs, $MgCl_2$) were purchased from Applied Biosystems. The annealing temperature was 55 °C. Thermal cycling was conducted using a Mastercycler personal from Eppendorf (Hamburg, Germany). All PCR products were purified by running a 1.5% agarose gel (TAE buffer, 100 V, 50 min) and a subsequent gel extraction (gel extraction kit, Qiagen). The standards were quantified using the PicoGreen reagent (Molecular Probes, Eugene, OR) and were checked by sequencing (Microsynth GmbH, Balgach, Switzerland). Standard curves were generated by a 10 fold serial dilution. 2.5 μL of the diluted standards were added per 25 μL TaqMan reaction.

Quantitative Real-Time PCR (TaqMan): Each TaqMan reaction contained 25 ng of sample cDNA in a total volume of 25 μL. We used 15.2 μL of Brilliant™ Quantitative PCR Core Reagent Kit components (10x Core PCR buffer, $MgCl_2$, dNTPs, reference dye, SureStart Taq) obtained from Stratagene (Amsterdam, Netherlands). The concentrations of primers and probes (Table 2) were 900 nM and 225 nM, respectively. Primers and probes were designed according to the guidelines of Applied Biosystems with help of the Primer Express 2.0 software. Primers were synthesised by Invitrogen and probes by Eurogentec (Seraing, Belgium). The TaqMan assay was performed on a Gene Amp 5700 Sequence Detector (Applied

Table 1. Primer sequences of MDR1 and MRP isoforms

Gene	Accession Number	Amplification Forward Primer	Reverse Primer	Area
MDR1	M14758	5'-ACAGTCCAGCTGATGCAGAGG-3'	5'-CCTTATCCAGAGCCACCTGAAC-3'	1730-2150
MRP1	NM_004996	5'-CACACTGAATGGCATCACCTTC-3'	5'-CCTTCTCGCCAATCTCTGTCC-3'	2173-2489
MRP2	NM_000392	5'-CCAATCTACTCTCACTTCAGCGAGA-3'	5'-AGATCCAGCTCAGGTCGGTACC-3'	3509-3981
MRP3	AF085690	5'-TCTATGCAGCCACATCACGG-3'	5'-GTCACCTGCAAGGAGTAGGACAC-3'	3419-3746
MRP4	AF071202	5'-AAGTGAACAACCTCCAGTTCCA-3'	5'-CCGGAGCTTTCAGAATTGAC-3'	2026-2543
MRP5	NM_005688	5'-CTAGAGAGACTGTGGCAAGAAGAGC-3'	5'-AAATGCCATGGTTAGGATGGC-3'	570-902

Primer sequences of MDR1 and MRP isoforms used for RT-PCR.

Biosystems), a combined thermocycler and fluorescence detector. Cycling conditions were 10 min 95 °C initial denaturation and activation of AmpliTaq Gold DNA polymerase, followed by 40 cycles of 15 s 95 °C denaturation, 1 min 60 °C combined annealing and primer extension. Each sample was run in triplicate. As a negative control, we used not-transcribed total RNA in duplicate. No significant amplification was observed in these samples.

Statistical Analysis: Gene expression was compared for each gene between the control group and the vasospastic patients by the two sided non-parametric Mann-Whitney U-test. The level of significance was to $p<0.05$. Correlation analysis was performed using the non-parametric Spearman's rank correlation.

RESULTS

The individual pattern of expression of MDR1 and MRP isoforms was qualitatively different between healthy controls and vasospastic patients. Whereas in healthy controls no systematic pattern of gene expression could be observed, each of the vasospastic patients showed a qualitatively similar expression pattern of MDR1 and MRP isoform genes (Figure 2). On average, vasospastic patients expressed about half as much of the MDR1 gene than controls and they showed a smaller inter-individual range of MDR1 expression than controls (Figure 3). This was significantly ($p=0.029$) smaller than in the control group.

Expression of the MRP1 gene was slightly but not significantly ($p=0.085$) higher in the vasospastic patients and, on average, almost doubled. Expression of the MRP2 and MRP5 genes decreased significantly ($p=0.003$ and $p=0.013$, respectively) in the vasospastic patients. No significant changes in gene expression was observed for the MRP3 and MRP4 genes, although a trend to lower expression could be stated.

Non-parametric rank correlation (Spearman's ρ) showed, with the exception of MRP1, a weak negative correlation to ET-1 levels ($\rho=-0.31$ to -0.59). Although correlation was significant for MDR1, MRP2, and MRP5, the values of ρ indicate only poor to moderate correlation.

The expression of the endothelin receptors ET_A and ET_B in mononuclear cells of control and vasospastic subjects was not detectable for absolute quantification by the standard-curve method. A borderline expression was obtained for both groups with mean threshold cycle (C_t) values between 38.9 and 39.6 (Table 3). The C_t value is defined as PCR cycle number, where the PCR product (represented by a corresponding fluorescence) reaches a predefined threshold value. Earlier cycle number corresponds to higher amounts of cDNA of the gene of interest in the sample. C_t values above 38 cycles were judged to represent no or only trace amount of gene expression.

As a positive control, prostate tissue was used, where C_t values of 25.15 and 27.24 for ET_A and ET_B receptors were obtained, respectively. Since in every PCR cycle the DNA amount is approximately doubled, a 14,000-fold and a 5,000-fold difference for ET_A and ET_B receptor expression was observed, respectively. Mononuclear cells seem only to express trace amounts of these receptors.

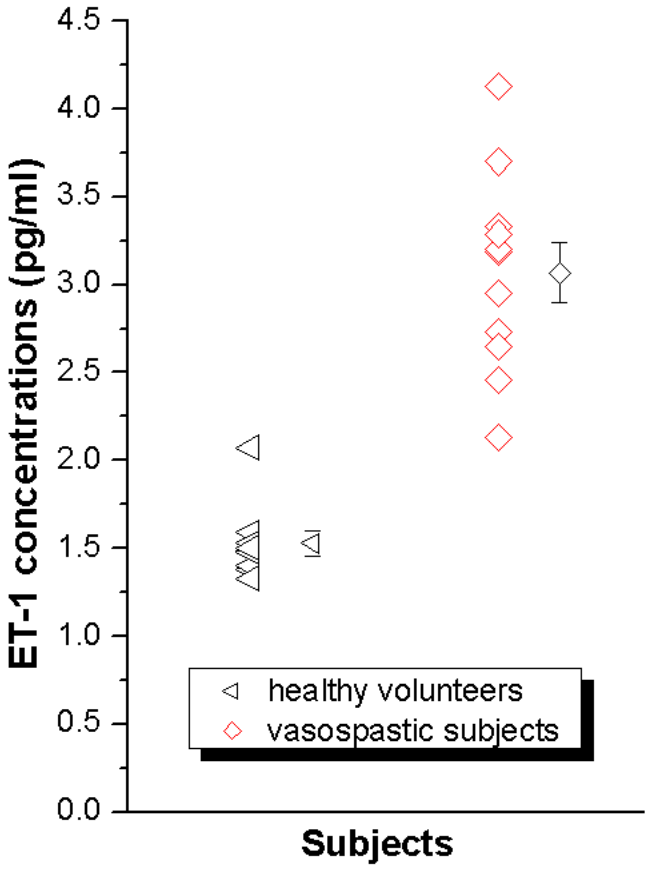

Figure 1. ET-1 Plasma Concentrations. Individual ET-1 concentrations as well as means and standard errors of the means in healthy and vasospastic subjects.

Table 2. Primer and probe sequences of MDR1 and MRP isoforms

Gene	Probe	Forward Primer	Reverse Primer
MDR1	5'-AAGCTGTCAAGGAAGCCAATGCCTATGACTT-3'	5'-CTGTATTGTTTGCCACCACGA-3'	5'-AGGGTGTCAAATTTATGAGGCAGT-3'
MRP1	5'-CCTCCACTTTGTCCATCTCAGCCAAGAG-3'	5'-GGGCTGCGGAAAGTCGT-3'	5'-AGCCCTTGATAGCCACGTG-3'
MRP2	5'-CTCAATATCACACAAACCCTGAACTGGCTG-3'	5'-ACTGTTGGCTTTGTTCTGTCCA-3'	5'-CAACAGCCACAATGTTGGTCTCTA-3'
MRP3	5'-CCAACCGGTGGCTGAGCATCG-3'	5'-GGTGGATGCCAACCAGAGAA-3'	5'-GCAGTTCCCCACGAACTCC-3'
MRP4	5'-CAAACCGAAGACTCTGAGAAGGTACGATTCCT-3'	5'-AAGTGAACAACCTCCAGTTCCAG-3'	5'-GGCTCTCCAGAGCACCATCT-3'
MRP5	5'-CTGACGGAAATCGTGCGGTCTTGG-3'	5'-CTGCAGTACAGCTTGTTGTTAGTGC-3'	5'-TCGGTAATTCAATGCCCAAGTC-3'

Primer and Probe sequences of MDR1 and MRP isoforms used for TaqMan analysis.

DISCUSSION

In addition to their contribution to the protection of the body against xenobiotics and to multidrug resistance in cancer, MDR proteins play an important but not yet fully understood physiological role.

The role of MDR proteins in the protection against toxic agents is supported by the wide substrate specificity of these transporters [2], the fact that MRP isoforms also mediate the transport of partially detoxified compounds, such as glutathione and glucuronide conjugates, and also by their tissue distribution. These transporters are present in important pharmacological barriers, such as in the blood-retina barrier by the retinal pigment epithelium [21] as well as the endothelial cells of the brain capillaries [22], and in the epithelial cells in the choroid plexus [23], both contributing to the blood-brain barrier. They could be also identified in the brush border membrane of intestinal cells [19], the biliary canalicular membrane of hepatocytes [24,25], and the luminal membrane in proximal tubules of the kidney [26]. So they are expressed as a consequence of differentiation triggers and in response to environmental challenges. Numerous studies revealed that MDR gene expression is not only influenced by harmful chemicals and metabolites, but also by stress-evoking stimuli. This stress response can either occur as an increase in MDR mRNA due to heat shock [27], UV, X and gamma irradiation [28-30] or genotoxic stress [21]. On the other hand induction of inflammatory response in experimental models of inflammation in rats and mice has been demonstrated to decrease the expression of PGP at the levels of mRNA [31]. Thereby, PGP expression is under the control of IL-6 [32].

Recently, acute inhibition of MDR1 (P-glycoprotein) and MRP2 function by the vasoactive hormone ET-1 [15,16] was demonstrated in sharks and killifish. In the present study we demonstrate changes in the expression of MDR1 (P-glycoprotein) and MRP isoform genes in leucocytes of vasospastic subjects manifested in a significant down-regulation of the mRNA levels of MDR1 (P-glycoprotein), MRP2 and MRP5 in vasospastic patients with elevated plasma concentrations of ET-1.

Endothelin is one of the most potent vasoconstrictors and was first discovered by Yanagisawa and co-workers in 1988 [33]. There exist three isoforms (ET-1, ET-2, and ET-3), each with 21 amino acid. ET-1 is present in many mammalian species, including humans. Although vascular endothelial cells

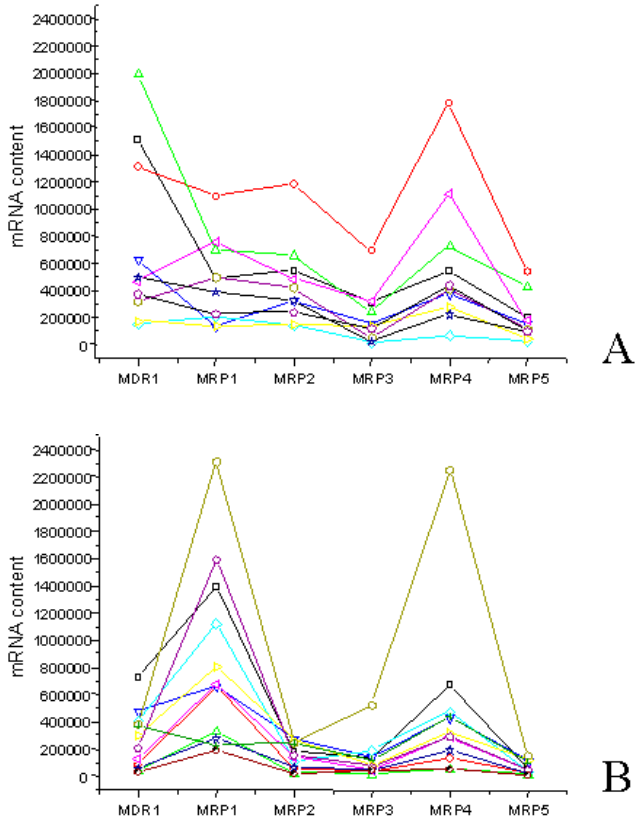

Figure 2. Individual gene expression of MDR1 and MRP isoforms. Individual gene expression of MDR1 (P-glycoprotein) and MRP isoforms (MRP1 to MRP5) genes: healthy controls (**A**), vasospastic persons (**B**).

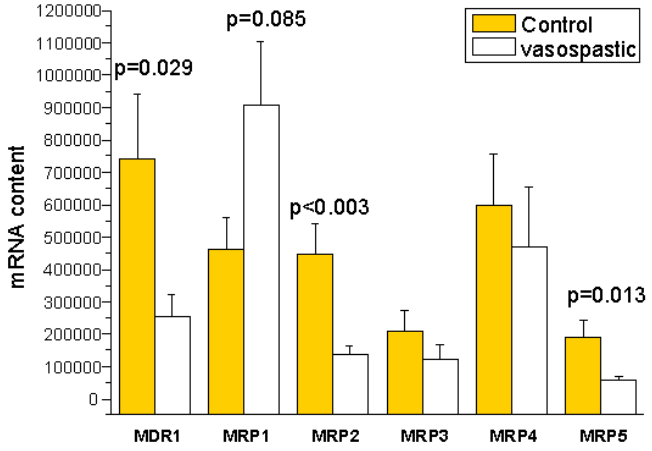

Figure 3. Gene expression of MDR1 and MRP isoforms. Mean gene expression of MDR1 and MRP isoform (MRP1 to MRP5) genes in healthy controls and vasospastic persons. Error bars represent the standard error of the mean.

Table 3. Mean C_T values obtained by TaqMan analysis

Sample	ET-A-Receptor	ET-B-Receptor
Controls	39.14	39.33
Vasospastic	38.90	39.60
Prostate	25.15	27.24

Threshold cycle (C_t) values and cDNA concentration are inversely correlated. A decrease by one in C_t values correspond approximately to a 2 fold cDNA concentration of the gene of interest. C_t values above 38 cycles are judged to represent no or only trace amount of gene expression.

are the major source of endothelin, it is also produced by a wide variety of cell types including renal tubular endothelium, glomerular mesangium, cardiac myocytes, glial cells, the pituitary, macrophages and mast cells [34]. Endothelins appear to act mainly as local paracrine/autocrine peptides, but circulating levels of endothelins also have great biological significance especially in pathological states of increased serum concentration [34,35].

Two receptors for endothelins have been characterized in humans, designated ET_A and ET_B [36,37]. The order of affinity of endothelins for ET_A receptor is endothelin-1>endothelin-2>endothelin-3. ET_B receptors show the same affinity for all 3 endothelins [34,35,37]. Both receptors are expressed in a wide variety of tissue types [38-41].

Concerning leukocytes, opposed results are available: in the human monocytic cell line THP-1 the presence of ET_B receptor mRNA was detected whereas another monocytic cell line (U937) lacked in its expression of the transcript [42].

The mechanisms of the changes in the expression of MDR1 (P-glycoprotein) and MRP isoforms in leucocytes of vasospastic persons are yet not understood. Although little is known about the signaling cascades that regulate MRPs, several pathways of their gene regulation appear to occur through stimulation of enviromental factors. While stress signals increase levels of MRP1 mRNA [30,43], MRP2 gene expression is down-regulated due to inflammatory cytokine release [44]. As mentioned above, in an animal model MDR1 (P-glycoprotein) and MRP2 mediated transport is under the control of ET-1, which acts here via protein kinase C [15,16].

There are indications in our experiments that the effect of elevated ET-1 levels on the expression of MDR proteins might be indirect: Although various studies provide evidence of the ET receptors in monocytic cell lines by using binding assays or receptor inhibition experiments [42,45], we could demonstrate that only trace amounts of mRNA transcripts of ET_A and ET_B receptor could be detected in isolated mononuclear cells of healthy controls and vasospastic persons. Compared to prostate tissue as positive control where both receptors are expressed, mononuclear cells showed a 14 fold and five thousand fold respectively less expression.

This view of an indirect mechanism is also corroborated by the poor intraindividual correlation between MDR protein expression and ET-1 plasma levels. The responsible mediating factors for MDR protein regulation are however not yet identified.

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