

INVESTIGATION OF ENDOTHELIN GENE EXPRESSION IN ENDOTHELIAL CELLS AND CARDIOMYOCYTES

PhD thesis

Dr. Keltai Katalin

Semmelweis University School of Ph.D. Studies

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of Cardiovascular Diseases



Supervisors: Dr. Béla Merkely, MD, DSc
Dr. László Cervenak, PhD

Official reviewers: Dr. József Tóvári, PhD
Dr. Péter Kempler, MD, DSc.

Head of the Final Examination Committee:
Dr. Anikó Somogyi, MD, DSc.

Members of the Final Examination Committee:
Dr. István Lőrincz, MD, PhD
Dr. Miklós Mózes, MD, PhD

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1. Introduction

Endothelin – an endothelium derived vasoconstrictor peptide was first described by *Yanagisawa* et al. in 1988. Further research in few years clarified that endothelin is not a single peptide, but a family of peptides with 3 members: endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3).

Physiological and pathological role of endothelins since then are subjects of intensive investigations, but a lot of details remained unclear. Endothelins have autocrine, paracrine and endocrine effects. . The most important place of endothelin biosynthesis is the vascular endothelium, but ET-1 is also produced in different tissues, by cardiac myocytes, brain cells, glomerular mesangial and epithelial cells and smooth muscle cells (SMC) of the aorta. The ET-1 gene is located on chromosome 6, its transcription is enhanced by hypoxia, shear-stress, angiotensin II, adrenaline, vasopressin, growth factors, insulin, oxidized LDL and thrombin through protein kinase-C, and inhibited by NO, prostacyclin and ANP (decrease of intracellular Ca^{2+} through increased cGMP level).

The active peptide is produced intracellularly in the Golgi apparatus and in the cellular membrane (on the surface of endothelial or smooth muscle cells). More than 75% of the produced endothelial ET-1 is secreted abluminally where it has access to its receptors on the vascular SMCs, therefore ET-1 is a paracrine rather than endocrine hormone. Distribution of receptors are cell- and tissue-specific. ET(A) receptors can be found on vascular SMCs, cardiomyocytes, cardiac fibroblasts, but not on endothelial cells. ET(A) mostly plays role in induction of vasoconstriction. ET(B) receptors are found predominantly on the endothelial cells and serve for the negative feedback of the ET-1 effect.

The endothelins activate complex, highly regulated intracellular signal pathways. Both short term (smooth muscle cell contraction or vasoactive peptide excretion) and long term (proliferation and migration) effects develop.

ET-1 is one of the most potent vasoconstrictors and plays a physiological role in maintaining basal vascular tone and normal blood pressure. It has proliferative, mitogenic effect on SMCs of the vascular media and on cardiomyocytes and effectively stimulates migration, phagocytosis and cytokine production (TNF, IL, GM-CSF) of monocytes, resulting in activation of macrophages and in triggering of inflammatory cellular response. ET-1 thought to have pathogenetic role in development of bone metastases, angiogenesis, in regulation of apoptosis and in production of free oxygen radicals.

ET-1 has dual effect in myocardial infarction; on the one hand, it has a causative role in the development of myocardial infarction through atherosclerosis, endothelial dysfunction and on the other hand it has stabilizing effect after infarction, it supports healing. After myocardial infarction fibroblast proliferation, expression of adhesion molecules and extracellular matrix deposition are enhanced by ET-1 resulting in myocardial fibrosis as part of postinfarct remodeling. Increased level of ET-1 is unfavorable for the coronary circulation, because it causes direct vasoconstriction, SMC proliferation, intimal hyperplasia, increased collagen-1 synthesis and stimulation of platelet aggregation.

The inner surface of the blood vessels are covered with the single cell layer endothelium. This layer is a physical barrier and it has also active haemodynamical and biochemical functions. Among others endothelium is responsible for maintaining vascular tone and structure, for regulation of vascular permeability and cell growth, thrombotic, fibrinolytic, inflammatory and redox processes, for influencing adhesions of white blood cells and platelets and for lipidoxidation. The endothelium is one of the largest organs in the human body and due to its direct contact with blood it is exposed to the toxic effects of intravenously administered compounds eg. chemotherapeutic agents. Anthracyclines (doxorubicin (DXR)) is a widely used cytostatic agent, but its administration is limited by its cardiovascular side effects. Few data are available on its acute or chronic effects on vessels and on endothelial dysfunction. Mode of action of anthracycline toxicity is not known completely, according to current knowledge, it is multifactorial. Suggested contributors to the toxic effects include direct DNA-damage (interkalation, alkylation, cross linking), direct membrane effects, formation of reactive oxygen species (ROS) and apoptosis induction.

In our previous study with lymphoma patients treated with DXR, we found a significant decrease in ET-1 plasma level after DXR treatment. The decrease of ET-1 level can contribute to the direct cytotoxic effect of DXR partly by inhibition of mRNA synthesis, partly causing endothelial dysfunction through production of superoxide agents. Decrease of ET-1 level can result in elimination of its cytoprotective effect, facilitating the cardiotoxic effect of anthracyclines. Based on these results we designed experiments to investigate the direct cytotoxic effects of anthracyclines to the ET-1 synthesis.

2. Objectives

Pathogenetic role of ET-1 in different cardiovascular diseases has been widely studied. Conclusions were drawn from animal studies and human plasma level measurements. Due to the abluminal secretion and the fast degradation of the peptide in the plasma, ET-1 plasma levels are not always reflecting the real processes. Based on our previous studies in DXR treated lymphoma patients we planned to directly investigate the role of ET-1 in cardiomyocytes. For this purpose we used a canine ischaemia/reperfusion model.

In this model plasma level of ET-1 is proportional to infarct size and also to the incidence of ischemia induced ventricular arrhythmias. It's also known that through inhibition of cardiomyocyte apoptosis, ET-1 has a role in tissue regeneration.

Objectives of the canine ischemia-reperfusion model experiment were:

- 1. To develop a method for the correct assessment of ET-1 mRNA production with the help of the reverse transcriptase-polymerase chain reaction (RT-PCR) method. Although ET-1 mRNA expression was already studied in a canine heart failure model by the ribonuclease protection assay, the RT-PCR method allows a more precise and sensitive measurement of the mRNA with the verification of the product.**
- 2. To investigate plasma levels of ET-1 and its precursor big ET-1 in an in vivo canine ischemia–reperfusion model.**
- 3. To investigate tissue levels of ET-1 mRNA in an in vivo canine ischemia–reperfusion model.**

Based on our previous studies in DXR treated lymphoma patients we conducted a second series of experiments to investigate the direct effects of DXR on the expression of endothelial cell genes known to be affected in cardiovascular diseases. Endothelial cells were chosen because they are the first to contact with the toxic agent (DXR).

Objectives of the DXR cytotoxicity experiments were:

- 1. To assess the DXR cytotoxicity on endothelial cells in different time points determining the optimal treatment time to investigate the direct effects of DXR.**
- 2. To determine the maximal cytotoxic concentration of DXR with different treatment length.**
- 3. To screen the gene expression pattern induced by DXR in HUVECs using an mRNA array (SA), consisting of genes expressed in the cardiovascular system and affected in several cardiovascular diseases.**
- 4. To assess the expression of ET-1 mRNA with real-time qPCR on endothelial cells.**
- 5. To validate our method, treatment of HeLa cells with DXR in the same setting.**
- 6. To validate our results with measurement of big ET-1 protein concentrations in DXR treated endothelial cells.**
- 7. To investigate the effect of DXR treatment on endothelial cells with use of microarray system assessing the expression profile of the whole genome. To map the connection network of ET-1 gene.**

3. Methods

3.1. Canine heart ischaemia/reperfusion modell

Nine consecutive mongrel dogs of either sex were studied. General anesthesia was performed. After endotracheal intubation, the dogs were ventilated with humidified room air. Left thoracotomy was performed and the left anterior descending artery (LAD) was isolated, and a snare occluder was placed around the LAD distal to its second diagonal branch for further ligation. Thirty-minute ligation of the LAD was performed and a 90-minute reperfusion period was observed thereafter. Blood and myocardium samples were collected before and after ligation and at 90 minutes of reperfusion. A myocardial biopsy was taken from the epicardial surface of the ischemic area. ET-1 and big ET-1 plasma levels were obtained by immuno-precipitation and detected by western blotting. The tissue level of ET-1 mRNA was assessed by RTPCR. The primers were designed online with the Primer 3 Design software. Identification of the PCR product was carried out first by molecular weight with gel electrophoresis and then by sequence analysis.

3.2. Experiments on endothelial cell culture

Preparation and culture of endothelial cells were performed according to our previously published method. Endothelial cytotoxicity was assayed according to Herczenik et al. LD50 was calculated from the two assays using a concentration gradient of untreated cells (from 10 000 to 1000 cells/ well) as standard curve.

For the SuperArray test the cells were lysed and stored in TRI® reagent. Total RNA (4 µg) was primed with oligo d(T)23 and reverse transcribed with Biotin-16-dUTP and M-MLV Reverse Transcriptase for GEArray system. The labeled cDNA was hybridized overnight on GEArray® Q Series Human Cardiovascular Disease I: Biomarkers (Cat.: HS-037) membrane. The chemiluminescent level was detected by ChemiImager 8900. Chemiluminescent data were analyzed with Genesis 1.5.0 software. mRNA levels were expressed as a ratio, compared to the corresponding housekeeping GAPDH. SA was validated by TNF α treated HUVECs, which showed significant elevation in the mRNA expression of IL-8, ICAM-1, VCAM-1, MCP-1 and E-Selectin (data not shown)—in accordance with previous publications.

For qPCR analysis the RNA–cDNA transcription was done with M-MLV reverse Transcriptase. LightCycler FastStartDNA Master SYBR Green I kit was used for quantification of cDNA on a LightCycler® 1.5. The ET-1 primers were designed from

database. The size of the PCR product was checked by electrophoresis. Identification of the PCR product was done by a sequence-analyzing device. To validate our qPCR assay further, we used HeLa cells as positive controls, since increased ET-1 mRNA expression has been shown on this cell type in response to DXR.

To validate mRNA results, ET-1 production was tested also with the commercially available big-ET-1 sandwich ELISA. ELISA was performed according to the instructions of the manufacturer. To investigate the effect of DXR treatment on endothelial cells with use of Agilent GE microarray system assessing the expression profile of the whole genome. The picture file was converted to data file by Agilent Feature Extraction program and using Agilent GeneSpring GX 11.5.1 statistical and orthology analyses were performed. Generally, microarrays are validated by qPCR method. In this case, we observed expression changes in several hundreds genes. It would have been uneasy and time consuming to perform qPCR for all the genes which showed expression change, so we used another microarray system (Affymetrix) to validate our results.

4. Results

Canine heart ischaemia/reperfusion modell

During ischemia there was a small, nonsignificant increase in both the plasma ET-1 and the big ET-1 levels (15.2 ± 4.1 fmol/mL to 17.9 ± 3.8 fmol/mL and from 14.7 ± 5.9 fmol/mL to 17.3 ± 5.1 fmol/mL) respectively. The ET-1 mRNA level in the myocardium decreased during ischemia to 57.8% of the baseline. In reperfusion a further and significant increase of the plasma level ET-1 was observed compared to the baseline and to the 30-minute ischemia level. ET-1 increased to 23.0 ± 5.1 fmol/mL ($P_{\text{to the baseline}} < 0.01$, $P_{\text{to 30-minute ischemia}} < 0.05$). Big ET-1 changed to 27.2 ± 7.0 fmol/mL ($P_{\text{to the baseline}} < 0.0004$, $P_{\text{to 30-minute ischemia}} < 0.01$). Simultaneously, an extensive increase in the ET-1 mRNA level (322% of the ischaemic, 214% of the baseline level) was detected.

Endothelial cell culture

DXR toxicity on endothelial cells

Extensive, non-specific breakdown of mRNA occurs both during apoptosis and during necrosis, and this can lead to the misinterpretation of altered gene expression. Therefore, it was necessary to determine the cytotoxicity of DXR at different time-points. Thus, HUVECs were treated with different doses of DXR for 2, 4, 6 and 24 h, respectively. Longer DXR treatment was not possible, due to the high apoptosis rate of endothelial cells. The cancer cell line HeLa, by contrast, is less sensitive and can be treated for longer periods. The half-maximal cytotoxic concentration (LD50) in HUVEC was 150 ng/ml at 48 h, 300 ng/ml at 24 h and above 10000 ng/ml at 6 h. Since high doses of DXR are used in the clinical treatment protocols administered in patients, and peak plasma concentration exceeds 1000 ng/ml for a short period, we exposed the cells to 1000 ng/ml DXR for 6 h.

Effects of DXR on mRNA expression pattern of endothelial cells.

The gene expression pattern induced by DXR in HUVECs was screened using an mRNA array (SA), consisting of genes expressed in the cardiovascular system and affected in several cardiovascular diseases. Out of the 96 representative cardiovascular genes of the array, only ET-1 mRNA expression was changed significantly by DXR treatment. Meanwhile, in the same experiments, TNF alpha – used as a positive control – induced mRNA expression of several adhesion molecules and cytokine.

ET-1 mRNA expression in DXR-treated endothelial cells

ET-1 mRNA expression decreased significantly and in a dose dependent manner. After 6 h, ET-1 mRNA expression in DXR treated (1000 ng/ml) endothelial cells was 10.9% of untreated controls ($p=0.0049$) measured during SA. In order to validate the results obtained by SA, endothelial cells were treated with DXR (300, 600 and 1000 ng/ml) and additionally, the expression of ET-1 mRNA was assessed with real-time qPCR after 6 h of treatment. The suppression of ET-1 expression was dose-dependent and very similar to that obtained with SA. However, because of the superior resolution of qPCR over SuperArray, even stronger suppression of ET-1 mRNA expression was found: HUVECs treated with 1000 ng/ml DXR expressed as low as 2.41% ET-1 mRNA, compared to controls ($p=0.0022$).

ET-1 mRNA expression of DXR treated HeLa cells

It is unknown how a sub-LD50 dose of DXR influences the expression of ET-1 in HUVECs. Studies using other cell types (human HeLa, rat ventricular myocytes, and murine HL-1) showed DXR dependent ET-1 induction, rather than the suppression that we found in HUVECs. To validate our method, we treated HeLa cells with DXR in the same setting. We observed dose- and time-dependent ET-1 mRNA induction in HeLa. This is in good accordance with other results obtained on this cell line. The effect on HUVEC, i.e. decreased ET-1 mRNA production, therefore, is probably a cell-specific reaction to DXR.

Effects of DXR treatment on ET-1 expression at protein level

ET-1 expression can be monitored quantitatively and accurately at mRNA level. However, it is nevertheless worthwhile to assess the ET-1 protein – the vasoactive agent itself, since mRNA levels do not always correctly reflect the corresponding protein concentrations. Therefore, we measured big ET-1 protein concentrations using big-ET-1 specific commercial ELISA. Endothelial cells were treated with less than half-lethal doses of DXR (50 and 200 ng/ml) and after 24 h, supernatants were collected and sandwich ELISA was performed. DXR significantly decreased the secretion of big ET-1 protein ($p=0.0101$).

Effects of DXR treatment on ET-1 expression at genom level –analysis of microarray assays

According to the above results we wanted to assess the effects of DXR on not only the ET-1 gene itself, but the network of genes regulating ET-1 and genes regulated by ET-1. Therefore,

we performed microarray assay, which is able to detect the expression pattern of the whole genom of the cell.

We sorted out the genes, which showed at least twofold expression change and a pathway analysis based on published connections between genes was run. We analyzed the genes of the main junctions of the network and collected their known connections to other genes. ET-1 junction has the fifth utmost connections in this system. However, this analysis does not take into account dose dependency, therefore we further selected those genes which showed dose dependent expression change by DXR treatment. As further criteria, we selected the genes where the changes of expression between 300 and 600 or 600 and 1000 $\mu\text{g/ml}$ treatments were at least 50% in both microarrays (Agilent és Affimetrix). After this 3-step selection we could highlight 24 genes with decreased and 24 genes with increased dose dependent mRNA expression. We observed that ET-1 has one of the most connections, which underlines that DXR affects the ET-1 system. Seven genes of the ET-1 gene network changed in parallel with ET-1.

5. Conclusions

5.1 Canine heart ischemia-reperfusion model

5.1.1. We developed an RT-PCR method for the correct, precise and sensitive assessment of ET-1 mRNA production in an in vivo canine ischemia–reperfusion model. The parallelly assessed plasma levels of ET-1 and its precursor big ET-1 made possible to have a better understanding of the processes during ischaemia and reperfusion.

5.1.2. In this canine ischemia–reperfusion model, ischemia resulted in higher big ET-1 and ET-1 levels.

5.1.3. There was a decrease of ET-1 mRNA during ischemia. This may be the consequence of degradation and decreased metabolism of the hypoxic cells locally, and the ET-1 detected in the plasma can be the product of healthy myocytes.

5.1.4. The rise of the ET-1 mRNA level after cessation of ligation indicates rapid big ET-1 synthesis. This is confirmed by the increased big ET-1 and ET-1 plasma levels during reperfusion. This latter can be associated with the generation of reperfusion arrhythmias or other complications of acute myocardial infarction.

5.2. DXR toxicity experiments

We conducted experiments on human endothelial cells to investigate the direct effects of sublethal doses of DXR on the expression of genes known to be affected in cardiovascular diseases.

5.2.1. It was found that short term DXR treatment inhibits ET-1 mRNA expression in a dose dependent manner. These results raise the possibility that the direct cardiotoxic effect of high DXR concentration during chemotherapy may be further enhanced by the initial suppression of ET-1 production, which leads to an enhanced susceptibility to apoptosis. The short-term decrease in ET-1 expression induced by DXR can also be deleterious to the cardiovascular system.

5.2.2. Out of the 96 representative cardiovascular genes of the array, only ET-1 mRNA expression was changed significantly by DXR treatment.

5.2.3. DXR significantly reduced the big ET-1 peptide level; however, not as efficiently as it was observed at mRNA level. This could be explained by the complex regulation of ET-1 peptide synthesis.

5.2.4. We screened the network of genes regulating ET-1 and genes regulated by ET-1 and investigated the effect of DXR treatment on endothelial cells with use of microarray system assessing the expression profile of the whole genome. Out of the genes which showed change in expression to DXR treatment ET-1 has one of the most connections underlining that DXR affects the ET-1 system.

We confirmed the central role of ET-1 system both in reaction to ischaemia in canine cardiomyocytes and in reaction to DXR treatment in human endothelial cells. Further experiments revealing the pathomechanism of the above processes are needed for better understanding the regulatory role of ET-1 in the cardiovascular system beyond regulation of blood pressure.

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