Peroxynitrite - matrix metalloproteinase and erythropoietin receptor signaling pathways in ischemic heart disease

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Summary of Ph.D. Thesis

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List of scientific publications related directly to the subject of the Thesis

I. Peter Bencsik, Viktor Sasi, **Krisztina Kiss**, Krisztina Kupai, Marton Kolossvary, Pal Maurovich-Horvat, Tamas Csont, Imre Ungi, Bela Merkely and Peter Ferdinandy: *Serum lipids and cardiac function correlate with nitrotyrosine and MMP activity in coronary artery disease patients*, Eur. J. Clin. Invest. 45 (2015) 692–701
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II. **Krisztina Kiss***, Csaba Csonka*, János Pálóczi, Judit Pipis, Anikó Görbe, Gabriella F Kocsis, Zsolt Murlasits, Márta Sárközy, Gergő Szűcs, Christopher P Holmes, Yijun Pan, Ashok Bhandari, Tamás Csont, Mehrdad Shamloo, Kathryn W Woodburn, Péter Ferdinandy, Péter Bencsik: *Novel, selective EPO receptor ligands lacking erythropoietic activity reduce infarct size in acute myocardial infarction in rats*, Pharm. Res. 113 (2016) 62-70
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List of full papers not related to the subject of the Thesis

I. **Krisztina Kiss**, Veronika Fekete, János Pálóczi, Márta Sárközy, Zsolt Murlasits, Judit Pipis, Irina A. Kheyfets, Julia L. Dugina, Svetlana A. Sergeeva, Oleg I. Epstein, Csaba Csonka, Tamás Csont, Péter Ferdinandy, Péter Bencsik: *Renin-Angiotensin-Aldosterone Signaling Inhibitors—Losartan, Enalapril, and Cardosten—Prevent Infarction-induced Heart Failure Development in Rats*, Altern. Ther. Health. Med. 22(2) (2016) 10-7
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II. Brockhoff B, Schreckenberg R, Forst S, Heger J, Bencsik P, **Kiss K**, Ferdinandy P, Schulz R, Schlüter KD: *Effect of nitric oxide deficiency on the pulmonary PTHrP system*, J. Cell. Mol. Med. (2016) doi: 10.1111/jcmm.12942
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Introduction

Epidemiology of cardiovascular diseases

Cardiovascular diseases (CVD) are responsible for approx. 45% of all death in Europe, which means more than 4 million deaths per year. Within CVD, coronary heart disease (CHD or coronary artery disease; CAD) represents the largest component. These epidemiological data support the relevance of extensive research in this field.

Pathomechanism and risk factors of coronary artery disease and myocardial ischemia

Atherosclerosis and its major consequence, CAD are considered as chronic inflammatory diseases. Leukocytes attached to the arterial endothelium initiate inflammatory cascade and calcification, and the atherosclerotic plaque is formed. The rupture of coronary atherosclerotic plaques leads to myocardial ischemia or acute myocardial infarction (AMI). Beside the complex pathomechanism of CAD, many risk factors (e.g. smoking) and comorbidities (e.g. hyperlipidemia) may influence the clinical appearance. Smoking is responsible for approx.

20% of deaths from CVD in men. Dyslipidemia is associated with high level of total cholesterol, low density lipoprotein (LDL) and triglyceride level, and decreased high density lipoprotein (HDL) levels. The contribution of increased nitrotyrosine (NTyr) formation to the development of atherosclerosis and CAD has been described in patients with hypercholesterolemia.

Diagnostic biomarkers and pharmacological treatment of CAD and AMI

Lipid parameters (e.g. cholesterol, LDL, HDL level) can be used as predictive biomarkers for atherosclerosis, while cardiac troponins or creatine-kinase MB are currently the best available biomarkers for the diagnosis of AMI. Control of risk factors is an essential component of the prevention in CAD (e.g. statins against hyperlipidemia). The medical treatment of AMI include ß-receptor antagonists, angiotensin-converting-enzyme inhibitors (ACEI), nitroglycerin, etc. However, in case of AMI, percutan coronary intervention (PCI) is the gold-standard therapy to reduce myocardial infarct size. Despite the large number of the biomarkers and drugs, the prediction of the prognosis of AMI is still hard, and there is still not an established drug to reduce myocardial infarct size. Therefore, there is an utmost need for seeking further molecules for prediction and diagnosis of CAD, and develop new cardioprotective drug candidates.

Signaling pathways for further analysis in CAD and myocardial ischemia

The role of nitro-oxidative stress in CAD and myocardial ischemia

Nitro-oxidative stress plays a pivotal role in the pathomechanism of several cardiovascular diseases including ischemic heart disease (IHD). The main effector of nitro-oxidative stress is peroxynitrite ($ONOO^-$), which forms different oxygen/nitrogen radicals contributing to radical reactions and to activation of enzymes including matrix metalloproteinases (MMPs) via S-nitrosylation/S-glutathiolation. Furthermore, peroxynitrite forms nitrotyrosine (NTyr) with free tyrosine residues, which is a widely used marker for peroxynitrite generation.

Matrix metalloproteinases in CAD and myocardial ischemia

Matrix metalloproteinases are calcium- and zinc-dependent endopeptidases, which have potent role in the pathology of tumor metastasis, inflammation or cardiovascular diseases. The most abundant MMPs in the human myocardium are MMP-2 and MMP-9 (gelatinase A and B, respectively), which play a crucial role in myocardial ischemia/reperfusion injury. Furthermore, it has been shown that serum MMP-2 predicts infarct size and ventricular dysfunction in ST-segment elevation myocardial infarction (STEMI) patients. The activation of MMP-2 and MMP-9 may occur not only by proteolysis but also via post-translational modification of pro-MMPs, mediated by $ONOO^-$. The correlations between serum

nitrotyrosine and MMP-2 and MMP-9 activities are still unknown in patients with CAD. Moreover, the correlation between serum nitrotyrosine, MMPs and lipid levels is not consistent in the literature.

Erythropoietin induced protective pathways in CAD and myocardial ischemia

Erythropoietin (EPO) and its analogue with a prolonged half-life, darbepoetin alpha (Dpa) are used in the treatment of anemia. However, EPO is a pleiotropic cytokine, and EPO receptors are present in the cardiomyocytes. Oxidative stress stimulates EPO receptors (EpoR) and beta-common receptors (ßcR) to form the heterocomplex, EpoR-ßcR. Via this complex, EPO stimulates protective signaling pathways, and thus contributes to tissue repair. Therefore, it is highly plausible that EPO or Dpa is able to reduce the effect of nitro-oxidative stress, and protect the heart against ischemia. Moreover, EPO reduces the expression of MMP-2 and -9, thus, EPO can attenuate collagen and extracellular matrix degradation in the ischemic heart.

Non-erythropoietic EPO analogues against myocardial ischemia

EPO and Dpa have previously been shown to decrease myocardial infarct size and improve cardiac ejection fraction after coronary artery occlusion in animal models. Based on the promising preclinical results, EPO and Dpa were clinically tested against AMI or heart failure. In most clinical trials, EPO or Dpa have failed to reduce infarct size in STEMI-patients. The lack of clinical efficacy likely arises from the immediate hematopoietic activity of EPO analogues. Thereby EPO or Dpa treatments may lead to thrombotic and/or thromboembolic adverse events, and increased mortality in IHD-patients.

Therefore, it is feasible that non-hematopoietic EPO analogues, such as asialoerythropoietin (asialoEPO) or carbamylated EPO (CEPO), may provide EpoR-mediated cardioprotection without concurrent hematopoietic safety issues. These analogues bind to EpoR-ßcR heteroreceptors, but not to the “classical” homodimeric form of EpoR, thereby maintaining their protective effects without stimulating hematopoiesis. However, since the structure of asialoEPO and CEPO mimics the three dimensional structure of EPO, clinical evaluation of potential immunogenicity and long-term stability is warranted. In contrast, a small peptide ligand may attenuate the aforementioned disadvantages and may serve as a suitable alternative compound to evoke cardioprotection by binding to EPO receptors.

Aims

Therefore, the aims of our present studies were

1) to characterize the correlations of the markers of $ONOO^-$ – MMP pathway in patients with single-vessel CAD subjected to elective PCI via the determination of
   - the correlation between serum nitrotyrosine and MMP-2 and MMP-9 activities;
   - the correlation of serum lipids (total, LDL and HDL cholesterol; triglyceride) with serum nitrotyrosine as well as with serum MMP-2 and MMP-9 activities, respectively;
   - the correlation between serum nitrotyrosine, MMP-2, MMP-9 activities and left ventricular ejection fraction (LVEF) as a marker of cardiac function;
   - the difference in serum nitrotyrosine, MMP-2 and MMP-9 activities in statin-naive and statin-treated patients;
   - the difference in serum nitrotyrosine, MMP-2 and MMP-9 activities in non-smoker and smoker patients
   - further correlations between renal function and nitrotyrosine levels and/or MMP activities.

2) to investigate the EpoR-mediated cardioprotection by testing two novel, selective EPO receptor ligand dimeric peptides (AF41676 and AF43136) in a rat model of AMI by investigating
   - the cytoprotective effects of EPO in isolated neonatal rat cardiomyocytes
   - the infarct size reducing effects of EPO and Dpa *in vivo*
   - the infarct size reducing effects of AF41676 and AF43136 compounds, which are structurally related to the peptide portion of peginesatide, but lacking erythropoietic activity *in vivo*.

Materials and methods

Characterization of the correlations of $ONOO^-$ and MMPs in patients with CAD

Patients and blood sampling

Patients with single-vessel CAD subjected to elective PCI were selected. The study was approved by the Ethics Committee of the University of Szeged. A written informed consent was obtained from all patients. Inclusion criteria were as follows: 1) Class II or III stable angina pectoris by the Canadian Cardiovascular Society grading system, 2) Single-vessel coronary artery disease defined as ≥70% diameter stenosis by visual assessment of the coronary angiogram. 36 patients were enrolled into this study. Echocardiographic

examinations were performed prior the PCI to measure cardiac function. PCI was performed using the standard femoral approach after premedication with aspirin and clopidogrel. Blood samples were collected 5 min before the PCI from femoral artery to determine the routine laboratory parameters, nitrotyrosine level and MMP-2, MMP-9 activity.

Determination of routine laboratory parameters

Serum levels of total, LDL and HDL cholesterol, triglyceride, and creatinine and urea nitrogen were quantitated by enzymatic methods (Institute of Laboratory Medicine, University of Szeged, Szeged, Hungary).

Measurement of MMP-2 and MMP-9 activity by gelatin zymography

To measure MMP-2 and MMP-9 activities, gelatin zymography was performed from serum samples. 8% polyacrylamide gels were copolymerized with gelatin and 40 µg of protein per lane was loaded. After electrophoresis, gels were washed with Triton X-100 and incubated for 20 h at 37°C in incubation buffer. Gels were then stained with Coomassie brilliant blue. For positive control, gelatinase zymography standard containing human MMP-2 and MMP-9 was used. For negative control, lanes containing serum samples were separately incubated in the presence of calcium chelator. Gelatinolytic activities were detected as transparent bands against the dark-blue background. Band intensities were quantified and presented in arbitrary units.

Measurement of nitrotyrosine in the serum by ELISA

To estimate systemic peroxynitrite formation, we measured free nitrotyrosine by ELISA in serum samples. Serum samples were deproteinized and incubated overnight with anti-nitrotyrosine rabbit IgG and nitrotyrosine acetylcholinesterase tracer in precoated (mouse anti-rabbit IgG) microplates followed by development with Ellman’s reagent. Serum nitrotyrosine levels were expressed in ng/ mL.

Measurement of cardiac function

Prior to PCI, echocardiographic examinations were performed on a GE Vivid 3 cardiac ultrasound device, using a 3.5-MHz transducer. Records were obtained in standard apical and parasternal two- and four-chamber views. LVEF was calculated using Simpson’s method.

Investigation of EpoR-mediated cardioprotection

The investigation conforms to the Guide for the care and use of laboratory animals published by the US National Institutes of Health (No. 85-23, revised 1996) and was approved by the Ethics Committee at the University of Szeged and, the Affymax Institutional Animal Care and Use Committee.

Animals and test compounds

Male Wistar rats (225–370 g) were used in the experiments. Test compounds (including acetate vehicle) were synthetized and provided by Affymax Inc. except for Erythropoietin (Procrit®, Amgen, Inc.), Darbepoetin alpha (Aranesp®, Amgen Europe), B-type natriuretic peptide (BNP; Sigma-Aldrich) and cyclosporine A (CsA; Sandimmun Neoral®, Novartis Hungária).

Characterization of non-erythropoietic EPO receptor ligands

Affymax Inc. developed and analyzed in preliminary experiments two selective EPO receptor ligand dimeric peptides, AF41676 and AF43136. The erythropoietic activity of the test compounds has been reduced by key amino acid substitutions. The amino acid sequence of AF41676 is $(AcYACHYGPITNalVCQPPK)_2$-IDA (SS:C3-C12) and the amino acid sequence of AF43136 is $(AcLYLCRYGRVHNalECQPLRK)_2$-DIG (SS:C4-C13). Nal is naphthylamine. To investigate EPO receptor binding ability of EPO, Dpa, AF41676 and AF43136, competition binding assay was carried out. HuEpoR-Fc was incubated with serial dilutions of competitor and 20,000 cpm ^{125}I EPO, using conditions based on a method for the detection of anti-EPO antibodies. To investigate the erythroid colony formation ability, human $CD34^+$ cells were incubated in the presence of EPO, Dpa, AF41676 or AF43136. Colonies containing over 50 cells were counted. To evaluate erythropoiesis stimulating effect, a single intravenous (iv.) dose of EPO (5000 U/kg), Dpa (10 µg/kg), AF41676 (10 mg/kg), AF43136 (10 mg/kg) and acetate vehicle (5 mL/kg) was administered for normocythemic rats, respectively. Reticulocyte number was determined in blood samples at baseline and 5 days following administration.

Study design

We investigated the cell-protective effect of EPO on isolated neonatal rat cardiomyocytes subjected to simulated ischemia/ reperfusion. Then, in 4 separate studies (study 1-4), we tested the infarct size reducing effect of EPO, Dpa and EPO-analogues (AF41676 and AF43136 compounds) administered before the onset of reperfusion, in an *in vivo* rat model of AMI.

Investigation of the direct cardio-cytoprotective effect by EPO

To determine the direct, non-erythropoietic effect of EPO, we tested different doses of EPO (1, 10, 100, 500 U/mL) on neonatal rat cardiomyocyte cell culture subjected to 150 min simulated ischemia followed by 120 min simulated reperfusion. BNP at 10^{-8} M was used as the positive control. Cell death was compared to acetate vehicle-treated cells.

Investigation of myocardial infarct size limiting effect of EPO and EPO analogues

In *in vivo* studies (studies 1–4), animals were randomly assigned and during the individual studies, all-cause mortality was registered to compare the survival among the controls and the drug-treated groups. *Study 1:* EPO was administered at 5000 U/kg bolus injection 5 min before the onset of reperfusion intraperitoneally (ip.) or iv. to compare different ways of administration. Positive control BNP was administered iv. at 10 nmol/kg bolus + 2 nmol/kg/min infusions for 15 min starting at the 25th min of ischemia. The acetate vehicle was used as the negative control and was administered iv. 5 min before the onset of reperfusion. *Study 2:* To investigate its dose dependence Dpa, a prolonged half-life EPO analogue, was administered iv. at the 15th min of ischemia, at 2.5, 5, 15 and 25 μg/kg boluses. The positive control BNP and the negative control acetate vehicle were administered identically to Study 1. *Study 3:* Dose dependence of AF41676 was tested at 0.3, 1, 3, 10 and 20 mg/kg doses administered as iv. bolus injection 5 min before the onset of reperfusion. Negative control acetate vehicle was administered according to Study 1. *Study 4:* In an exploratory study, we tested AF43136 in two doses in order to monitor potential hypotensive effects. AF43136 was administered at 10 and 30 mg/kg doses iv. as a bolus infusion for 5 min starting at the 25th min of ischemia. Saline, the positive control CsA (10 mg/kg, diluted in saline) and acetate vehicle were administered identically as test compounds.

Simulated ischemia/reperfusion in cultured neonatal cardiomyocytes

Newborn rat hearts were excised and digested in trypsin solution. After fibroblasts elimination, cardiomyocytes were plated into 24-well plates: 10^5 cells/well supplemented with 1 mL growth medium and incubated under normoxic conditions (37°C, 95% air and 5% CO_2 gas mixture). To simulate tissue ischemia, combination of hypoxic chamber (37°C, 95% N_2 and 5% CO_2) and hypoxic solution was used for 150 min. In treated groups, EPO, BNP or acetate vehicle was administered into the hypoxic solution. Following treatments, 120 min simulated reperfusion was applied in growing medium and in normoxic incubator. At the end of the experiments, viability tests were performed with trypan blue staining. Cells were counted in a Burker chamber and the ratio of dead (blue) cells to total cell count was calculated and plotted.

In vivo rat model of acute myocardial infarction

Rats were anesthetized with pentobarbital and ventilated with room air. Blood pressure, surface-lead ECG, and body core temperature were monitored. Hemodynamic data were collected at baseline, 29th min of ischemia and 120th min of reperfusion. Right femoral vein was cannulated for the administration of the test compounds. After thoracotomy, left

descending coronary artery was occluded for 30 min by a suture, then 120-min reperfusion was followed. Then, hearts were excised and infarct size was determined by standard triphenyltetrazolium-chloride (TTC) staining. Digital planimetry (InfarctSize™ v.2.5, Pharmahungary, Szeged, Hungary) was used for evaluation. Area at risk zone (AAR) was expressed as the percentage of total left ventricular area (LV), and infarct size (IS) was expressed as a percentage of the AAR.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) or standard error of the mean (SEM), respectively. Statistical significance was accepted at $p< 0.05$. Univariate correlations were analyzed using Pearson correlation coefficient, bootstrapped confidence intervals and linear regression analysis. Two-tailed Student's t-test or variance analysis (ANOVA) followed by post hoc tests were used as appropriate. Mortality was analyzed by using Chi-square test followed by Fisher's exact test.

Results

Characterization of the correlations of $ONOO^-$ and MMPs in patients with CAD

Correlation between serum nitrotyrosine and MMP-2 or MMP-9 activities

MMP-9 activity correlated positively with nitrotyrosine ($r= 0.535$, $p= 0.010$); however, there was no significant correlation between serum nitrotyrosine and MMP-2 activity.

Correlation between serum lipids and nitrotyrosine or MMPs activity

Serum total cholesterol, LDL cholesterol and triglyceride levels correlated positively ($r= 0.582$, $p= 0.003$; $r= 0.552$, $p= 0.008$; $r= 0.471$, $p= 0.023$, respectively), while HDL cholesterol negatively ($r= -0.455$, $p= 0.033$) with serum nitrotyrosine. Serum MMP-2 activity correlated positively with serum total cholesterol and LDL cholesterol levels ($r= 0.550$, $p= 0.015$ and $r= 0.448$, $p= 0.028$, respectively); however, neither serum triglyceride nor HDL cholesterol showed a significant correlation with MMP-2 activity. None of the serum lipid parameters correlated with MMP-9 activity in patients with CAD.

Correlation between myocardial function and serum nitrotyrosine or MMPs activity

Serum nitrotyrosine level correlated negatively with LVEF ($r= -0.548$, $p= 0.010$). MMP-2 activity did not correlate with LVEF, however, MMP-9 activity also showed a significant negative correlation with LVEF ($r= -0.732$, $p= 0.0002$). The negative correlation indicates that deteriorated cardiac function associates with elevated nitrotyrosine level and MMP-9 activity.

Serum nitrotyrosine or MMPs activity in statin-treated patients

Fifteen of the 36 patients with CAD were treated with different types of statins. In statin-treated patients, a significantly reduced serum nitrotyrosine level was found as compared to statin-naive patients (13.6 ± 5.1 ng/mL vs. 23.5 ± 4.5 ng/mL; $p< 0.05$). MMP-2 and MMP-9 activities did not show any difference between statin-treated and statin-naive patients.

Serum nitrotyrosine or MMPs activity in smoking patients

Eleven patients were active smoker during the study, and 17 patients never smoked in their life. Serum nitrotyrosine level did not show any difference between non-smoker and smoker patients. MMP-2 activity was significantly increased in smoker patients as compared to the non-smokers (45.5 ± 8.5 vs. 22.4 ± 4.6 arbitrary unit; $p< 0.05$). MMP-9 activity did not show any difference between non-smokers and smokers.

Correlation between renal function and serum nitrotyrosine or MMPs activity

We tested the correlation of renal function parameters with markers of peroxynitrite–MMP pathway and with LVEF, since renal dysfunction may be a severe complication of CAD. Serum creatinine correlated positively with nitrotyrosine ($r= 0.422$, $p= 0.036$), but not with MMP-2 and MMP-9 activities. Urea nitrogen level correlated positively with MMP-9 activity ($r= 0.423$, $p= 0.035$), but not with nitrotyrosine or MMP-2 activity. Serum creatinine correlated negatively with LVEF ($r= -0.503$, $p= 0.009$); however, urea nitrogen did not correlate with LVEF.

Investigation of EpoR-mediated cardioprotection

Erythropoietic activity of test compounds

As described previously, erythropoietic activity of test compounds was analyzed by Affymax Inc. EPO, Dpa and AF43136 were bound to the EPO receptor with high affinity (EC_{50}= 7, 12, 27 pM, respectively), AF41676 was bound with modest affinity (EC_{50}= 1240 pM). EPO and Dpa increased the differentiation of $CD34^+$ cells to erythroid precursors with high efficiency (EC_{50}= 2 to 14 pM), whereas AF41676 and AF43136 required vastly greater amounts (approx. 3–4 orders of magnitude) for the same effect. Reticulocytosis, a predictive marker of erythropoiesis, was significantly stimulated by EPO and Dpa (762±146, 1056±104 vs. $373±24.3×10^9$; $p< 0.05$), but not by AF41676 and AF43136 (284±51.3, $286±57.0×10^9$ vs. $373±24.3×10^9$) as compared to acetate vehicle.

The effects of EPO on cell death

EPO at 100 U/mL significantly decreased the ratio of dead cells (by approx. 16%) compared to the vehicle control group in cultured neonatal rat cardiomyocytes subjected to

simulated ischemia/reperfusion. The positive control BNP reduced cell death compared to the vehicle by approx. 30%.

The effects of EPO on infarct size and hemodynamics (Study1)

EPO at 5000 U/kg administered iv. before the onset of reperfusion decreased infarct size significantly compared to the vehicle by approx. 24%, while ip. administration was less effective (approx. 11% decrease, non-significant). The positive control BNP decreased infarct size significantly compared to the vehicle by approx. 47%. BNP reduced mean arterial blood pressure (MABP) significantly during ischemia compared to the vehicle, whereas EPO did not show any significant effect on MABP. There was no significant difference in survival among the groups.

The effects of darbepoetin on infarct size and hemodynamics (Study 2)

Dose-response relationship of Dpa showed maximal infarct size limiting effect at 5 µg/kg compared to the vehicle. The infarct size-limiting effect of Dpa was comparable to that of the positive control BNP (approx. 33% for both). There was no significant difference in the infarct-size limiting effect of the other Dpa doses (2.5, 5 or 25 µg/kg, respectively) as compared to the vehicle. BNP and Dpa at 5 µg/kg reduced MABP significantly during ischemia, while Dpa at 15 µg /kg significantly improved MABP at the end of reperfusion compared to the vehicle. There was no significant difference in survival among the groups.

The effects of AF41676 on infarct size and hemodynamics (Study 3)

In the dose-response curve, AF41676 at 0.3, 1, 3 and 10 mg/kg decreased infarct size significantly, where the 3 mg/kg dose showed the most potent infarct size limiting effect compared to the vehicle (approx. 46% reduction). BNP also reduced infarct size significantly compared to the vehicle (approx. 25% reduction). BNP as well as 10 and 20 mg/kg AF41676 reduced MABP during ischemia compared to the vehicle. AF41676 at 20 mg/kg reduced heart rate (HR) at the 29^{th} min of ischemia and at 10 mg/kg reduced MABP at the end of reperfusion. In spite of the transient hypotensive effect of the 10 and 20 mg/kg dose of AF41676, there was no significant difference in survival among the groups.

The effects of AF43136 on blood pressure, survival and infarct size (Study 4)

AF43136 at 10 mg/kg reduced infarct size significantly by approx. 30% compared to the acetate vehicle, however, infarct size data of 30 mg/kg AF43136 were equivocal due to the high mortality ratio and the consequent small sample size at the end of the experiments (n= 2). The positive control CsA decreased infarct size significantly by approx. 23% compared to the saline control. AF43136 at 30 mg/kg decreased MABP significantly during ischemia compared to the acetate vehicle, but it did not reduce blood pressure at 10 mg/kg. In addition,

AF43136 at 10 mg/kg increased MABP at the end of 120 min reperfusion compared to the acetate vehicle. AF43136 at 30 mg/kg reduced survival rate significantly compared to acetate vehicle.

Discussion

Summary of major findings of our studies

The aims of the present experiments were to analyze the $ONOO^-$ – MMP and EPO receptor-mediated signaling pathways in ischemic heart diseases. We found that serum nitrotyrosine positively correlated with MMP-9 activity, but did not correlate with MMP-2. We performed a comprehensive investigation to establish the correlation between serum NTyr, MMP-2, MMP-9 and the individual components of the lipid panel, respectively. We found negative correlation between serum NTyr, MMP-9 activity and cardiac function. In our study, statin-treatment or active smoking influenced serum nitrotyrosine level or MMPs activity. Finally, we found correlations between serum NTyr level, the activity of MMPs or cardiac function and parameters of renal function, respectively.

The receptor binding ability and non-erythropoietic effect of AF41676 and AF43136 compounds were assessed by Affymax Inc. We confirmed that EPO induced cardio-cytoprotection is independent from its erythropoietic activity against simulated ischemia/reperfusion. Furthermore EPO and its derivative, Dpa decreased infarct size in an *in vivo* rat model of AMI. The main novelty of this study was that AF41676 reduced infarct size in a dose-dependent manner in a rat model of AMI, and AF43136 was also able to evoke cardioprotection, however, its higher dose could have potential adverse effects.

Serum nitrotyrosine correlates with MMP activity

We found a positive correlation of serum NTyr with MMP-9 activity, but not with MMP-2 activity. This may suggest that peroxynitrite can activate MMPs in humans. However, the reason why we have not found a significant correlation between peroxynitrite and MMP-2 activity is not known. A plausible explanation is, while MMP-9 is an abundant enzyme in leukocytes, MMP-2 is an intracellular enzyme in contractile tissues that can be released, for example, due to acute myocardial ischemia/reperfusion, and our CAD patient population did not have severe ischemia at the time of tissue sampling.

Serum lipids correlate with nitrotyrosine and MMPs activity

Hyperlipidemia is a well-characterized risk factor for cardiovascular diseases. Here, we have shown that NTyr correlated positively with triglyceride, total and LDL cholesterol levels, and negatively with HDL cholesterol. In a previous study, positive correlation between

LDL cholesterol and NTyr was reported in hypercholesterolemic patients. Here, we have found that serum MMP-2 activity correlated positively with total and LDL cholesterol levels. However, HDL cholesterol, or triglyceride levels did not correlate with MMP-2 activity. According to our best knowledge, only one previous study found a negative correlation between serum MMP-2 activity and HDL cholesterol in patients with CAD. In our present study, serum MMP-9 activity did not show a correlation with any of the serum lipids. In contrast, two studies in patients with CAD and acute coronary syndrome showed that MMP-9 protein level positively correlated with LDL cholesterol or triglyceride level. However, in these studies, the authors did not measure MMP-9 activities.

Myocardial function correlates with serum nitrotyrosine and MMPs activity

We have demonstrated first time in the literature that cardiac function characterized by LVEF showed a significant negative correlation with serum nitrotyrosine in patients with CAD. This may suggest that nitro-oxidative stress is detrimental to cardiac function in humans, which is in accordance with preclinical studies. Furthermore, we have found here that LVEF correlated negatively with MMP-9 activity; however, there was no correlation with MMP-2 activity. Previous findings showed that MMP-2 and MMP- 9 protein levels correlate with LVEF in patients with AMI or heart failure.

Decreased serum nitrotyrosine in statin-treated patients

Statins are inhibitors of endogenous cholesterol synthesis, and they attenuate nitro-oxidative stress. Accordingly, we found here a reduced serum nitrotyrosine level in statin-treated patients with CAD, but we have not found difference in MMP-2 and MMP-9 activities related to statin-treatment. In contrast, it has previously been shown in patients with CAD that activities of MMP-2 and MMP-9 were reduced after 2-month pravastatin therapy.

Increased MMP-2 activity in smoker patients

Here we found that MMP-2 but not MMP-9 activity was significantly higher in the serum samples of smoker patients. In clinical studies, increased gene expression of MMP-2 and -9 or elevated proMMP-2 and proMMP-9 levels were found in smoker patients, however the active forms of MMPs were not detectable in that studies. Although cigarette smoke contains peroxynitrite and peroxynitrite-generating species suggesting that it contributes to the increased nitro-oxidative stress, we could not demonstrate any difference in serum $ONOO^-$ level between smokers and non-smokers.

Renal function correlates with serum nitrotyrosine and MMPs activity

It has previously been described that renal dysfunction may contribute to the mortality in patients with myocardial infarction. Although uremic patients and patients with severe left

ventricular dysfunction were not enrolled into the present study, we have shown a significant negative correlation between serum creatinine level and LVEF for the first time in the literature in patients with CAD. Moreover, we have found a positive correlation between serum creatinine and NTyr as well as between urea nitrogen and MMP-9 activity in patients with CAD. These results suggest that increased nitrotyrosine level and MMP-9 activity may indicate an incipient renal dysfunction before myocardial infarction.

Cardioprotective effects of EPO

We demonstrated that EPO, independently from its erythropoietic activity, protected cardiomyocytes against simulated ischemia/reperfusion. Our findings are consistent with previous studies that EPO has cytoprotective- and anti-apoptotic effects on cardiomyocytes. In *in vivo* coronary occlusion/reperfusion experiments, we found that iv., but not ip. administration of the same dose of EPO decreased infarct size in rats, although it has been previously demonstrated that EPO reduces infarct size by both ip. or iv. administration. This discrepancy could derive from pharmacokinetic differences or different duration of treatments.

Dose-response effect of Dpa in AMI

Here, we also reported first time in the literature the dose-response effects of Dpa on infarct size, when administered before reperfusion. Although, the infarct size-limiting dose of Dpa varies in a wide range (from 2.5 to 30 µg/kg) in the literature, our results point out that 5 µg/kg dose of Dpa is sufficient to reduce infarct size in a rat model of AMI.

Cardioprotective effect of AF41676 and AF43136

One of the major novelty of our present study is that two novel selective non-erythropoietic EPO receptor ligand dimeric peptides, AF41676 and AF43136, reduced infarct size administered before reperfusion in an *in vivo* rat model of AMI. AF41676 was effective at a wide dose-range (0.3–10 mg/kg). Although, high doses (10 and 20 mg/kg) of AF41676 decreased MABP, they did not influence survival. In contrast, AF43136 reduced infarct size, but it was lethal in higher doses due to a rapid decline in MABP. AF41676 and AF43136 exhibit reliable binding to the extracellular portion of the EPO receptor, however, do not stimulate erythropoiesis suggesting that they may act via the EpoR-mediated cytoprotective pathways.

It has been previously demonstrated that the modified EPO analogues, CEPO and asialoEPO, or pyroglutamate helix B surface peptide (pHBSP or ARA 290) consisting of 11 amino acids of the beta-chain of EPO, preserve the tissue protective effects of EPO, such as infarct size reduction without any erythropoietic safety problems, but they may have potential

for high antigenicity. In contrast, AF peptides were structured via the linker and disulfide bonds to wrap around each other, and present a surface that is highly complementary to the EPO receptor. These features support our findings, that non-erythropoietic EPO analogue dimeric peptides may be promising cardioprotective agents against acute myocardial infarction.

Conclusions

Novel findings

We have demonstrated for the first time in the literature that (i) serum nitrotyrosine correlates with MMP-9 activity, (ii) serum lipid parameters correlate with nitrotyrosine level and MMP-2 activity, (iii) myocardial function correlates with nitrotyrosine, MMP-9 activity as well as with creatinine, and (iv) creatinine correlates with nitrotyrosine and urea nitrogen correlates with MMP-9 activity in patients with CAD. This is the first demonstration that two novel selective non-erythropoietic EPO receptor ligand dimeric peptides, AF41676 and AF43136, reduce infarct size in a rat model of acute myocardial infarction.

Future perspectives

These findings suggest that nitrotyrosine and MMP-2 and MMP-9 activities may have predictive or diagnostic values in cardiac and renal function of patients with CAD, and attenuation of nitro-oxidative stress and MMP activities may provide therapeutic benefits in this patient population. Therefore, large prospective clinical studies would be necessary. Moreover, non-erythropoietic EPO receptor ligands may be potential cardioprotective agents in acute myocardial infarction. Further safety and translational studies are needed to ensure the safety and efficacy of these agents as potential alternative clinical treatments.

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