

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

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Abbreviations

AAR	area at risk
ACEI	angiotensin-converting-enzyme inhibitors
AMI	acute myocardial infarction
AsialoEpo	asialo-erythropoietin
β cR	beta common receptor
BFU-E	erythroid blood forming unit
BNP	B-type natriuretic peptide
BUN	blood urea nitrogen
CAD	coronary artery disease
CEPO	carbamylated erythropoietin
CHD	coronary heart disease
$\text{CO}_3^{\bullet-}$	carbonate radical
CsA	cyclosporine A
CVD	cardiovascular disease
Dpa	darbepoetin alpha
EC_{50}	half maximal effective concentration
ECM	extracellular matrix
EPO	erythropoietin
EpoR	erythropoietin receptor
IHD	ischemic heart disease
ip	intraperitoneal
IS	infarct size
iv	intravenous
HDL	high density lipoprotein
HR	heart rate
LDL	low density lipoprotein
LVEF	left ventricular ejection fractions
MABP	mean arterial blood pressure
MMP	matrix metalloproteinase
*NO_2	nitrogen dioxide radical
NT-proBNP	N-terminal pro-brain natriuretic peptide
NTyr	nitrotyrosine
NYHA	New York Heart Association
OH^{\bullet}	hydroxyl radical
ONOO^-	peroxynitrite
ONOOH	peroxynitrous acid
PCI	percutan coronary intervention
PHBSP	pyroglutamate helix B surface peptide
SI/ R	simulated ischemia/ reperfusion
SR	survival ratio
STEMI	ST-segment elevation myocardial infarction
TTC	2,3,5-triphenyltetrazolium-chloride
TIMP	tissue inhibitors of matrix metalloproteinase

Summary

In the industrialized world, cardiovascular diseases (CVD) are responsible for almost 50% of the total cause of death, and among CVD, ischemic heart disease (IHD) represents the highest subpopulation. In spite of huge number of many preclinical and clinical studies, there is still no cardioprotective drug on the market to reduce infarct size in IHD.

In the pathomechanism of myocardial ischemia/reperfusion injury, peroxynitrite (ONOO⁻) – matrix metalloproteinase (MMP) signaling as well as erythropoietin (EPO)-induced cytoprotective pathways have well-established roles. ONOO⁻ – MMP pathway is influenced by hyperlipidemia, which is one of the most common comorbidity of IHD. Moreover, in spite of the promising preclinical findings with EPO against acute myocardial infarction (AMI), EPO failed to reduce infarct size in clinical trials and showed significant safety problems.

Therefore, our aims were (i) to obtain novel information on the role of ONOO⁻ – MMP and (ii) EPO receptor- mediated signaling pathways in IHD. Therefore, we investigated i) the correlation of markers of peroxynitrite–MMP signaling with hyperlipidemia and with cardiac function in patients with significant coronary stenosis and ii) the cardioprotective effects of EPO, the prolonged half-life EPO analogue, darbepoetin alpha (Dpa), and two novel selective non-erythropoietic EPO receptor ligand dimeric peptides (AF41676 and AF43136) in a rat model of AMI.

We found in coronary artery disease patients, that serum nitrotyrosine (NTyr), a widely accepted marker of ONOO⁻ generation, positively correlated with MMP-9 activity, but not with MMP-2 activity. NTyr positively correlated with total and LDL cholesterol, serum triglyceride, and creatinine, while it negatively correlated with HDL cholesterol and with left ventricular ejection fraction (LVEF). MMP-2 activity correlated positively with total and LDL cholesterol. In statin-treated patients, a significantly reduced serum NTyr level was found compared to statin-naïves; however, MMP activities were not different in statin-treated and in statin-naïve patients. MMP-2 activity was significantly increased in smoker patients compared to the non-smokers, while NTyr or MMP-9 activity did not show any difference related to smoking. MMP-9 activity correlated positively with urea nitrogen and negatively with LVEF, respectively. Serum creatinine correlated negatively with LVEF.

In a pilot cell culture study, EPO at 100 U/mL significantly decreased cell death compared to vehicle in rat neonatal cardiomyocytes subjected to simulated ischemia/reperfusion. In further studies (studies 1–4), *in vivo* AMI was induced by 30 min coronary occlusion and 120 min reperfusion in male Wistar rats. Test compounds and positive controls (B-type natriuretic peptide, BNP or cyclosporine A, CsA) were administered *iv.* before the onset of reperfusion. Infarct size (IS) was measured by standard TTC staining. In study 1, 5000 U/kg EPO reduced infarct size significantly compared to vehicle. In study 2, Dpa showed maximal infarct size-reducing effect at 5 µg/kg compared to the vehicle. In study 3, in the dose-response curve of AF41676, 3 mg/kg was the most effective dose compared to the vehicle. The positive control BNP significantly decreased infarct size in studies 1–3 by approximately 35%. In study 4, AF43136 at 10 mg/kg decreased infarct size, similarly to the positive control CsA compared to the appropriate vehicle.

We demonstrated for the first time in the literature that (i) serum NTyr correlates with MMP-9 activity, (ii) lipid parameters correlate with NTyr and MMP-2 activity, (iii) myocardial function correlates with creatinine, NTyr and MMP-9 activity, and (iv) creatinine correlates with NTyr and urea nitrogen with MMP-9 activity in patients with CAD. Therefore, studying the biomarkers of peroxynitrite–MMP pathway in large prospective trials may reveal their predictive or diagnostic avails.

Moreover, we proved first time in the literature, that selective, non-erythropoietic EPO receptor ligand dimeric peptides AF41676 and AF43136 administered before reperfusion are able to reduce infarct size in a rat model of AMI. Non-erythropoietic EPO receptor peptide ligands may be promising cardioprotective agents in IHD.

1. Introduction

1.1 Epidemiology of cardiovascular diseases

Cardiovascular diseases (CVD) are still the most common cause of death worldwide, and responsible for approx. 45% of all death in Europe [2]. It means more than 4 million people across Europe year by year, and a quarter of these deaths (1.4 million people) belong to the premature death, before the age of 75 years (Fig. 1) [2]. Within CVD, coronary heart disease (CHD or coronary artery disease (CAD)) represents the largest component (Fig. 1) [2].

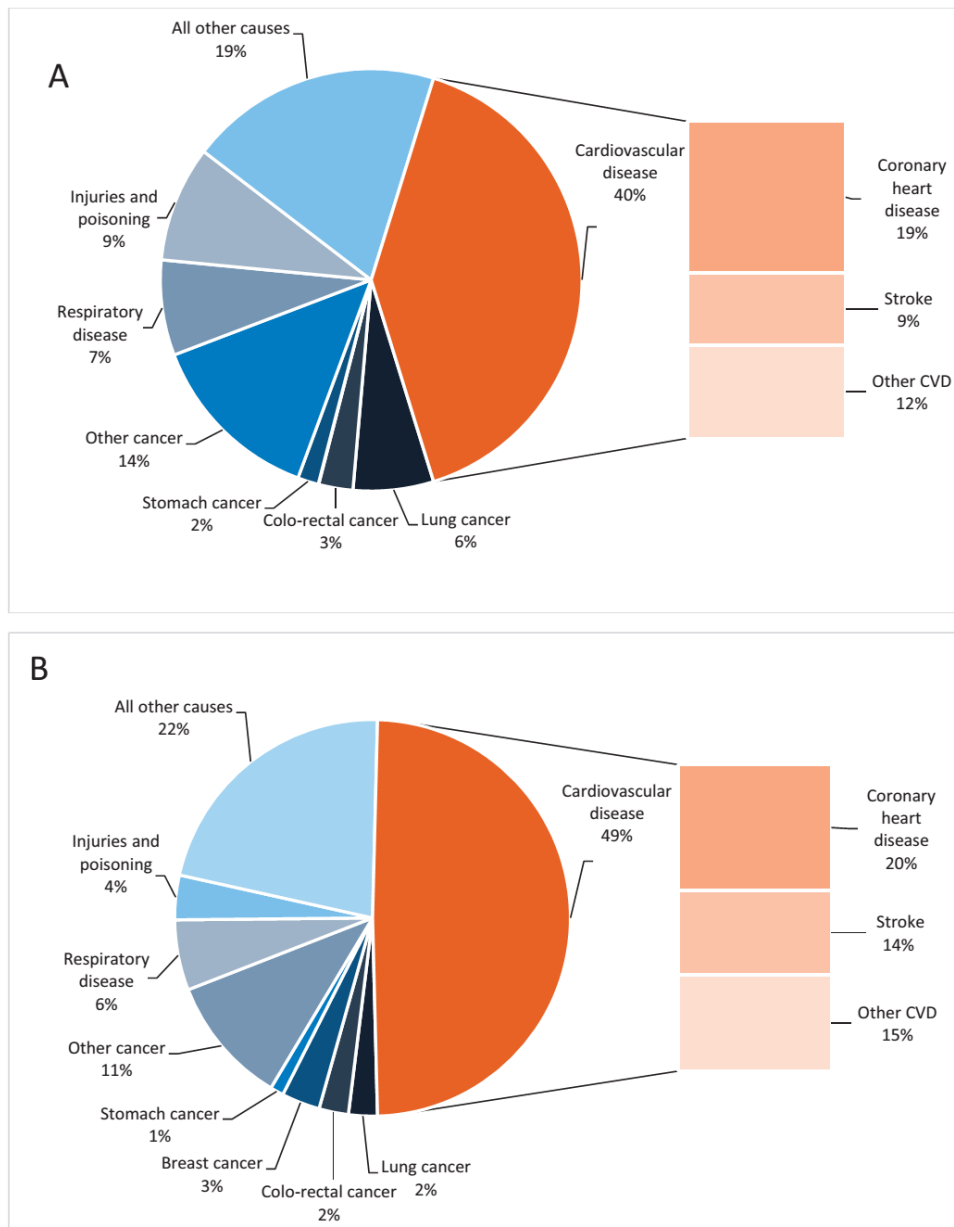


Figure 1. Proportion of all deaths due to major causes in Europe. Last available data (after 2014) in WHO Mortality Database, among men (A) and women (B) [2].

Although, the death rate from CHD shows a mild improvement in the last 15 years in most European countries including Hungary (Fig. 2) [3], the epidemiological data above support the relevance of extensive further research in this field.

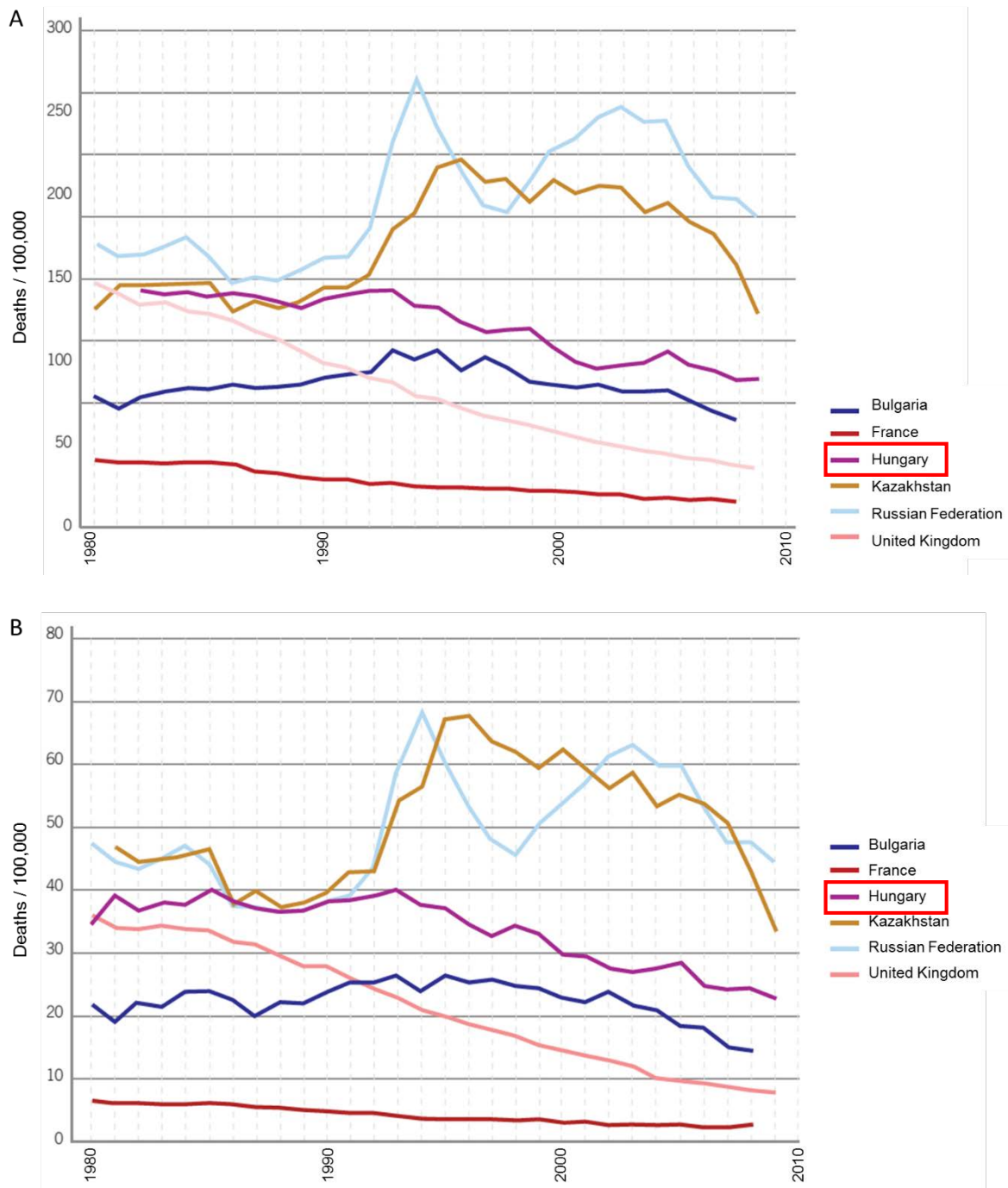


Figure 2. Death rates from CHD. Men (A) and women (B) aged under 65, from 1980 to 2010, selected countries. Hungary is labelled in red frame and with purple line [3].

1.2 Pathomechanism of coronary artery disease and myocardial ischemia

Previously, atherosclerosis and its major consequence, CAD, were considered as cholesterol storage diseases, but recently, they are considered as chronic inflammatory

diseases [see for review 4]. When the arterial endothelium and risk factors (dyslipidemia, bacterial end products, etc.) coincide, leukocytes attach to the arterial wall and secrete proinflammatory cytokines and matrix metalloproteinases (MMPs) [4]. MMPs destroy the extracellular matrix (ECM) of the artery and components of the ECM bind lipoproteins, and make them more sensitive to oxidation [4]. Oxidized phospholipids contribute to the exacerbation of the inflammatory response [4]. During the progression, calcification may occur and the atherosclerotic plaque with a lipid-rich core is formed [4]. The rupture of coronary atherosclerotic plaques leads to myocardial ischemia or even acute myocardial infarction (AMI) [4]. Besides the novel pathways of the pathomechanism of CAD, there are novel studies investigating other aspects, e.g. microRNAs in coronary disease [5] or the genetic background of CAD [6], whose aim is the personalized medicine in the future [6]. However, further investigation of the known molecules of CAD's pathomechanism has still high relevance to characterize novel prognostic or diagnostic biomarkers and develop new molecules as therapeutic targets in ischemic heart disease (IHD).

1.3 Smoking and dyslipidemia as risk factors in CAD and myocardial ischemia

Beside the complex pathomechanism of CAD, many risk factors (e.g. smoking, inactivity, excess alcohol intake) and comorbidities (e.g. hyperlipidemia, obesity, diabetes mellitus, hypertension, etc.) may influence the clinical appearance of CAD and make the diagnostic or therapeutic possibilities even harder [3, 7].

Smoking remains one of the most important modifiable risk factors for premature mortality. About 20% of deaths from CVD in men and 3% in women are due to smoking [3].

Dyslipidemia is a well-characterized risk factor of CVD, which consists of elevated triglyceride and decreased high density lipoprotein (HDL) levels and it is associated with high level of total cholesterol and low density lipoprotein (LDL) [7]. Patients with dyslipidemia have increased risk for CVD [8]. Hyperlipidemia due to enhanced nitro-oxidative stress [9, 10] and deteriorate the endogenous cardioprotective mechanisms, such as ischemic preconditioning [11, 12]. The contribution of increased nitrotyrosine (NTyr) formation to the development of atherosclerosis and thereby to CAD has been described in patients with hypercholesterolemia-combined CAD [13].

1.4 Predictive and diagnostic biomarkers applied clinically in CAD and myocardial ischemia

There are numerous biomarkers used by clinicians in the daily practice [14]. Measurement of lipid parameters (e.g. cholesterol, LDL, HDL, oxidized-LDL) can be used as predictive biomarkers for atherosclerosis development [14]. In case of myocardial necrosis, cardiac troponin T or I, creatine-kinase MB or myoglobin are currently the best available biomarkers of choice for the diagnosis of AMI [14]. In chronic states, e.g. in heart failure, N-terminal pro-brain natriuretic peptide (NT-proBNP) is the most valuable prognostic marker [14].

Despite the large number of the biomarkers, the presence of myocardial ischemia and simultaneously prediction the individual prognosis of AMI is still hard [14]. Therefore, there is an utmost need for seeking further molecules, which can complement the prediction or diagnosis of CAD, e.g. nitrotyrosine or MMPs, which are not established and standardized yet [14]. 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.

1.5 Pharmacological treatment of CAD and AMI

Similarly to the biomarkers, a broad spectrum of pharmacological therapy is available in case of CAD or AMI. Control of risk factors is an essential component of the prevention in CAD including the life-style habits (exercise, diet) or medical possibilities (anti-hypertensive drugs or statins against hyperlipidemia) [4]. In case of AMI, percutan coronary intervention (PCI) is the gold-standard therapy to reduce myocardial infarct size [15]. Guidelines for the medical treatment of patients with AMI include β -receptor antagonists, angiotensin-converting-enzyme inhibitors (ACEI), statins, nitroglycerin, oxygen or morphine [15]. Moreover, there are novel anti-inflammatory drugs against atherosclerosis, ‘the root of all evil’, e.g. interleukin-1 β , P-selectin and 5-lipoxygenase, which mean promising therapy [16].

However, clinicians still do not have an established cardioprotective drug on the market to reduce the size of the infarcted area, attenuate the consequences of myocardial ischemia/reperfusion injury thereby to improve survival and/or the quality of life for AMI patients. Furthermore, because of the high prevalence of CAD (see above) and the diversity of patient’s comorbidities, which may strongly influence the pharmacokinetic properties as well as the effects of the potentially cardioprotective drugs, there is an immense demand for the discovery of novel cardioprotective approaches, and for the development of new cardioprotective drug candidates.

1.6. Signaling pathways for further analysis in CAD and myocardial ischemia

1.6.1 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 [17]. The main effector of nitro-oxidative stress is peroxynitrite (ONOO^-), which is able to form different radicals and contribute to many radical reactions (see details in Fig. 3 d-f-g arrows) [17]. Moreover, ONOO^- may activate several enzymes including MMPs via S-nitrosylation/S-glutathiolation of their tyrosine and/or cysteine residues [17, 18] (Fig. 3 b arrow) [17]. Furthermore, peroxynitrite forms nitrotyrosine with free tyrosine residues, which is a widely used marker for peroxynitrite generation [19].

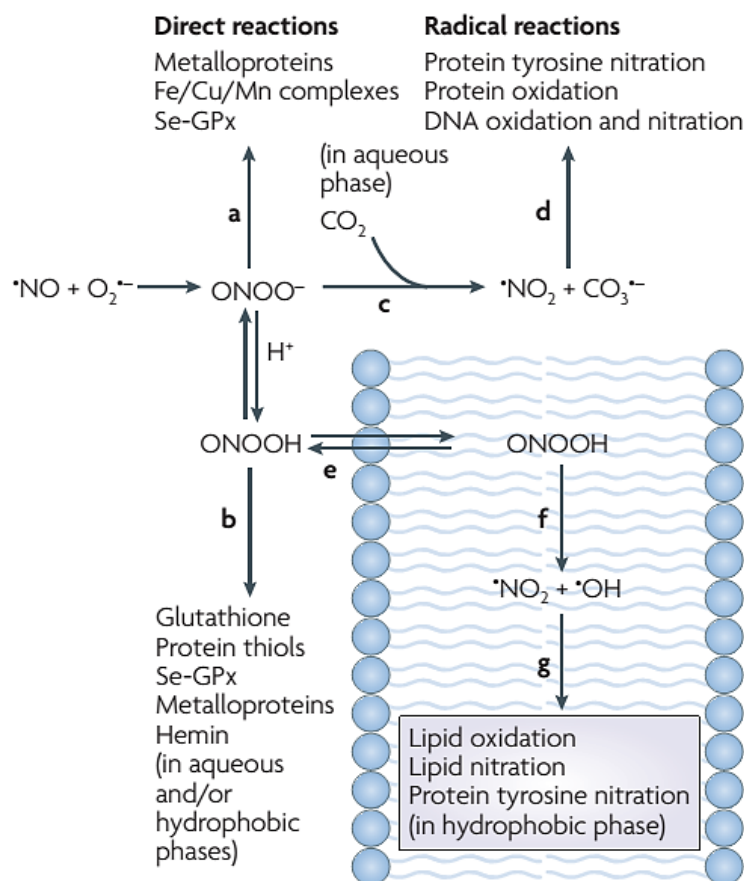


Figure 3. Biochemistry of peroxynitrite: reaction targets and fate. Peroxynitrite anion (ONOO^-) is in equilibrium with peroxynitrous acid (ONOOH) and both of them can undergo direct reactions with biomolecules (a; b). ONOO^- with carbon dioxide forms carbonate ($\text{CO}_3^{\bullet-}$) and nitrogen dioxide ($^*\text{NO}_2$) radicals (c), which are good oxidants (d), that can readily oxidize amino acids such as cysteine and tyrosine in biological systems. As an alternative pathway, ONOOH can undergo homolytic fission to generate hydroxyl ($^*\text{OH}$) and $^*\text{NO}_2$ radicals (g). However, this reaction is slow in biological systems, and therefore is a modest component of the *in vivo* reactivity of peroxynitrite in aqueous compartments. However, ONOOH is able to cross lipid bilayers (f) and $^*\text{OH}$ and $^*\text{NO}_2$ radicals seems to become relevant in hydrophobic phases to initiate lipid peroxidation and lipid and protein nitration processes (g) [17].

1.6.2 Matrix metalloproteinases in CAD and myocardial ischemia

Matrix metalloproteinases are endopeptidases, which were discovered during the degradation of extracellular matrix protein in tadpole tail [20]. The family of MMPs contain approx. 30 members classified according to their major substrates (e.g. collagenases, gelatinases, stomelysins, etc.) [21]. MMPs play a crucial role in the physiology of embryogenesis or angiogenesis, but also have a potent role in the pathology of tumor metastasis or inflammation [see for review 22]. MMPs are major targets of drug and diagnostic development in several pathologies including cardiovascular diseases [21, 23].

The most abundant MMPs in the human myocardium are MMP-2 and MMP-9 (gelatinase A and B), which have been shown to play a crucial role in many cardiovascular diseases, (e.g. atherosclerosis, ischemic heart disease, stroke, or heart failure) [22], especially in myocardial ischemia/reperfusion injury in several animal models [24]. Furthermore, it has been shown that serum MMP-2 predicts infarct size and ventricular dysfunction in ST-segment elevation myocardial infarction (STEMI) patients [25].

The activation of MMP-2 and MMP-9 may occur not only by proteolysis but also due to nitro-oxidative stress, mediated by ONOO^- as one of the most potent activator, via post-translational modification of pro-MMPs and the formation of transcriptional complexes [26, 27] (Fig. 4 [22]).

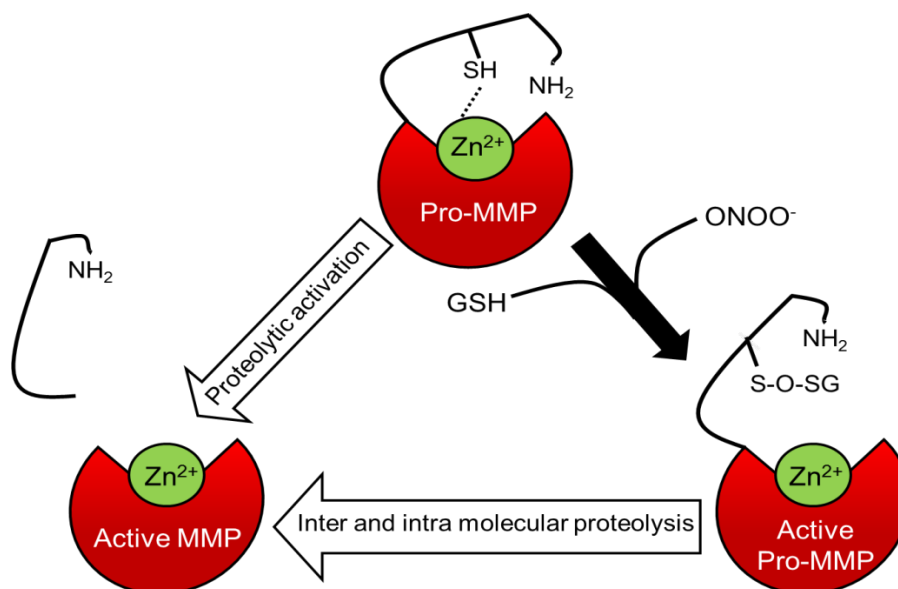


Figure 4. Activation mechanisms of MMPs. The full-length MMP can be activated in two ways. Proteolytic activation of MMP occurs by removal of the autoinhibitory propeptide domain (left arrow) resulting in an active truncated MMP. The simultaneous presence of oxidative stress (ONOO^-) and cellular glutathione (GSH) may lead to S-glutathiolation of the critical cysteine residue in the propeptide domain, disrupting its binding to the catalytic Zn^{2+} ion, resulting in an active full-length enzyme [22].

1.6.3 Erythropoietin induced protective pathways in CAD and myocardial ischemia

Erythropoietin (EPO) and its analogue with prolonged half-life, darbepoetin alpha (Dpa) are the main components of anemia treatment in chronic kidney disease and in cancer patients with anemia [28]. However, EPO is a pleiotropic cytokine, as EPO receptors (EpoR) are expressed in several different tissues (e.g. bone marrow, skeletal muscle, kidney, pancreas, heart, etc. [for review see 29]). It is well known that EPO receptors are present in rat cardiomyocytes [30], and also in human atrial and ventricular cardiomyocytes, as well as in cardiac endothelial cells [31].

Oxidative stress (namely hypoxia via hypoxia-inducible factor family) induces externalization or enhanced expression of EPO receptors and beta-common receptors (β cR; also known as CD131, which provides increased ligand-binding affinity to the receptor complex) to form the heterocomplex, EpoR- β cR [32-35]. Via this complex, EPO is able to stimulate protective signaling pathways and reduce apoptosis, inflammation, and thus contributes to tissue repair [32, 34] (Fig. 5).

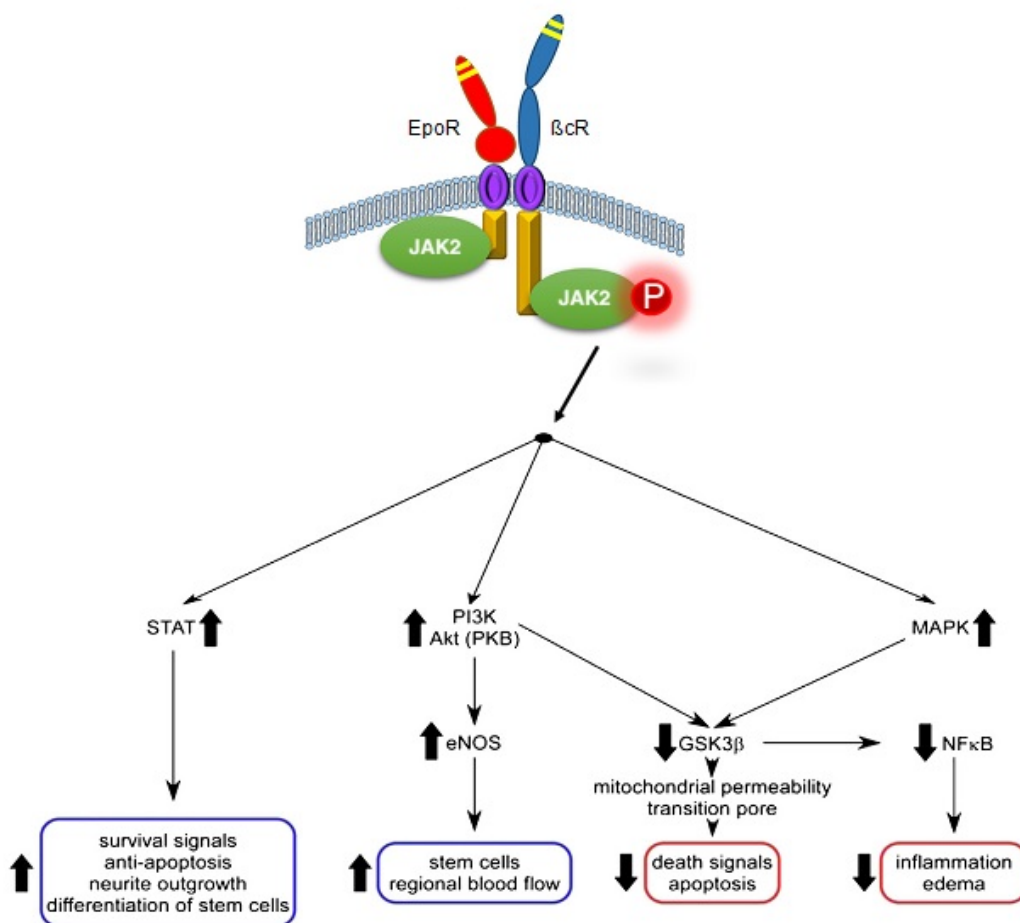


Figure 5. Intracellular signaling pathways in the biological activity of EpoR- β cR heterocomplex. EpoR- β cR complex phosphorylate and activate Janus kinase-2 (JAK2). Subsequently, signal transducers and transcription factors, including STAT, Akt, AMPK,

NFkB and the mitogen-activated protein kinase systems are variably activated depending upon the tissue. Downstream sustained activation in survival, tissue reparative, and anti-inflammatory pathways leads to tissue protection and repair [34.]

Therefore, it is highly plausible that EPO or Dpa has the capacity to reduce the effect of the nitro-oxidative stress in the myocardium and protect the heart against acute or chronic myocardial ischemia [29, 36, and 37]. Moreover, EPO reduces the expression of MMP-2 and -9 and simultaneously increases the tissue inhibitors of MMP (TIMPs) level, therefore ameliorates the shifted imbalance of MMP-2/TIMP-2 ratio during ischemia/reperfusion injury [38, 39, and 40]. Thus, EPO is able to attenuate collagen and extracellular matrix degradation in the ischemic heart [38, 39, and 40].

1.6.4 Non-erythropoietic EPO analogues against myocardial ischemia

EPO and Dpa were previously shown to decrease myocardial infarct size, prevent myocyte loss and hypertrophy, improve cardiac contractility and ejection fraction, and increase capillary density after transient or permanent coronary artery occlusion in different animal models [41, 42].

Based on the promising results of preclinical experiments with EPO and Dpa for cardioprotection, they were clinically tested against AMI or heart failure. Although, EPO improved left ventricular ejection fraction and New York Heart Association (NYHA) functional class in patients heart failure combined with anemia, Dpa had no effect on myocardial function in this population [43]. In most clinical trials, EPO or Dpa administered before or after percutaneous coronary intervention have failed to reduce infarct size in STEMI patients [29, 44]. This lack of clinical effectiveness likely arises from the immediate hematopoietic activity of EPO analogues. Hematopoiesis is activated by association with homodimeric (i.e. erythropoietic) receptors before erythropoietic EPO analogues would exert their tissue-protective effects via EpoR-βcR heterocomplex receptors [29]. Thereby, due to enhanced erythropoiesis, high-dose or long-term EPO or Dpa treatments may lead to thrombotic and/or thromboembolic adverse events (e.g. stent thrombosis, stroke, etc.) [29], and increased mortality in ischemic heart disease patients [44-46]. Another synthetic erythropoiesis-stimulating agent, peginesatide is a dimeric peptide without sequence homology to EPO [47]. Peginesatide binds selectively and with high affinity to the EPO receptor [47].

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 [29]. These analogues bind to EpoR- β cR heteroreceptors, but not to the “classical” homodimeric form of EpoR, thereby maintaining their protective effects without stimulating hematopoiesis [29]. In preclinical studies, asialoEpo or CEPO were shown to protect different tissues including myocardium by reducing apoptosis or inducing cardiac neovascularization with less adverse effects on procoagulant/haemopoetic activity [48-51]. 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 [34, 52]. In contrast, a small peptide ligand may attenuate the aforementioned disadvantages (i.e. via reduction in the number of possible epitopes) and may serve as a suitable alternative compound to evoke cardioprotection by binding to EPO receptors.

2. 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 percutaneous coronary intervention 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 fractions (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 cell-protective 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*.

3. Materials and methods

3.1 Characterization of the correlations of ONOO⁻ and MMPs in patients with CAD

3.1.1 Study design

Patients with single-vessel CAD subjected to elective PCI were selected. Echocardiographic examinations were performed prior the PCI to measure cardiac function. Blood samples were collected from patients 5 min before PCI to determine routine laboratory parameters, NTyr level and MMPs activities.

3.1.2 Patients and blood sampling

The study was approved by the Ethics Committee of the University of Szeged. A written informed consent was obtained from all patients enrolled in the study. The investigations were carried out in consecutive patients with single-vessel coronary disease elected for PCI.

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 $\geq 70\%$ diameter stenosis by visual assessment of the coronary angiogram.

In order to collect a homogenous population, patients with previous myocardial ischemic events, with some remedies or with certain comorbidities had to be excluded from this study.

Exclusion criteria were as follows:

- 1) Angina pectoris or other signs of myocardial ischemia within 1 week before intervention.
- 2) History of myocardial infarction or baseline ST-segment abnormalities.
- 3) Severe left ventricular dysfunction ($< 35\%$ LVEF or NYHA functional circulatory stages III–IV).
- 4) Low 3–4 ventricular arrhythmias before the intervention.
- 5) Angiographically visible collateral vessels interfering with the treated coronary artery.
- 6) Treatment with ATP-sensitive potassium channel inhibitors.
- 7) Serum electrolytes out of the physiologic range.
- 8) Serum creatinine level $> 150 \mu\text{mol/L}$.

9) Hypothyroidism.

According to inclusion and exclusion criteria, 36 patients were enrolled into this study. PCI was performed using the standard femoral approach in all patients after premedication with 100 mg of aspirin and 75 mg of clopidogrel once a day for 2 days prior to PCI.

Blood samples were collected from patients 5 min before the percutaneous coronary intervention from femoral artery. Blood samples were used immediately to determine the routine laboratory parameters. Additional blood samples were centrifuged at 1000 g for 15 min, at 4°C, and then, serum samples were collected into polypropylene microcentrifuge tubes and stored at -20°C until the performance of nitrotyrosine and MMP-2, MMP-9 activity measurements.

3.1.3 Biochemical analyses

3.1.3.1 Determination of routine laboratory parameters

Serum levels of total, LDL and HDL cholesterol, triglyceride, and creatinine and urea nitrogen (BUN) were quantitated by enzymatic methods (Institute of Laboratory Medicine, University of Szeged, Szeged, Hungary) on a Roche Hitachi Modular P800 automated analyzers (Roche Diagnostics, Indianapolis, IN, USA).

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

To measure circulatory MMP-2 and MMP-9 activities, gelatin zymography was performed from serum samples (for a representative zymogram see Fig. 6). Gelatinolytic activities of MMPs were examined as previously described [53]. 8% polyacrylamide gels were copolymerized with gelatin (2 mg/mL, type A from porcine skin; Sigma-Aldrich, Budapest, Hungary), and 40 µg of protein per lane was loaded. An internal standard (American Type Culture Collection, Manassas, VA, USA) was loaded into each gel to normalize activities between gels. After electrophoresis (150 V, 1.5 h), gels were washed with 2.5% Triton X-100 for 3×15 min and incubated for 20 h at 37°C in incubation buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂ and 0.05% NaN₃, pH 7.4). Gels were then stained with 0.05% Coomassie brilliant blue (G-250; Sigma-Aldrich) in a mixture of methanol-acetic acid-water [2.5:1:6.5 (vol/vol)] and destained in aqueous 4% methanol- 8% acetic acid (vol/vol). For positive controls, gelatinase zymography standard containing human MMP-2 and MMP-9 (Cat. No.: CC73, Chemicon Europe Ltd., South-ampton, UK) and MMP-2

standard (Cat. No.: CC071; Chemicon Europe Ltd.) were used. For negative control, lanes containing serum samples were cut off after renaturation of the gel and were separately incubated for 20 h at 37°C in development buffer in the presence of the calcium chelator EGTA (ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid; 10 mM). Gelatinolytic activities were detected as transparent bands against the dark-blue background. Band intensities were quantified, expressed as the ratio to the internal standard and presented in arbitrary units.

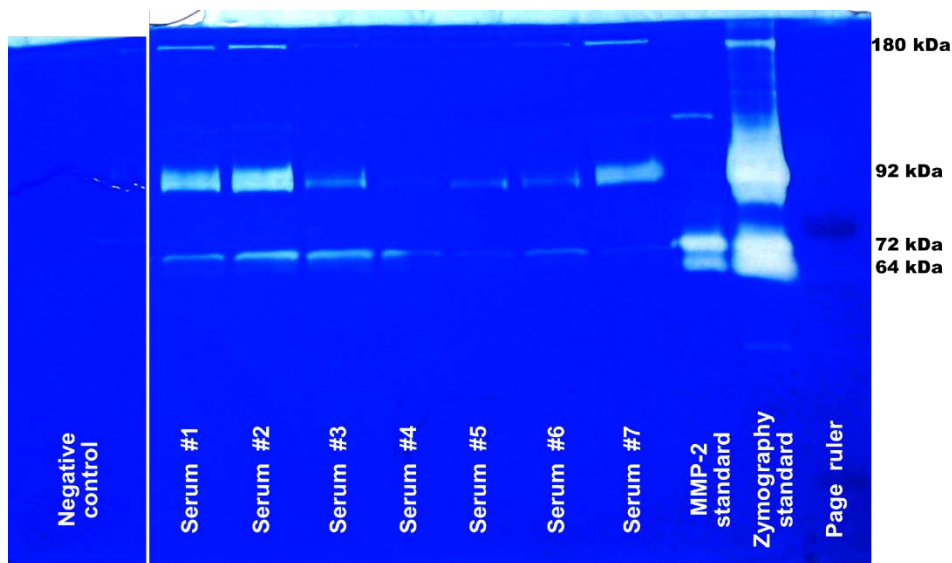


Figure 6. Representative gelatin zymogram from serum samples of CAD patients.

3.1.3.3 Measurement of nitrotyrosine in the serum by ELISA

To estimate systemic peroxynitrite formation, we have measured free nitrotyrosine by ELISA in serum samples as previously described [54, 55]. Serum samples were deproteinized by addition of four volumes of ice-cold ethanol. After centrifugation, supernatants were separated and evaporated under nitrogen flow, and then, they were dissolved in phosphate buffer. Deproteinized sera were incubated overnight with anti-nitrotyrosine rabbit IgG and nitrotyrosine acetylcholinesterase tracer in pre-coated (mouse anti-rabbit IgG) microplates followed by development with Ellman's reagent. Serum nitrotyrosine levels were expressed in ng/ mL.

3.1.4 Measurement of cardiac function

Prior to PCI, echocardiographic examinations were performed on a GE Vivid 3 cardiac ultrasound device (GE Healthcare, Milwaukee, WI, USA), and data were digitally recorded. Images were acquired using a 3.5-MHz transducer. Images and video records were obtained in standard apical and parasternal two- and four-chamber views. To assess LVEF, calculations were performed according to recommendations for chamber quantification in consensus. LVEF was calculated using Simpson's method ($LVEF = (\text{end diastolic volume} - \text{end systolic volume}) / \text{end diastolic volume} \times 100$).

3.2 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 (NIH publication No. 85-23, revised 1996) and was approved by the Ethics Committee at the University of Szeged and, when necessary, the Affymax Institutional Animal Care and Use Committee.

3.2.1 Animals and test compounds

Male Wistar rats (225–370 g) (purchased from Toxi-Coop Toxicological Research Center Zrt., Dunakeszi, Hungary and Charles-River, Budapest, Hungary) were housed in a room maintained at 12 h light-dark cycles and at a constant temperature of $22 \pm 2^\circ\text{C}$. The animals had free access to standard laboratory chow and drinking water.

Test compounds (including acetate vehicle) were synthesized and provided by Affymax Inc. except for Erythropoietin (Procrit®, Amgen, Inc., Thousand Oaks, CA), Darbepoetin alpha (Aranesp®, Amgen Europe b.v., ZK Breda, The Netherlands), B-type natriuretic peptide (BNP; Sigma-Aldrich, Budapest, Hungary) and cyclosporine A (CsA; Sandimmun Neoral®, Novartis Hungária Budapest, Hungary). Acetate vehicle consisted of 40 mM acetate and 4.3% sorbitol, pH 5.0.

3.2.2 Characterization of non-erythropoietic EPO receptor ligands

Two selective EPO receptor ligand dimeric peptides, AF41676 and AF43136 were developed and analyzed in preliminary experiments by Affymax Inc. The analysis of the two test compounds consisted of the determination of amino acid sequence, receptor binding assay and erythroid colony assay *in vitro*, and investigation of the erythropoietic activity *in vivo*.

3.2.2.1 Structure of non-erythropoietic EPO receptor ligands

AF41676 and AF43136 were synthesized by Affymax Inc. as their acetate salts using a combination of solid phase peptide techniques to prepare the individual peptide chains followed by solution phase conjugation to the bifunctional linkers. The peptides were purified by reverse-phase preparative chromatography and characterized by mass spectrometry. Their erythropoietic activity has been significantly 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 1-naphthylamine. Peptide dimer linkers are iminodiacetic acid (IDA) and 1,1'-[oxybis[(1-oxo-2,1-ethanediyl)oxy]]bis-2,5-pyrrolidinedione (DIG), and the parentheses indicate two cyclic peptide chains linked to their respective linkers via the epsilon amine on the side chain of lysine.

3.2.2.2 Investigation of erythropoietic activity of test compounds

To investigate EPO receptor binding ability of EPO, Dpa, AF41676 and AF43136, competition binding assay was carried out by Affymax Inc. by incubating 100 pg of HuEpoR-Fc (R&D Systems) with serial dilutions of competitor and 20,000 cpm ¹²⁵I EPO, followed by incubation, addition of Protein G beads, washing, and counting, using conditions based on a method for the detection of anti-EPO antibodies [56]. The specificity of the binding properties of EPO, Dpa, AF41676, and AF43136 were evaluated by MDS Pharma Services (Taipei, Taiwan) in a screen of 66 diverse receptors, including both homo- and heterodimeric EPO receptors (LeadProfilingScreen).

To investigate the erythroid colony formation ability of EPO, Dpa, AF41676 and AF43136, erythroid colony assay was performed as described previously [57]. Human CD34⁺ cells (Cambrex, Walkersville, MD, USA) were resuspended at 15,000 cells/mL in Iscove's Modified Dulbecco's Medium (IMDM) with 10% FBS. EPO, Dpa, AF41676 and AF43136 were added to a methylcellulose-based colony assay media (MethoCult GF H4534, StemCell Technologies, and Vancouver, BC, Canada) supplemented with rhSCF, rhGM-CSF, and rhIL-3. Cells were added to the methylcellulose and plated with subsequent incubation at 37°C, in a 5% CO₂ incubator for 12–14 days. Colonies containing over 50 cells were counted by phase-contrast microscopy.

To evaluate erythropoiesis stimulating effect, EPO, Dpa, AF41676 and AF43136 were examined for their potential to induce reticulocytosis in normocythemic male Sprague-

Dawley rats (n= 4). Colleagues at Affymax Inc. administered a single intravenous (iv.) dose of EPO at 5000 U/kg, Dpa at 10 µg/kg, AF41676 at 10 mg/kg, and AF43136 at 10 mg/kg and acetate vehicle (5 mL/kg) via the tail vein, respectively. Blood (0.5 mL) was collected for determination of reticulocyte number by retroorbital puncture of metoxyflurane-anesthetized rats into EDTA-containing tubes at baseline (pre-dose) and 5 days following administration as described previously [57].

3.2.3 Study design

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

3.2.3.1 Investigation of direct cardio-cytoprotective effect by EPO

To determine the direct, non-erythropoietic effect of EPO on cardiomyocytes, 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 (Table 1).

Model	Protocol	n	Experimental groups				End points
			negative controls	positive controls	test compounds & applied doses		
<i>In vitro</i> SI/R	150 min SI/ 120 min R	7-8	acetate vehicle	BNP 10-8 M	EPO (U/mL)	1 10 100 500	cell viability
<i>In vivo</i> rat myocardial infarction	30 min ischemia / 120 min reperfusion	9-11	acetate vehicle	BNP 10 nmol/kg bolus + 2 nmol/kg/min	EPO (U/kg)	5000 ip iv	infarct size, survival, hemodynamics
		8-11	acetate vehicle	BNP 10 nmol/kg bolus + 2 nmol/kg/min	Dpa (µg/kg)	2.5 5 15 25	
		8-13	acetate vehicle	BNP 10 nmol/kg bolus + 2 nmol/kg/min	AF41676 (mg/kg)	0.3 1 3 10 30	
		8-10	saline, acetate vehicle	CsA 10 mg/kg	AF43136 (mg/kg)	10 30	

Table 1. Study design. n: sample size/ group; SI/R: simulated ischemia/ simulated reperfusion; BNP: B-type natriuretic peptide; EPO: Erythropoietin; Dpa: darbepoetin alpha; CsA: cyclosporine A; iv: intravenous; ip: intraperitoneal

3.2.3.2 Investigation of myocardial infarct size limiting effect of EPO, Dpa and EPO analogues

In a further series of *in vivo* studies (studies 1–4, see below), animals were randomly assigned to the different experimental groups with a total number of 8–13 animals per group. Each test compound was administered before the onset of reperfusion (Table 1). During the individual studies, all-cause mortality was registered to compare the survival among the controls and the drug-treated groups.

Study 1: We have administered EPO at 5000 U/kg [used successfully for cardioprotection several times in the literature; for review see [58] bolus injection 5 min before the onset of

reperfusion intraperitoneally (ip) or intravenously (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. This dose was derived from a previous publication where 10 nM BNP was cardioprotective *ex vivo* [59], and due to its short half-life [60] a maintenance infusion (2 nmol/kg/min) was applied in our *in vivo* model. The vehicle was used as the negative control and comprised 40 mM acetate with 4.3% sorbitol, pH 5.0 and was administered iv. 5 min before the onset of reperfusion.

Study 2: To investigate the dose dependence of the EPO analogue, darbepoetin (Dpa) was administered at the 15th min of ischemia, in 2.5, 5, 15 and 25 µg/kg boluses administered iv. The positive control BNP and the negative control acetate vehicle were administered identically to Study 1. The reason for Dpa administration 10 min earlier than EPO (i.e. at the 15th min of ischemia) was its prolonged half-life.

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 accordingly to Study 1.

Study 4: In an exploratory study, we tested AF43136, a slightly modified version of AF41676 (see above for structural differences) in two doses in order to monitor potential hypotensive effects, which have occurred previously with EPO analogues. 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 at a final volume load of 6 mL/kg due to the high concentration being administered (30 mg/kg). Saline and the positive control CsA (10 mg/kg, diluted in saline) were administered iv. as a bolus infusion for 5 min starting at the 25th min of ischemia at a final volume load of 1 mL/kg. Acetate vehicle was administered same as test compounds at a final volume load of 6 mL/kg.

3.2.4 Simulated ischemia/reperfusion in cultured neonatal cardiomyocytes

Neonatal cardiomyocyte isolation and culture method was described previously [61, 62]. Newborn Wistar rats were disinfected with 70% ethanol and sacrificed by cervical dislocation. Hearts were excised and transferred to cold, sterile phosphate buffered saline (PBS) pH 7.2. Ventricles were separated from atria, minced gently with sterile forceps and collected in 0.25% trypsin solution (Invitrogen, Life Technologies Hungary Ltd., Budapest, Hungary). At the end of digestion, cell suspension was centrifuged for 15 min at 2000 rpm at 4°C, supernatant was removed gently and the pellet was re-suspended in complete growth

medium and plated onto 6-well plates to eliminate fibroblasts (pre-plating). Supernatants were collected in fresh Falcon tubes and cells were counted manually in a Burker chamber. Then cells of the supernatant were re-plated into 24-well fresh plates: 10^5 cells/well supplemented with 1 mL growth medium and incubated under normoxic conditions (37°C, in 95% air and 5% CO₂ gas mixture) in a standard CO₂ incubator. Growth medium was changed the following day. Cultures were used for treatment at the 3-day-old stage. The simulated ischemic group was treated during the experimental protocol using a combination of hypoxic chamber and hypoxic solution [62] to simulate tissue ischemia. In treated groups, EPO (1, 10, 100, 500 U/mL) and BNP (10^{-8} M) or acetate vehicle were administered into the hypoxic solution and plates were kept in a hypoxic chamber (gassed with 95% N₂ and 5% CO₂ at 37°C) for 150 min. Following treatments, 120 min of simulated reperfusion was applied in growing medium (mixed with treating compounds) and the plates were kept in a normoxic incubator.

3.2.5 Cell viability measurement

At the end of the experiments, viability tests were performed in all groups with trypan blue staining after simulated reperfusion. Treating solutions were removed and cells were detached with trypsin. Single cell suspensions were collected into Eppendorf tubes and centrifuged at 2000 rpm for 15 min at 4°C. Supernatants were removed and the pellets were re-suspended in 50 µL PBS solution and mixed with 50 µL 0.4% trypan blue and incubated for 5 min. Cells were counted in a Burker chamber and the ratio of dead (blue) cells to total cell count was calculated and plotted.

3.2.6 *In vivo* rat model of acute myocardial infarction

Myocardial infarction in rats was described previously [63]. Rats were anesthetized by intraperitoneal injection of pentobarbital (Euthasol, Produlab Pharma b.v., Raamsdonksveer, The Netherlands; 60 mg/kg bolus dose followed by 15–20 mg/kg when required during the experiment). The rats were weighed and stomach and chest area shaved. Body temperature was maintained using a constant-temperature heating pad. The trachea was intubated with a cannula connected to a rodent ventilator. The animals were ventilated with room air (6.2 mL/kg, 70 ± 5 breath/min). Blood pressure, surface-lead ECG, and body core temperature were monitored throughout the experiments to ensure the stability of the preparation (Haemosys data acquisition system, Experimetria, Budapest, Hungary), while hemodynamic

data were collected at baseline, 29th min of ischemia and 120th min of reperfusion. The right carotid artery was cannulated for measurement of blood pressure and the right femoral vein was cannulated for the administration of the test compounds. A thoracotomy was performed at the 5th inter-costal space and the heart was exposed through the fifth intercostal space. The pericardium was incised and a 5-0 Prolene suture was placed around the left descending coronary artery. The coronary artery was then occluded for 30 min by placing the ligature through a small piece of plastic tubing and pulling the snare tightly in place using a hemostat. After 30 min of coronary occlusion, reperfusion was initiated by releasing the snare and then continued for 120 min. All-cause mortality was monitored throughout the entire study, i.e. from the anesthesia to the end of reperfusion. The cause of death was determined as follows: irreversible ventricular arrhythmias or hypotension.

3.2.7 Infarct size measurement

At the end of the 120 min reperfusion, the heart was excised and infarct size was determined [64]. The left anterior descending coronary artery was re-occluded and the heart was perfused with 4 mL of 0.3% (w/v) Evans-blue dye (Sigma-Aldrich, Budapest, Hungary) with a constant 100 cmH₂O pressure into the aorta to delineate the area at risk zone. Stained hearts were rapidly frozen (at -20°C for at least 30 min), cut into 2 mm thick slices, and each slice was incubated at 37°C in 1.5 mL of 1% (w/v) 2,3,5-triphenyltetrazolium-chloride (TTC, Merck Biosciences, Budapest, Hungary) dissolved in 50 mM phosphate buffer (pH 7.4) for 10 min. Slices were then transferred into 10% formalin solution for 5–10 min, rinsed, and then scanned between glass plates. The differently stained areas of the images were quantified by digital planimetry (InfarctsizeTM2.5, Pharmahungary, Szeged, Hungary). Evaluation of all images was carried out in a blinded manner by an experienced investigator throughout the study. Area at risk zone (AAR) was expressed as the percentage of total ventricular area (LV), and infarct size (IS) was expressed as a percentage of the AAR.

3.3 Statistical analysis

Data are expressed as mean \pm standard deviation (SD) or mean \pm standard error of the mean (SEM). Statistical significance was accepted at $p < 0.05$.

Univariate correlations between lipid parameters and MMP activities as well as nitrotyrosine were analyzed using the Pearson correlation coefficient. Because of the relatively small sample sizes in the current study, bootstrapped confidence intervals were

calculated, using 1000 samples with replacement, to make robust estimates of the correlation coefficients as described elsewhere [65, 66]. Linear regression analysis was used to estimate the prediction of the selected variables. The effects of statin treatment on variables were analyzed by unpaired two-tailed Student's t-test.

Differences among means of the ratio of dead cells in cell-culture experiments, and among means of IS/AAR (%) in *in vivo* experiments were analyzed by One-way analysis of variance (ANOVA) followed by Fisher LSD post hoc test to compare each group to the control when appropriate. To analyze the reticulocyte stimulation test, One-way ANOVA was used followed by Tukey post hoc test, where means of reticulocyte number/L of EPO and Dpa were compared to the vehicle, while means of the AF test compounds were compared to EPO. Mortality was analyzed with Chi-square test followed by Fisher's exact test. CsA group was compared to the saline control group by Student's t-test.

4. Results

4.1 Characterization of the correlations of ONOO⁻ and MMPs in patients with CAD

Baseline characteristics of the 36 patients with coronary artery disease are summarized in Table 2.

Biographic and anamnestic variables	Mean \pm SD or Occurrence (number of individuals)
Age (years)	67.4 \pm 10.5
Gender, m/f	19/17
Type 1 diabetes	0/36
Type 2 diabetes	5/36
Statin treatment	15/36
ACE inhibitor treatment	31/36
ARB treatment	1/36
Nitrate treatment	18/36
Hemodynamic variables	
Systolic blood pressure (mmHg)	141.7 \pm 11.1
Diastolic blood pressure (mmHg)	89.8 \pm 21.4
Heart rate (beats/min)	70.4 \pm 12.1
LVEF (%)	56.0 \pm 10.5
Coronary stenosis (%)	63.5 \pm 8.0
Occurrence of hypertension	29/36
Serum variables	
Total cholesterol (mmol/L)	4.6 \pm 0.9
LDL cholesterol (mmol/L)	2.5 \pm 0.8
HDL cholesterol (mmol/L)	1.3 \pm 0.4
Triglycerides (mmol/L)	1.8 \pm 0.8
3-nitrotyrosine (ng/mL)	17.4 \pm 11.3
Urea nitrogen (mmol/l)	5.9 \pm 2.4
Creatinine (μ mol/l)	86.3 \pm 27.5

Table 2. Baseline characteristics of the 36 coronary artery disease patients.

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

We have determined whether serum nitrotyrosine correlates with MMP-2 and MMP-9 activities in patients with CAD. Nitrotyrosine correlated positively with MMP-9 activity (Fig. 7A; $r = 0.535$, $p = 0.010$); however, there was no significant correlation between serum nitrotyrosine and MMP-2 activity (Fig. 7B).

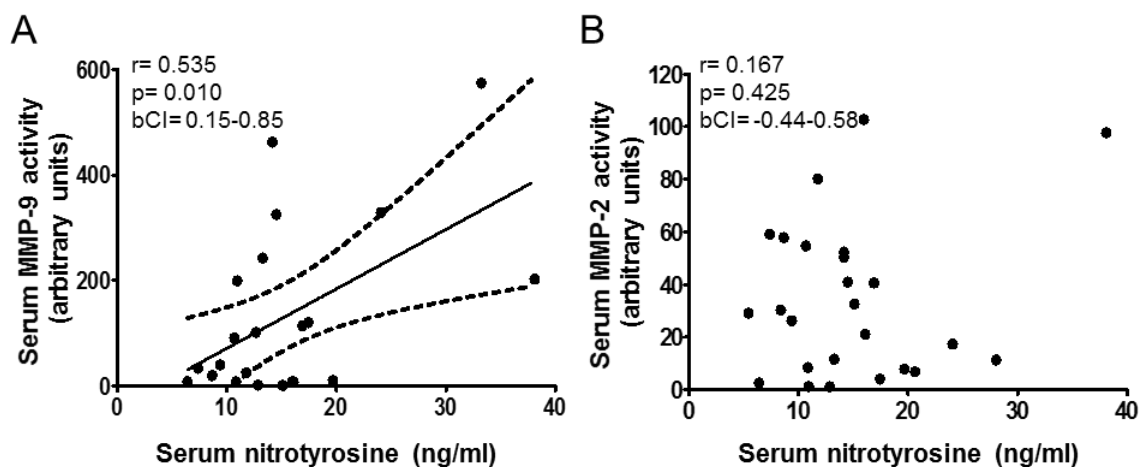


Figure 7. Correlation of serum nitrotyrosine content with MMP-2 and MMP-9 activities. Serum MMP-9 activity correlated positively with serum nitrotyrosine (A). Serum MMP-2 activity (B) did not correlate with serum nitrotyrosine. Continuous line: linear regression of data; dotted line: 95% confidence interval. r : Pearson's correlation coefficient, p : significance, bCI : bootstrapped confidence interval of correlation coefficient.

4.1.2 Correlation between serum lipids and nitrotyrosine or MMPs activity

In patients with CAD, serum total cholesterol (Fig. 8A), LDL cholesterol (Fig. 8B) and triglyceride (Fig. 8C) 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 (Fig. 8D; $r = -0.455$, $p = 0.033$) with serum nitrotyrosine. Serum MMP-2 activity correlated positively with serum total cholesterol (Fig. 8E, $r = 0.550$, $p = 0.015$) and LDL cholesterol levels (Fig. 8F, $r = 0.448$, $p = 0.028$, respectively); however, neither serum triglyceride (Fig. 8G) nor HDL cholesterol (Fig. 8H) showed a significant correlation with MMP-2 activity. None of the serum lipid parameters correlated with MMP-9 activity in patients with CAD (Table 3).

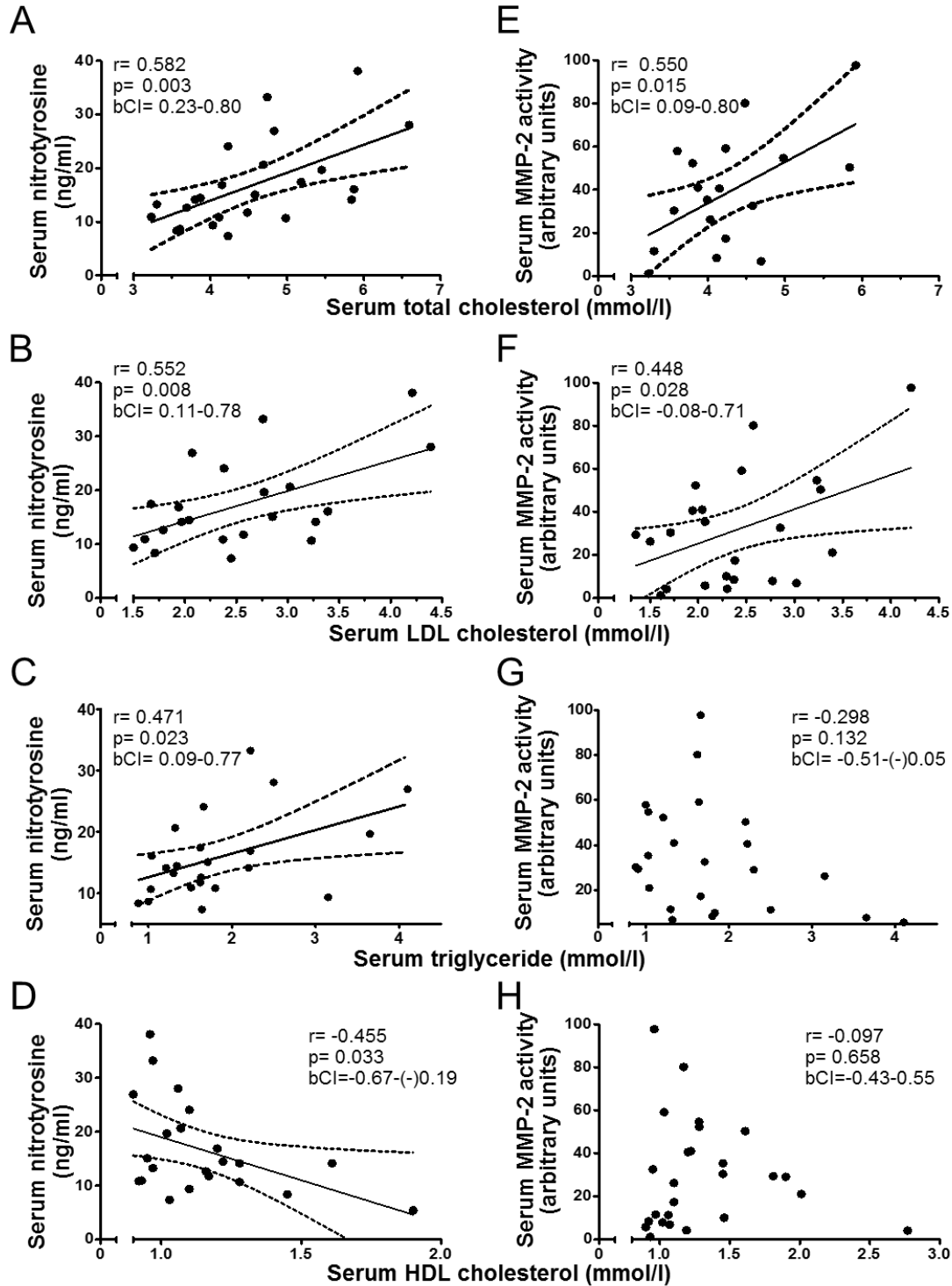


Figure 8. Correlation of serum nitrotyrosine and MMP-2 activities with serum lipids. Serum nitrotyrosine level correlated positively with total (A) and LDL cholesterol (B) as well as with triglyceride (C) and correlated negatively with HDL cholesterol (D). Serum MMP-2 activity correlated positively with serum total (E) and LDL cholesterol levels (F). Neither serum triglyceride (G) nor HDL cholesterol (H) showed a significant correlation with serum MMP-2 activity. Continuous line: linear regression of data; dotted line: 95% confidence interval. r : Pearson's correlation coefficient, p : significance, bCI : bootstrapped confidence interval of correlation coefficient.

Variables		Pearson correlation coefficient	Bootstrapped confidence intervals of correlation coefficient	p
MMP-9 /	total cholesterol	0.171	-0.27-0.49	0.436
MMP-9 /	LDL cholesterol	0.020	-0.38-0.30	0.939
MMP-9 /	HDL cholesterol	0.050	-0.31-0.59	0.840
MMP-9 /	triglyceride	0.024	-0.34-0.40	0.913

Table 3. Correlations between serum MMP-9 activity and lipid parameters.

4.1.3 Correlation between myocardial function and serum nitrotyrosine or MMPs activity

Serum nitrotyrosine level correlated negatively with LVEF (Fig. 9A, $r = -0.548$, $p = 0.010$). MMP-2 activity did not correlate with LVEF (Fig. 9B), however, MMP-9 activity also showed a negative correlation with LVEF (Fig. 9C, $r = -0.732$, $p = 0.0002$, similarly to the nitrotyrosine level), in patients with CAD. The negative correlation indicates that deteriorated cardiac function associates with elevated nitrotyrosine level and MMP-9 activity.

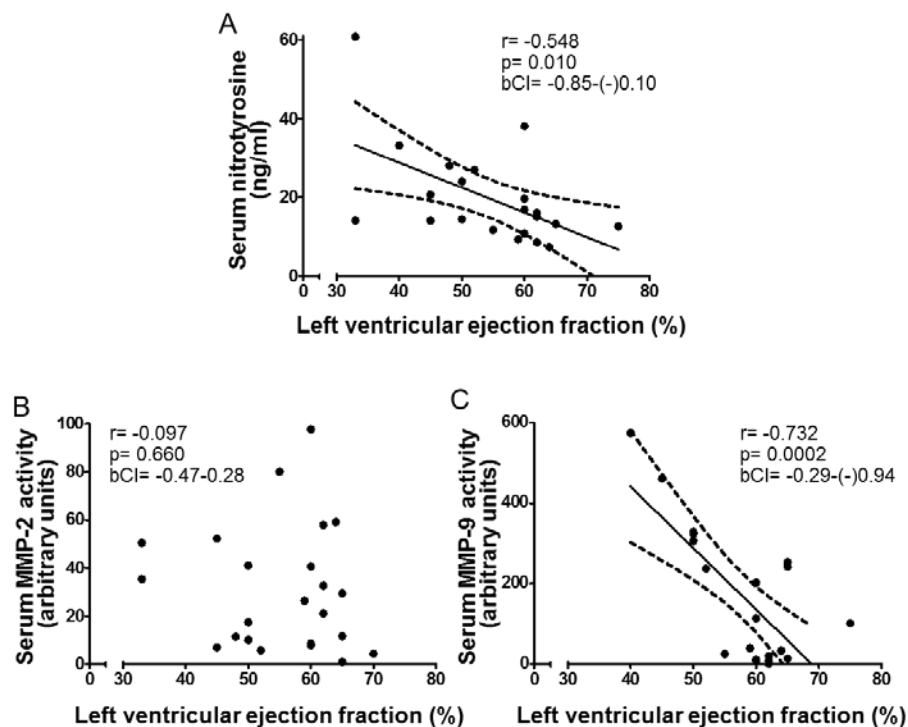


Figure 9. Correlation of left ventricular ejection fraction (LVEF) with serum nitrotyrosine, and serum MMP-2 and MMP-9 activities. Serum nitrotyrosine (A) and MMP-9 (C) activity correlated negatively with LVEF. Serum MMP-2 activity did not correlate with LVEF (B). Continuous line: linear regression of data; dotted line: 95% confidence interval. r : Pearson's correlation coefficient, p : significance, bCI : bootstrapped confidence interval of correlation coefficient.

4.1.4 Serum nitrotyrosine or MMPs activity in statin-treated patients

Fifteen of the 36 patients with CAD involved in this study were treated with different types of statins. In statin-treated patients, a significantly reduced serum nitrotyrosine level was found as compared to statin-naïve patients (Fig. 10A, 23.5 ± 4.5 ng/mL vs. 13.6 ± 5.1 ng/mL; $p < 0.05$). MMP-2 and MMP-9 activities did not show any difference between statin-treated and statin-naïve patients (Fig. 10B, C).

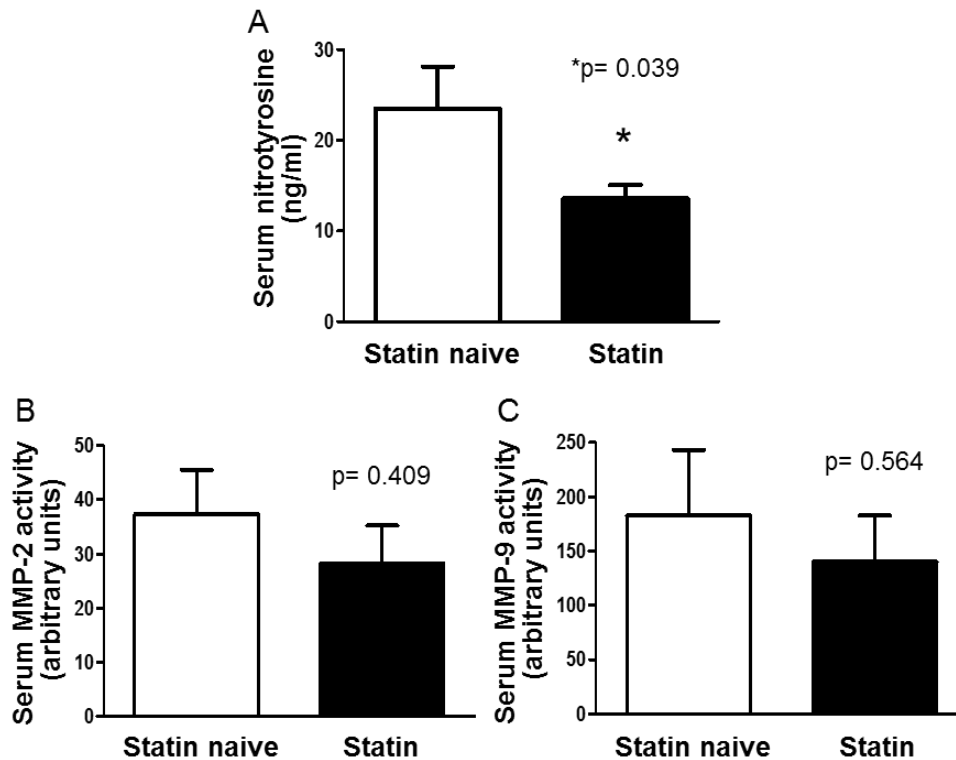


Figure 10. Serum nitrotyrosine levels (A) and serum MMP-2 (B) and MMP-9 (C) activities in statin-treated and statin-naïve patients with CAD. Serum nitrotyrosine was significantly reduced in statin-treated patients with CAD as compared to statin-naïve patients ($*p < 0.05$). MMP-2 and MMP-9 activities were not changed in statin-treated patients with CAD as compared to statin-naïve ones. Data are shown as mean \pm SEM

4.1.5 Serum nitrotyrosine or MMPs activity in smoking patients

Eleven of the 36 patients with CAD were active smoker during the study and seventeen patients did not smoke (we got no information about smoking in case of eight patients). Serum nitrotyrosine level did not show any difference between non-smoker and smoker patients (Fig. 11A, 16.0 ± 1.7 ng/mL vs. 16.7 ± 3.3 ng/mL; $p = 0.7$). MMP-2 activity was significantly increased in smoker patients as compared to the non-smokers (Fig. 11B, 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 (Fig. 11C, 123.0 ± 31.6 vs. 130.0 ± 45.2 arbitrary unit, $p=0.9$).

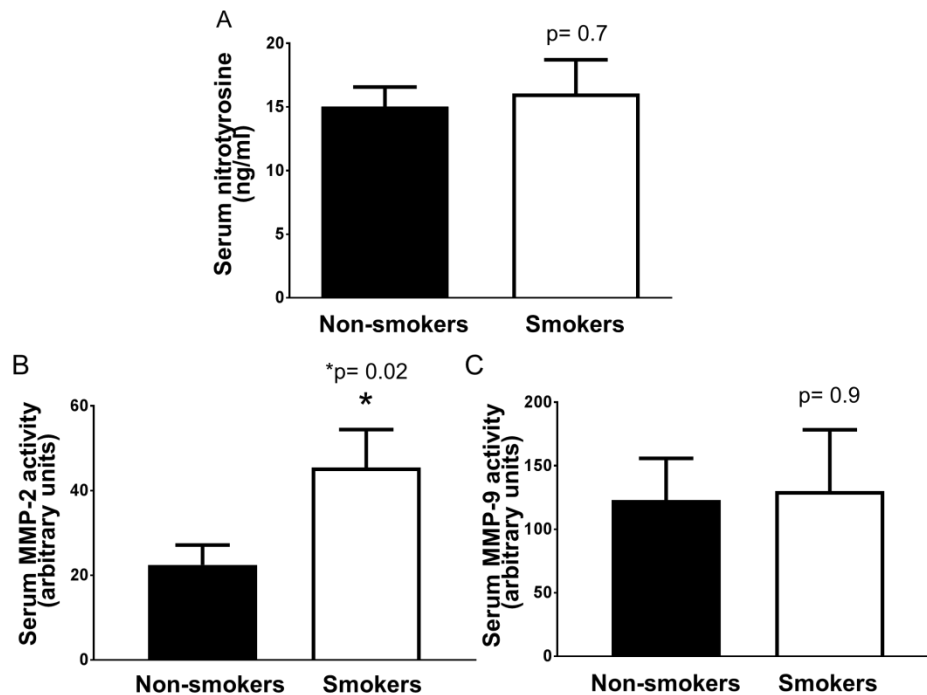


Figure 11. Serum nitrotyrosine levels (A) and serum MMP-2 (B) and MMP-9 (C) activities in non-smoker and smoker patients with CAD. Serum MMP-2 was significantly increased in smoker patients with CAD as compared to non-smoker patients ($*p < 0.05$). NTyr and MMP-9 activities were not changed in non-smoker patients with CAD as compared to smoker ones. Data are shown as mean \pm SEM, $n = 11-17$.

4.1.6 Correlation between renal function and serum nitrotyrosine or MMPs activity

In addition, we tested the correlation of renal function parameters with markers of peroxynitrite–MMP pathway and also with LVEF, since renal dysfunction may be a severe complication in CAD. Serum creatinine correlated positively with nitrotyrosine ($r = 0.422$, $p = 0.036$; Fig. 12A), but not with MMP-2 and MMP-9 activities (Table 4). Urea nitrogen level correlated positively with MMP-9 activity ($r = 0.423$, $p = 0.035$; Fig. 12B), but not with nitrotyrosine or MMP-2 activity (Table 4). Serum creatinine correlated negatively with LVEF ($r = -0.503$, $p = 0.009$; Fig. 12C); however, urea nitrogen did not correlate with LVEF (Table 4).

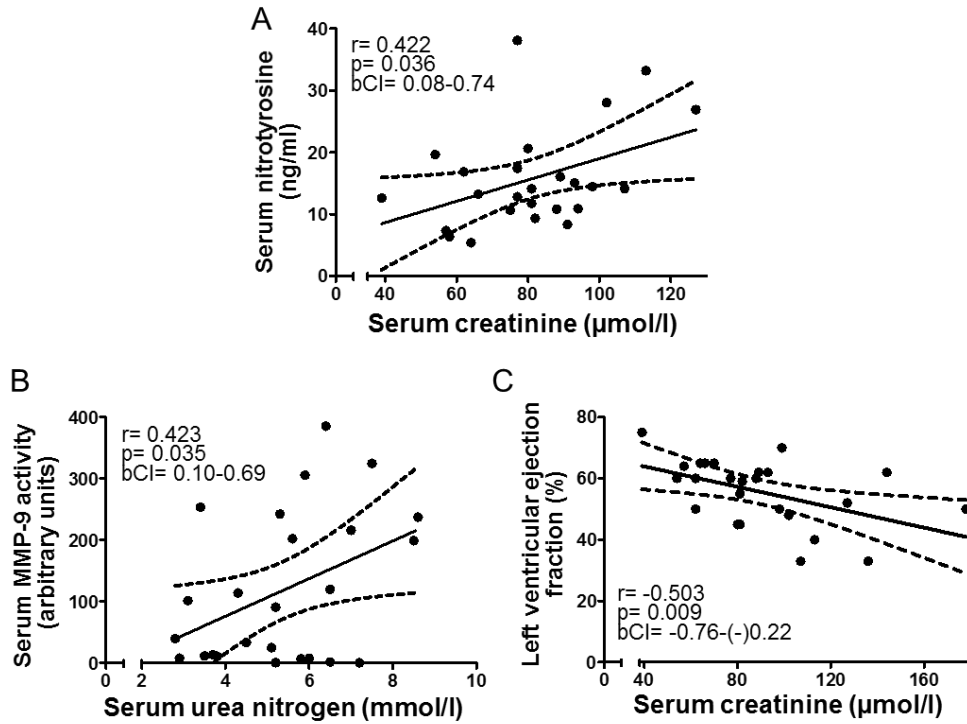


Figure 12. Correlation of renal function with serum nitrotyrosine, MMP activities and myocardial function. Serum nitrotyrosine correlated positively with serum creatinine (A). Serum MMP-9 activity correlated positively with urea nitrogen level (B). Continuous line: linear regression of data; dotted line: 95% confidence interval, r : Pearson's correlation coefficient, p : significance, bCI : bootstrapped confidence interval of correlation coefficient.

Variables		Pearson correlation coefficient	Bootstrapped confidence intervals of correlation coefficient	p
MMP-2 /	creatinine	0.125	-0.20-0.45	0.504
MMP-9 /	creatinine	0.118	-0.30-0.48	0.550
NTyr/	BUN	0.105	-0.22-0.39	0.602
MMP-2 /	BUN	0.071	-0.20-0.42	0.700
LVEF/	BUN	-0.250	-0.57-0.00	0.217

Table 4. Correlations between serum nitrotyrosine level, MMPs activities, or LVEF and renal function parameters. MMP: matrix metalloproteinase, NTyr: nitrotyrosine, BUN: blood urea nitrogen, LVEF: left ventricular ejection fraction.

4.2 Investigation of EpoR-mediated cardioprotection

4.2.1 Erythropoietic activity of test compounds

As described previously, erythropoietic activity of test compounds was analyzed by Affymax Inc.

EPO, Dpa and AF43136 bound to the EPO receptor with high affinity (AF41676 exhibiting with modest affinity), AF41676 and AF43136 did not show *in vitro* erythropoietic activity (Table 5A).

EPO and Dpa differentiated CD34⁺ cells into erythroid precursors with high efficiency (EC₅₀= 2 to 14 pM), whereas AF41676 and AF43136 required vastly greater amounts (approx. 3–4 orders of magnitude, Table 5A).

Reticulocytosis, a predictive marker of erythropoiesis, was significantly stimulated 5 days following administration of EPO at 5000 U/kg and Dpa at 10 µg/kg, but not by administration of AF41676 and AF43136 compared to acetate vehicle (Table 5B, Fig. 13).

A			B	
Test compounds	Binding (EC ₅₀ , pM)	BFU-E (pM)	Test compounds	Reticulocytes (10 ⁹ /L)
EPO	7	2	Acetate vehicle	373±24
Dpa	12	14	EPO (5,000 U/kg)	762±146*
AF41676	1240	34680	Dpa (10 µg/kg)	1056±104*
AF43136	27	2623	AF41676 (10 mg/kg)	284±51 [#]
			AF43136 (10 mg/kg)	286±57 [#]

Table 5. Data for erythroid colony assay and EPO receptor binding assay of the test compounds (A). Data for reticulocyte stimulation test measured 5 days after administration of the test compounds (B). Data are expressed as mean ± SD. AF41676 and AF43136 did not induce reticulocytosis while EPO and Dpa produced a robust erythropoietic response. *p< 0.05 vs. acetate vehicle, [#]p< 0.05 vs. EPO. BFU-E: Erythroid blood forming unit; EC₅₀: Half maximal effective concentration; EPO: Erythropoietin; Dpa: darbepoetin alpha.

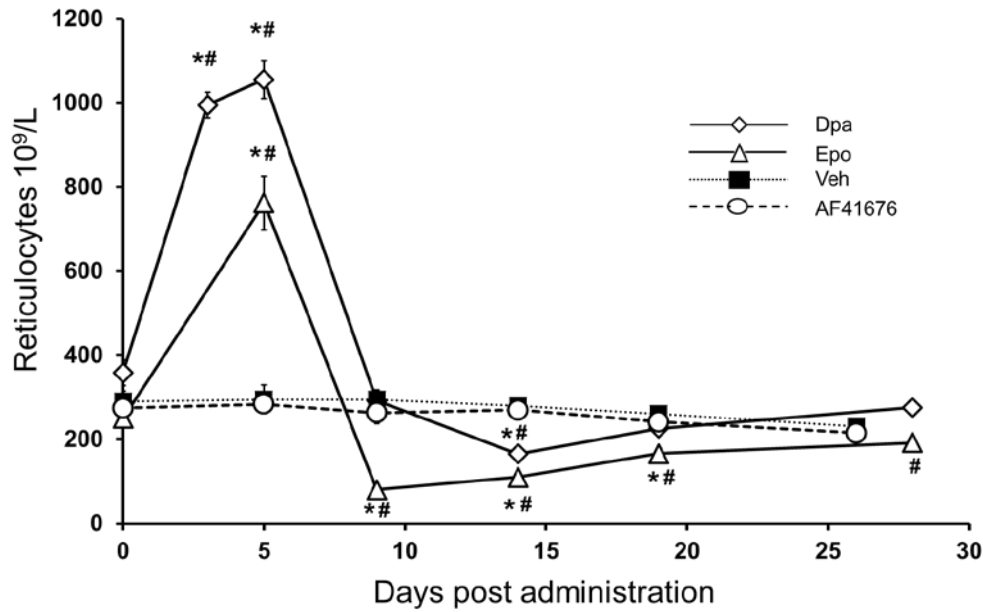


Figure 13. The effects of 5,000 Units/kg EPO, 10 µg/kg Dpa, 10 mg/kg AF41676, and acetate vehicle on reticulocytes number following administration of single iv. boluses. Data are expressed as mean ± SD, * $p < 0.05$ vs. vehicle, # $p < 0.05$ vs. baseline analyzed by repeated measures ANOVA, $n = 4$ rats per time point/ group.

4.2.2 The effects of EPO on cell death

Erythropoietin showed a U-shaped response curve on cell viability in cultured neonatal rat cardiomyocytes subjected to simulated ischemia/reperfusion (Fig. 14B). EPO at 100 U/mL significantly decreased the ratio of dead cells (by approx. 16%) compared to the vehicle control group. Similarly to lower doses (1 or 10 U/mL), highest tested dose (500 U/mL) of EPO did not reduce cell death as compared to the vehicle-treated cells. The positive control BNP reduced cell death compared to the vehicle by approx. 30% (Fig. 14B).

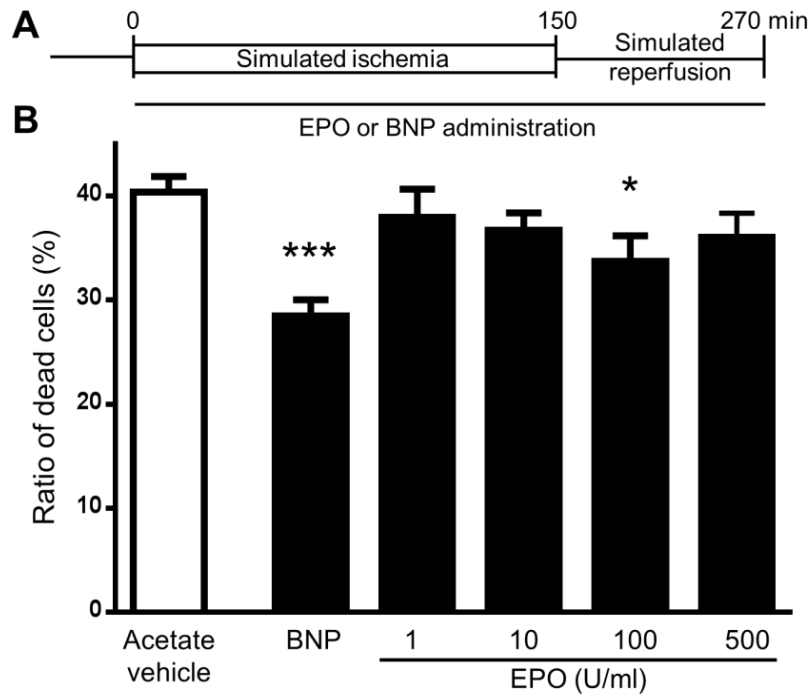


Figure 14. Panel A: Experimental protocol for the pilot study. Panel B: Dose-response relationships for EPO and the effect of BNP on cell death in cultured neonatal rat cardiomyocytes subjected to simulated ischemia/reperfusion. Data are expressed as mean \pm SEM, * p < 0.05, *** p < 0.001 vs. simulated ischemia/reperfusion (SI/R) control; n = 7-8 wells.

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

Different EPO dosing regimens were evaluated. EPO at 5000 U/kg administered intravenously, before the onset of reperfusion decreased infarct size significantly compared to the vehicle by approx. 24%, while intraperitoneal administration was less effective (approx. 11% decrease, non-significant) (Fig. 15B, Table 6). The positive control BNP (10 nmol/kg + 2 nmol/kg infusion for 15 min) decreased infarct size significantly compared to the vehicle by approx. 47% (Fig. 15B, Table 6). There was no difference in area at risks among the groups (Table 6). BNP reduced mean arterial blood pressure (MABP) significantly during ischemia compared to the vehicle, whereas EPO did not show any significant effect on MABP or heart rate (HR) (Table 6). There was no significant difference in survival among the groups (Table 6).

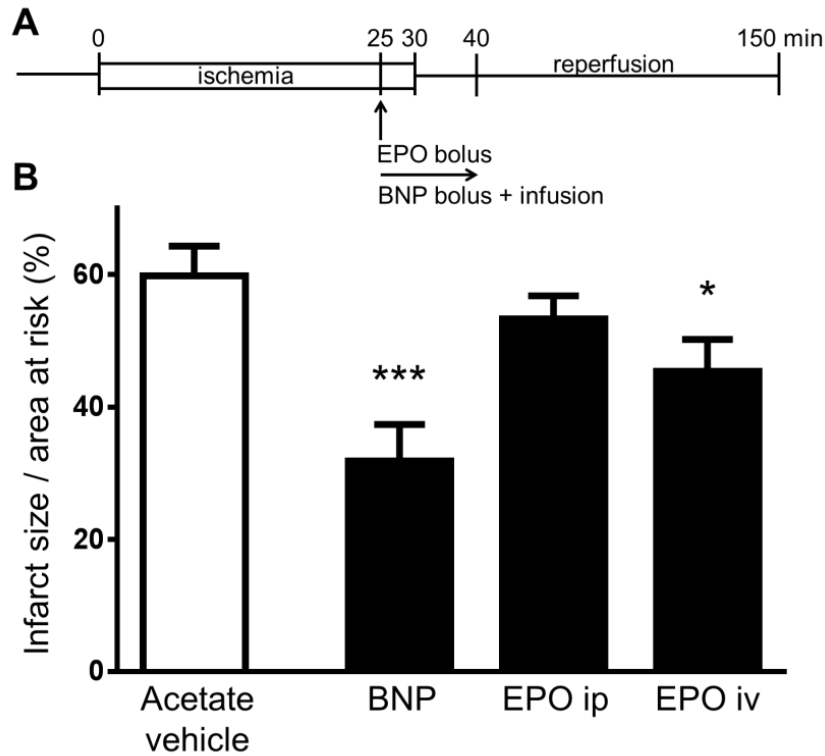


Figure 15. Panel A: Experimental protocol for Study 1. Panel B: The effects of BNP and EPO on infarct size in rats subjected to *in vivo* 30-min coronary occlusion followed by 120 min reperfusion. EPO was administered either intraperitoneally (EPO ip) or intravenously (EPO iv) for rats. Data are expressed as means \pm S.E.M., * $p < 0.05$, *** $p < 0.001$ vs. vehicle control; $n = 8-9$.

Exp. Group	n	AAR/LV (%)	mean arterial blood pressure (mmHg)			heart rate (beats/min)			SR (%)
			BL	ISCH	REP	BL	ISCH	REP	
Vehicle acetate	9	34.3 \pm 2.7	142 \pm 7	119 \pm 7	107 \pm 6	404 \pm 10	399 \pm 14	400 \pm 14	82
BNP	8	38.8 \pm 5.5	124 \pm 10	89 \pm 5*	91 \pm 6	401 \pm 14	405 \pm 6	413 \pm 11	89
Epo ip	8	33.9 \pm 2.2	151 \pm 7	118 \pm 5	108 \pm 9	418 \pm 20	410 \pm 18	399 \pm 15	89
Epo iv	8	37.3 \pm 3.7	126 \pm 11	115 \pm 8	111 \pm 9	427 \pm 6	396 \pm 13	403 \pm 12	80

Table 6. Hemodynamic parameters, area at risk and survival ratio for study 1. Data are expressed as means \pm S.E.M, * $p < 0.05$, *** $p < 0.001$ vs. vehicle. BNP: B-type natriuretic peptide; Epo: Erythropoietin; ip: intraperitoneal; iv: intravenous; AAR/LV: area at risk/ left ventricle; BL: baseline; ISCH: 29th min of ischemia; REP: 120th min of reperfusion; SR: survival rate

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

Different doses of darbepoetin were administered iv. at the 15th min of ischemia. Dose-response relationship of darbepoetin showed maximal infarct size limiting effect at 5 $\mu\text{g}/\text{kg}$ compared to the vehicle (Fig. 16B, Table 7). The infarct size-limiting effect of darbepoetin was comparable to that of the positive control BNP (approx. 33% for both, Fig. 16B, Table 7). There was no significant difference in the infarct-size limiting effect of the other Dpa doses (2.5, 5 or 25 $\mu\text{g}/\text{kg}$, respectively). There was no significant difference in the area at risks among groups (Table 7). BNP and Dpa at 5 $\mu\text{g}/\text{kg}$ reduced MABP significantly during ischemia, while Dpa at 15 $\mu\text{g}/\text{kg}$ significantly improved MABP at the end of reperfusion compared to the vehicle. Neither Dpa nor BNP had any effect on HR (Table 7). There was no significant difference in survival among the groups (Table 7).

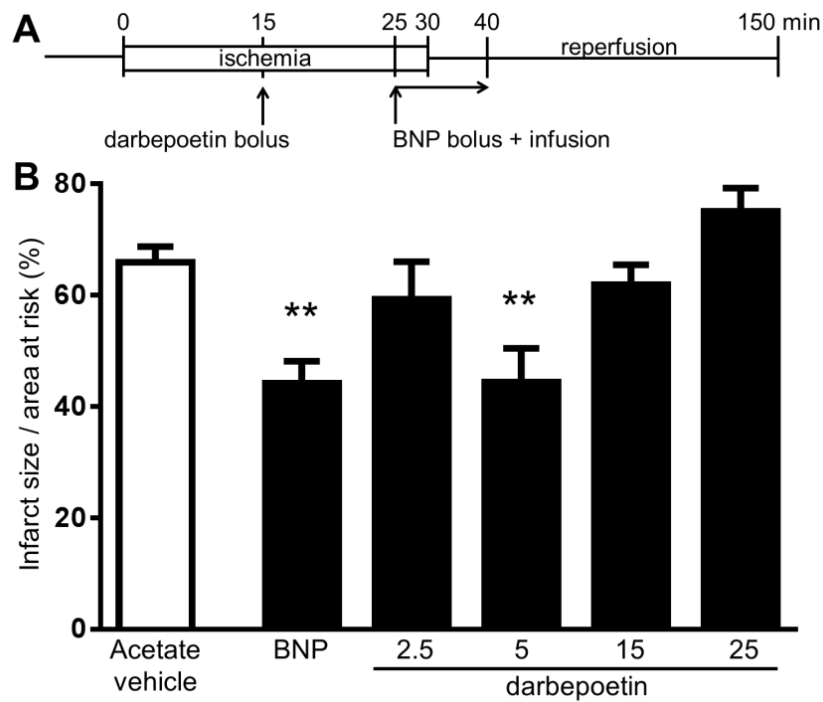


Figure 16. Panel A: Experimental protocol for Study 2. Panel B: Dose-response relationships for darbepoetin (doses are given in $\mu\text{g}/\text{kg}$) and the effect of BNP on infarct size in rats subjected to *in vivo* 30-min coronary occlusion followed by 120 min reperfusion. Data are expressed as means \pm SEM, ** $p < 0.01$ vs. vehicle control; $n = 7-8$.

Exp. Group	n	AAR/LV (%)	mean arterial blood pressure (mmHg)			heart rate (beats/min)			SR (%)
			BL	ISCH	REP	BL	ISCH	REP	
Vehicle acetate	8	41.5±3.3	115±4	95±7	75±3	411±11	422±13	389±10	90
BNP	7	51.6±1.5	107±5	71±4*	75±4	386±15	389±18	386±14	100
Dpa 2.5 µg/kg	8	51.5±3.0	112±3	91±5	80±4	408±13	437±11	387±17	100
Dpa 5 µg/kg	8	43.3±5.3	123±2	62±4*	76±6	430±3	378±9	398±16	100
Dpa 15 µg/kg	7	41.3±4.1	126±11	80±5	91±4*	368±14*	418±8	416±8	88
Dpa 25 µg/kg	7	49.9±2.5	127±1	104±4	74±4	396±14	420±11	406±14	88

Table 7. Hemodynamic parameters, area at risk and survival ratio for study 2. Data are expressed as means ± SEM, *p< 0.05 vs. vehicle. BNP: B-type natriuretic peptide; Dpa: darbepoetin alpha; AAR/LV: area at risk/left ventricle; BL: baseline; ISCH: 29th min of ischemia; REP: 120th min of reperfusion; SR: survival rate

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

Dose-dependent efficacy of AF41676 was investigated by five different intravenously-administered doses in a bolus injection 5 min before the onset of reperfusion. On the dose-effect curve, AF41676 at 0.3, 1, 3 and 10 mg/kg decreased infarct size significantly, of which the 3 mg/kg dose showing the most potent infarct size limiting effect compared to the vehicle (approx. 46% reduction, Fig. 17B, Table 8). BNP also reduced infarct size significantly compared to the vehicle (approx. 25% reduction; Fig. 17B, Table 8). There was no significant difference in the area at risks among the groups (Table 8). BNP as well as 10 and 20 mg/kg AF41676 reduced MABP during ischemia compared to the vehicle (Table 8). AF41676 at 20 mg/kg reduced HR at the 29th min of ischemia and at 10 mg/kg reduced MABP at the end of reperfusion (Table 8). 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 (Table 8).

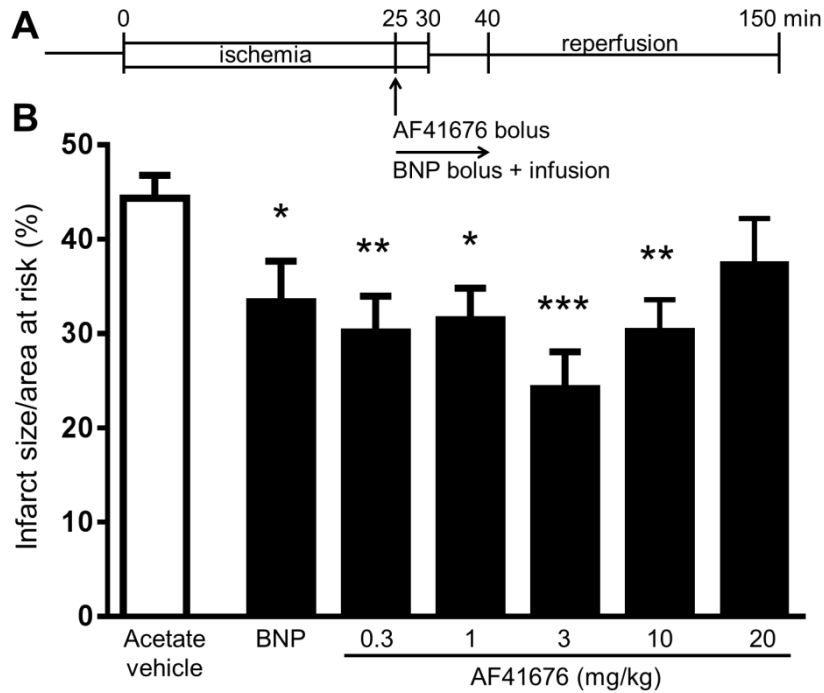


Figure 17. Panel A: Experimental protocol for Study 3. Panel B: Dose-response curve for AF41676 on infarct size in rats subjected to *in vivo* 30-min coronary occlusion followed by 120 min reperfusion. Data are expressed as means \pm SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 vs. vehicle control; n = 7-8

Exp. Group	n	AAR/LV (%)	mean arterial blood pressure (mmHg)			heart rate (beats/min)			SR (%)
			BL	ISCH	REP	BL	ISCH	REP	
Vehicle (acetate)	8	33.0 \pm 3.4	118 \pm 9	116 \pm 11	100 \pm 9	418 \pm 14	427 \pm 16	402 \pm 10	100
BNP	8	34.9 \pm 2.4	103 \pm 9	73 \pm 6*	98 \pm 6	396 \pm 16	405 \pm 10	490 \pm 18*	89
AF41676 0.3 mg/kg	8	43.3 \pm 4.0	109 \pm 6	111 \pm 4	94 \pm 3	429 \pm 5	450 \pm 4	423 \pm 8	100
AF41676 1 mg/kg	8	36.7 \pm 4.0	114 \pm 9	104 \pm 6	80 \pm 8	427 \pm 8	433 \pm 11	410 \pm 9	100
AF41676 3 mg/kg	8	44.5 \pm 2.6	125 \pm 5	116 \pm 10	104 \pm 5	408 \pm 17	418 \pm 11	411 \pm 4	77
AF41676 10 mg/kg	8	34.1 \pm 3.5	122 \pm 5	75 \pm 8*	75 \pm 8*	443 \pm 6	393 \pm 17	413 \pm 17	90
AF41676 20 mg/kg	7	40.9 \pm 2.4	125 \pm 8	84 \pm 1*	83 \pm 9	413 \pm 11	361 \pm 26*	438 \pm 16	80

Table 8. Hemodynamic parameters, area at risk and survival ratio for study 3. Data are expressed as means \pm SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 vs. vehicle. BNP: B-type natriuretic peptide; AAR/LV: area at risk/left ventricle; BL: baseline; ISCH: 29th min of ischemia; REP: 120th min of reperfusion; SR: survival rate

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

AF43136 was administered in 10 and 30 mg/kg doses iv. as a bolus infusion for 5 min starting at the 25th min of ischemia at a final volume load of 6 mL/kg. AF43136 at 10 mg/kg dose reduced infarct size significantly by approx. 30% compared to the acetate vehicle control, however, infarct size data of 30 mg/kg AF43136 is equivocal due to the high mortality ratio and the consequent small sample size at the end of the experiments (n= 2; Fig. 18B, Table 9). The positive control CsA used in study 4 decreased infarct size significantly by approx. 23% compared to the saline control (Fig. 18B, Table 9). There was no difference in the area at risks among the groups (Table 9). AF43136 at 30 mg/kg decreased mean arterial blood pressure significantly during ischemia compared to the acetate vehicle, but it did not reduce blood pressure at 10 mg/kg (Table 9). In addition, AF43136 at 10 mg/kg increased mean arterial blood pressure at the end of 120 min reperfusion compared to the acetate vehicle (Table 9). AF43136 at 30 mg/kg reduced survival rate significantly compared to acetate vehicle (Fig. 18C).

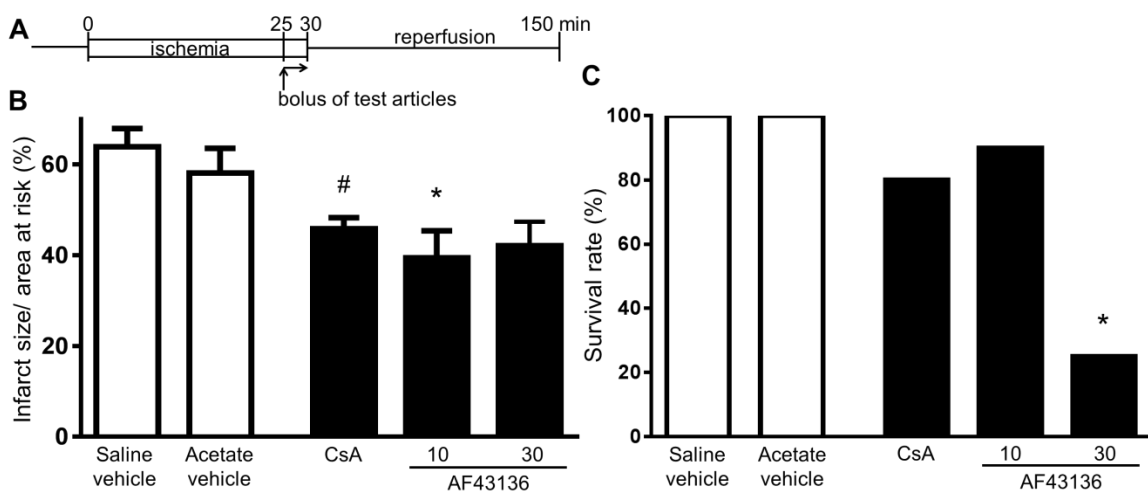


Figure 18. Panel A: Experimental protocol for Study 4. Panel B: The effect of 2 doses of AF43136 and cyclosporine A (CsA) on infarct size in rats subjected to *in vivo* 30-min coronary occlusion followed by 120 min reperfusion. Data are expressed as means \pm SEM, * $p < 0.05$ vs. vehicle control, # $p < 0.05$ vs. saline control; n= 7–8. Infarct size data of 30 mg/kg AF43136 contains only 2 samples because of high mortality rate (see in Panel C). Panel C: The effect of 2 doses of AF43136 and cyclosporine A (CsA) on survival. Data are expressed as means \pm SEM, * $p < 0.01$ vs. vehicle control.

Exp. Group	n	AAR/LV (%)	mean arterial blood pressure (mmHg)			heart rate (beats/min)		
			BL	ISCH	REP	BL	ISCH	REP
Vehicle (saline)	8	27.9±2.6	110±5	91±4	72±3	416±9	432±23	440±21
Vehicle (acetate)	8	30.6±3.1	106±4	90±6	67±4	433±15	427±20	415±17
CsA	7	34.5±2.5	99±6	82±6	76±5	445±12	425±22	434±12
AF43136 10 mg/kg	8	28.2±5.5	111±6	105±3	88±5*	394±11	413±12	426±13
AF43136 30 mg/kg	8	20.3±4.8 [§]	96±6	47±9*	54±18 [§]	438±15	383±19	437±15 [§]

Table 9. Hemodynamic parameters, area at risk for study 4. Data are expressed as means ± SEM, *p < 0.05 vs. vehicle; [§]n = 2 due to high mortality. CsA: cyclosporine A; AAR/LV: area at risk/left ventricle; BL: baseline; ISCH: 29th min of ischemia; REP: 120th min of reperfusion.

5. Discussion

The aims of the present experiments were to analyze further the ONOO⁻ – MMP and EPO receptor-mediated signaling pathways in ischemic heart diseases by investigating: i) the correlations of the markers of ONOO⁻ – MMP signaling with hyperlipidemia as well as cardiac function in patients with significant coronary stenosis and ii) EpoR-mediated cardioprotective effects of two novel selective non-erythropoietic EPO receptor ligand dimeric peptides (AF41676 and AF43136) in an *in vivo* rat model of AMI.

5.1 Summary of major findings of our studies

5.1.1 Characterization of ONOO⁻ – MMP signaling

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 nitrotyrosine, MMP-2, MMP-9 and the individual components of the lipid panel, respectively. Here we found, that serum nitrotyrosine correlated positively with serum total- and LDL- cholesterol as well as with triglyceride levels, while it correlated negatively with HDL cholesterol. MMP-2 activity showed a significant positive correlation with total- and LDL cholesterol as well, but did not correlate with triglyceride or HDL cholesterol. MMP-9 activity showed no correlation with any components of the lipid panel.

Besides the lipid panel, we used the left ventricular ejection fraction to examine the correlation between serum nitrotyrosine, MMPs and cardiac function. The nitrotyrosine level and the activity of MMP-9 correlated negatively with LVEF, respectively, while MMP-2 activity showed no correlation with this parameter.

In patients treated with statin, the nitrotyrosine level was significantly lower, than in statin-naive patients. There was no significant difference in MMP-2 and MMP-9 activity in the statin-treated population. In smoker patients, MMP-2 activity was significantly higher than in non-smoker patients. There was no significant difference in serum nitrotyrosine level and MMP-9 activity between smoker and non-smoker CAD patients.

Finally, we investigated the connection among serum nitrotyrosine levels, the activity of MMPs or the cardiac function and parameters of renal function. We found that serum nitrotyrosine correlates positively with serum creatinine, and MMP-9 activity correlates positively with serum urea nitrogen, while LVEF correlates negatively with serum creatinine.

5.1.2 Cardioprotection by two selective non-erythropoietic EPO receptor ligand dimeric peptides (AF41676 and AF43136)

During the preliminary *in vitro* experiments, the receptor binding ability and non-erythropoietic effect of AF41676 and AF43136 compounds were confirmed. AF43136 bound with high affinity and AF41676 bound with modest activity to the EPO receptor. Moreover, AF41676 and AF43136 did not stimulate erythropoiesis either *in vitro* or *in vivo*.

We confirmed that EPO was able to induce cardio-cytoprotection independently from its erythropoietic activity in cultured neonatal cardiomyocytes subjected to simulated ischemia/reperfusion injury, furthermore EPO as well as its well-known derivative, Dpa were shown to decrease 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.

5.2 Serum nitrotyrosine correlates with MMP activity

We found a positive correlation of serum nitrotyrosine with MMP-9 activity, but not with MMP-2 activity. This may suggest that peroxynitrite can activate MMPs in humans as well. Previously, a positive correlation between nitrotyrosine content and MMP-9 expression was found in instable atherosclerotic plaques derived from patients with ischemic heart disease, which supports our present results [67]. However, the reason why we have not found a significant correlation between peroxynitrite and MMP-2 activity is not known. A plausible explanation is that MMP-2 activation and release can be less sensitively detected in the serum than that of MMP-9. While MMP-9 is an abundant enzyme in different cells and tissues including macrophages and leukocytes, MMP-2 is an intracellular enzyme occurring mainly in contractile tissues that can be activated and released; for example, due to acute myocardial ischemia/reperfusion and infarction [18, 68-71]. However, our CAD patient population did not have severe ischemia at the time of tissue sampling.

5.3 Serum lipids correlate with nitrotyrosine and MMPs activity

Hyperlipidemia is a well-characterized risk factor for cardiovascular diseases, and it has been also demonstrated that it contributes to elevated nitro-oxidative stress in humans [13, 72]. We have previously shown in rats and in apoB-100 transgenic mice that hypercholesterolemia increases nitro-oxidative stress, which leads to myocardial functional

deterioration [73, 74]. Here, we have also examined the correlation pattern of serum lipids with nitrotyrosine in patients with CAD and shown that nitrotyrosine correlated positively with triglyceride, total and LDL cholesterol levels, and negatively with HDL cholesterol. In a previous study, an increased serum nitrotyrosine level was reported in hypercholesterolemic patients, wherein only LDL cholesterol showed significant positive correlation with nitrotyrosine [75]. In patients with CAD, a minor correlation was found between only serum protein bound nitrotyrosine and triglyceride [13]. A similar correlation was shown by others in patients with metabolic syndrome [76]. Here, we have found that serum MMP-2 activity correlated positively with total and LDL cholesterol levels. However, neither HDL cholesterol, nor triglyceride levels correlated with MMP-2 activity. According to our best knowledge, MMP-2 activity has been investigated by only one previous study in patients with CAD, in which a negative correlation between serum MMP-2 activity and HDL cholesterol was found; however, other correlations were not assessed [77]. In another study, plasma MMP-2 protein level was shown to correlate negatively with HDL in patients with CAD [78]. Noji et al. measured plasma MMP-2 in patients with CAD several days before coronary intervention and interestingly found a positive correlation between MMP-2 protein level and HDL cholesterol; however, they did not assess MMP-2 activity [79]. In our present study, serum MMP-9 activity did not show correlation with any of the serum lipids. Nilsson et al. found negative correlation between MMP-9 level and serum HDL in hypercholesterolemia patients [80]. Two independent studies in patients with CAD and acute coronary syndrome showed that MMP-9 protein level positively correlated with LDL cholesterol [78, 79]. Furthermore, correlation of plasma MMP-9 protein content with triglyceride level has been also shown [78]. However, in these studies, the authors did not measure MMP-9 activities, only MMP-9 protein immune reactivity.

5.4 Myocardial function correlates with serum nitrotyrosine and MMPs activity

Here, we have demonstrated first time in the literature in patients with CAD that cardiac function characterized by LVEF showed a significant negative correlation with serum nitrotyrosine. 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. This is partly in accordance with previous findings showing that MMP-2 and MMP-9 protein levels correlate with LVEF or left ventricular end diastolic volume index in cardiac patients with acute myocardial infarction or heart failure [81-83]. We have also

examined here the correlation of serum cholesterol and triglyceride levels with LVEF, however, we did not observe any significant correlation among these data. In contrast, Wang et al. described a positive correlation of LVEF with serum HDL cholesterol and a negative correlation with serum triglyceride in patients with angina [84].

5.5 Decreased serum nitrotyrosine in statin-treated patients

Most CAD patients are treated with various statins. Statins are well-characterized inhibitors of endogenous cholesterol synthesis, and it has been also described that they attenuate nitro-oxidative stress [85] as assessed by plasma nitrotyrosine level in patients with CAD [13]. Accordingly, we found here a reduced serum nitrotyrosine level in statin-related CAD patients as compared to statin naive ones. Here, we have found that MMP-2 and MMP-9 activities were not significantly different in statin-treated patients with CAD as compared to statin naives, although a tendency of decrease was found. In contrast, it has been shown in 48 patients with CAD undergone coronary artery bypass surgery that protein levels and activities of MMP-2 and MMP-9 measured both in plasma and in pericardial fluid were reduced after 2-month pravastatin therapy as compared to untreated patients [86]. Other authors have also reported a reduction in plasma MMP-2 and/or MMP-9 protein levels after statin treatment of cardiac patients; however, in these studies, only MMP protein levels were measured [81-83, 87].

5.6 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 compared to the non-smokers. Previously, significant increase in proMMP-9 and modest increase in proMMP-2 activity was found in rat aortic vascular smooth muscle cells treated with cigarette-smoke extracts [88]. In clinical studies, increased gene expression of MMP-2 and MMP-9 was found in venous [89] and aortic wall samples [90] of smoker patients. In human serum samples, the amount of proMMP-2 and proMMP-9 was higher in smokers, however the active forms of MMPs were not detectable in those samples [91]. Although cigarette smoke contains peroxynitrite and peroxynitrite-generating species suggesting that it contributes to the pathogenesis of atherosclerosis via increased nitro-oxidative stress [92, 93], we could not demonstrate any difference of serum ONOO⁻ level between smokers and non-smokers.

5.7 Renal function correlates with serum nitrotyrosine and MMPs activity

It has been previously described that renal dysfunction may develop and contribute to mortality in patients with myocardial infarction [94]. It is also well known that ischemic coronary disease is a leading cause of death in uremic patients [95]. 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 nitrotyrosine as well as between urea nitrogen and MMP-9 activity in patients with CAD. These results suggest that both increased nitrotyrosine level and MMP-9 activity may indicate an incipient renal dysfunction before myocardial infarction even when the patients have creatinine and/or urea nitrogen levels near or within the normal range.

5.8 Cardioprotective effects of EPO

In the present study, we demonstrated that EPO, independently from its erythropoietic activity, protected cultured neonatal cardiomyocytes against simulated ischemia/reperfusion. Our findings confirm previous studies that EPO has cytoprotective- [96] and anti-apoptotic effects on cardiomyocytes [97]. In *in vivo* coronary occlusion/reperfusion experiments, we found that iv., but not ip. administration of the same dose of EPO before the onset of reperfusion decreased infarct size in rats, although it has been previously demonstrated that EPO reduces infarct size by both ip. [97, 98] or iv. [99] administration. This discrepancy could be due to pharmacokinetic differences or different duration of treatments [100].

5.9 Dose-response effect of Dpa in AMI

Here, we also reported first time in the literature about the dose-response relationship of Dpa administered before the onset of reperfusion. Although, the infarct size-limiting dose of Dpa administered before reperfusion varies in a wide range in the literature (from 2.5 [101] to 30 $\mu\text{g}/\text{kg}$ [102]), our results point out that 5 $\mu\text{g}/\text{kg}$ dose of Dpa is sufficient to reduce infarct size in a rat model of AMI.

5.10 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, reduce infarct size significantly when administered before reperfusion in an *in vivo* rat model of AMI. AF41676 was effective in reducing infarct size at a wide dose-range (0.3–10 mg/kg). Although, high doses (10 and 20 mg/kg) of AF41676 decreased mean arterial blood pressure, they did not influence survival, and we did not register any severe side effects during the experiments. In contrast, AF43136 reduced infarct size, but it was lethal in higher doses due to a rapid decline in mean arterial blood pressure during its administration. As analogs of the peptide portion of peginesatide, AF41676 and AF43136 were initially prepared as an attempt to define non-erythropoietic agents. These analogs 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, preserve the tissue protective effects of EPO, such as infarct size reduction, prevention of cardiac hypertrophy and improvement of cardiac function [35,48-51], without any erythropoietic safety problems [29]. However, these EPO analogues are unfavorable due to their potential for high antigenicity, inadequate long-term stability and relatively high costs of production [34, 52]. Therefore, previously, a small EPO analogue, pyroglutamate helix B surface peptide (pHBSP or ARA 290) consisting of 11 amino acids of the beta-chain of EPO, was generated to substitute EPO or its large peptide analogues to achieve cardioprotection without inducing erythropoiesis [103]. PHBSP is a small fragment of EPO, and one would assume that when excised out of the cytokine, it would only randomly adopt the appropriate conformation and thus be a very poor binder to the receptor. So far, only one study has demonstrated that pHBSP reduces infarct size in acute coronary ligation in rats [104]. 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. The two AF peptides are structurally related to the peptide portion of peginesatide and therefore expected to bind EpoR tightly. These features support our findings, that non-erythropoietic EPO analogue dimeric peptides may be promising cardioprotective agents against acute myocardial infarction.

5.11 Study limitations

In the present human study the number of the enrolled patients was relatively low. However, bootstrap analysis of the correlation coefficients was performed, which is a widely accepted statistical method of making robust estimates of the coefficients in case of small sample sizes. The correlations we found were relatively weak, but statistically significant. This may show that there might be several other players in the interactions between the parameters we correlated in the present study.

Direct translational conclusion to human cardioprotection cannot be drawn from the preclinical results of EPO analogues. However, modulation of EPO receptor stimulation by novel receptor ligands should be a target of continued investigations as a promising cardioprotective strategy.

6. Conclusion

6.1 Novel findings

Here, we investigated the correlations of the ONOO⁻ – MMP pathway and analyzed EpoR-mediated cardioprotective signaling in ischemic heart disease.

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.

Here, we have shown 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. This is the first demonstration that these EPO receptor ligand peptides are cardioprotective in a rat model of AMI, and their effects are comparable with that of EPO and Dpa.

6.2 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 nitrooxidative stress and MMP activities may provide therapeutic benefits in this patient population. Thus, to study markers of peroxynitrite–MMP pathway in large prospective studies may reveal their diagnostic avails.

Moreover, non-erythropoietic EPO receptor ligands may be potential cardioprotective agents in acute myocardial infarction. Further translational studies will be needed to ensure the safety and efficacy of these agents as potential alternative clinical treatments.

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Appendix**I.**

Serum lipids and cardiac function correlate with nitrotyrosine and MMP activity in coronary artery disease patients

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ABSTRACT

Aims Peroxynitrite-matrix metalloproteinase (MMP) signalling has been shown to contribute to myocardial ischaemia/reperfusion injury and heart failure and to be influenced by hyperlipidaemia in preclinical models. Therefore, here we investigated the correlation between the markers of peroxynitrite–MMP signalling and hyperlipidaemia in patients with significant coronary stenosis.

Methods Five minutes before percutaneous coronary intervention (PCI), arterial blood samples were collected from 36 consecutive patients with coronary artery disease (CAD) selected for elective PCI.

Results Serum nitrotyrosine positively correlated with MMP-9 activity ($r = 0.54$, $P = 0.01$), but not with MMP-2 activity. Nitrotyrosine positively correlated with total ($r = 0.58$; $P < 0.01$) and LDL cholesterol ($r = 0.55$; $P < 0.01$), serum triglyceride ($r = 0.47$; $P < 0.05$), and creatinine ($r = 0.42$; $P < 0.05$) and negatively correlated with HDL cholesterol ($r = -0.46$; $P < 0.05$) and with left ventricular ejection fraction (LVEF; $r = -0.55$; $P < 0.05$), respectively. MMP-2 activity correlated positively with total ($r = 0.55$; $P < 0.05$) and LDL cholesterol ($r = 0.45$; $P < 0.05$). In statin-treated patients, a significantly reduced serum nitrotyrosine was found as compared to statin naives; however, MMP activities and serum cholesterol levels were not different. MMP-9 activity correlated with urea nitrogen ($r = 0.42$; $P < 0.05$) and LVEF ($r = -0.73$; $P < 0.01$). Serum creatinine correlated negatively with LVEF ($r = -0.50$, $P < 0.01$).

Conclusions This is the first demonstration that (i) serum nitrotyrosine correlates with MMP-9 activity, (ii) lipid parameters correlate with nitrotyrosine and MMP-2 activity, (iii) myocardial function correlates with creatinine, nitrotyrosine and MMP-9 activity, and (iv) creatinine correlates with nitrotyrosine and urea nitrogen with MMP-9 activity in patients with CAD. Studying the biomarkers of peroxynitrite–MMP pathway in large prospective trials may reveal their diagnostic avails.

Keywords Cardiac function, coronary artery disease, lipids, matrix metalloproteinase, nitro-oxidative stress, nitrotyrosine.

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Introduction

Matrix metalloproteinases (MMPs) are major targets of drug and diagnostic development in several pathologies including cardiovascular diseases [1,2]. The most abundant MMPs in the human myocardium are MMP-2 and MMP-9, which have been shown to play a crucial role in myocardial ischaemia/reperfu-

sion injury in several animal models [3]. Furthermore, it has been shown that serum MMP-2 predicts infarct size and ventricular dysfunction in ST-elevation myocardial infarction patients [4]. The activation of MMP-2 and MMP-9 may occur not only by proteolysis but also due to nitro-oxidative stress via post-translational modification of pro-MMPs and the formation of transcriptional complexes [5,6].

Nitro-oxidative stress plays a pivotal role in the patho-mechanism of several cardiovascular diseases including ischaemic heart disease [7]. The main effector of nitro-oxidative stress is peroxynitrite, which may activate several enzymes including MMPs via S-nitrosylation/S-glutathiolation of their tyrosine and/or cysteine residues [7,8]. Furthermore, peroxynitrite nitrates free tyrosine residues to form nitrotyrosine, which is a widely used marker for peroxynitrite generation [9].

The contribution of increased nitrotyrosine formation to the development of atherosclerosis and thus to coronary artery disease (CAD) has been described in patients with hypercholesterolaemia-combined CAD [10]. Hyperlipidaemia is well-characterized risk factor for cardiovascular diseases at least in part due to its nitro-oxidative stress-enhancing effect [11,12] and deterioration of cardioprotective mechanisms [13,14]. Nevertheless, the correlation between serum nitrotyrosine and lipid levels is not consistent in the literature. Moreover, correlations between serum nitrotyrosine and MMP-2 and MMP-9 activities are still unknown in patients with CAD.

Therefore, we have conducted a pilot clinical study to determine the correlation between serum nitrotyrosine and MMP-2 and MMP-9 activities in patients with single-vessel CAD subjected to elective percutaneous coronary intervention. Furthermore, we have investigated 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. To investigate whether serum nitrotyrosine or MMP-2 and MMP-9 activities may correlate with cardiac function, we determined left ventricular ejection fractions (LVEF). Finally, we have examined whether there is a difference in serum MMP-2 and MMP-9 activities in statin-naive and statin-treated patients with CAD.

Materials and methods

Patients and blood sampling

The study was approved by the Ethics Committee of the University of Szeged. A written informed consent was obtained from all patients enrolled in the study. The investigations were carried out in consecutive patients with single-vessel coronary disease elected for percutaneous coronary intervention (PCI). Blood samples were collected from patients 5 min before the PCI.

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 $\geq 70\%$ diameter stenosis by visual assessment of the coronary angiogram.

Exclusion criteria were as follows:

- 1 Angina pectoris or other signs of myocardial ischaemia within 1 week before intervention.
- 2 Lown 3–4 ventricular arrhythmia before the intervention.
- 3 Severe left ventricular dysfunction ($<35\%$ LVEF or New York Heart Association functional circulatory stages III–IV).
- 4 Treatment with ATP-sensitive potassium channel inhibitors.
- 5 History of myocardial infarction or baseline ST-segment abnormalities.
- 6 Serum electrolytes out of the physiologic range.
- 7 Serum creatinine level $>150 \mu\text{mol/L}$.
- 8 Hypothyreosis.
- 9 Angiographically visible collateral vessels interfering with the treated coronary artery.

According to inclusion and exclusion criteria, 36 patients were enrolled into this study.

PCI was performed using the standard femoral approach in all patients after premedication with 100 mg of aspirin and 75 mg of clopidogrel once a day for 2 days prior to PCI. Serum levels of total, LDL and HDL cholesterol, triglyceride, creatinine and urea nitrogen were quantitated by enzymatic methods on a Roche Hitachi Modular P800 automated analyzers (Roche Diagnostics, Indianapolis, IN, USA). Additional blood samples were collected from patients 5 min before the percutaneous coronary intervention from femoral artery. Blood samples were centrifuged at 1000 g for 15 min, at 4 °C, and then, serum samples were collected into polypropylene microcentrifuge tubes and stored at $-20 \text{ }^\circ\text{C}$ until the performance of nitrotyrosine and MMP-2, MMP-9 activity measurements.

Biochemical analyses

To measure circulatory MMP-2 and MMP-9 activities, gelatin zymography was performed from serum samples (for a representative zymogram see Fig. 1). Gelatinolytic activities of MMPs were examined as previously described [15]. Briefly, 8% polyacrylamide gels were copolymerized with gelatin (2 mg/mL, type A from porcine skin; Sigma-Aldrich, Budapest, Hungary), and 40 μg of protein per lane was loaded. An internal standard (American Type Culture Collection, Manassas, VA, USA) was loaded into each gel to normalize activities between gels. After electrophoresis (150 V, 1.5 h), gels were washed with 2.5% Triton X-100 for 3×15 min and incubated for 20 h at 37 °C in incubation buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl_2 and 0.05% NaN_3 , pH 7.4). Gels were then stained with 0.05% Coomassie brilliant blue (G-250; Sigma-Aldrich) in a mixture of methanol-acetic acid-water [2.5:1:6.5 (vol/vol)] and destained in aqueous 4% methanol-8% acetic acid (vol/vol). For positive controls, gelatinase zymography standard containing human MMP-2 and MMP-9 (Cat. No.: CC73, Chemicon Europe Ltd., South-

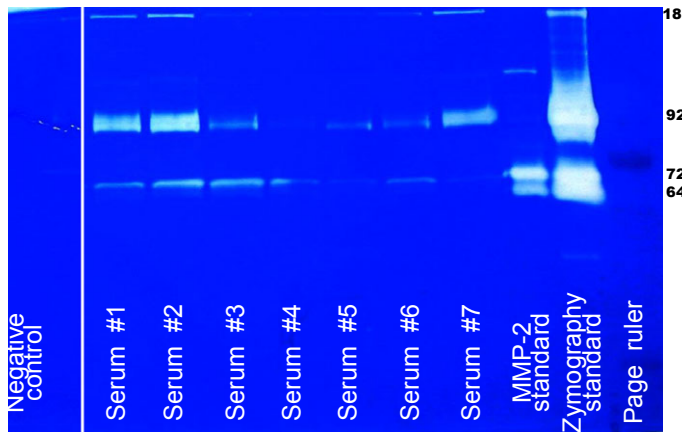


Figure 1 Representative gelatin zymogram from serum samples of patients with CAD.

ampton, UK) and MMP-2 standard (Cat. No.: CC071; Chemicon Europe Ltd.) was used. For negative control, lanes containing serum samples were cut off after renaturation of the gel and were separately incubated for 20 h at 37 °C in development buffer in the presence of the calcium chelator EGTA (ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; 10 mM). Gelatinolytic activities were detected as transparent bands against the dark-blue background. Band intensities were quantified, expressed as the ratio to the internal standard and presented in arbitrary units.

To estimate systemic peroxynitrite formation, we have measured free nitrotyrosine by ELISA in serum samples as previously described [16,17]. Briefly, serum samples were deproteinized by addition of four volumes of ice-cold ethanol. After centrifugation, supernatants were separated and evaporated under nitrogen flow, and then, they were dissolved in phosphate buffer. Deproteinized sera were incubated overnight with antinitrotyrosine 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 (GE Healthcare, Milwaukee, WI, USA), and data were digitally recorded. Images were acquired using a 3.5-MHz transducer. Images and cines were obtained in standard apical and parasternal two- and four-chamber views. To assess LVEF, calculations were performed according to recommendations for chamber

quantification in consensus. LVEF was calculated using the modified Simpson's method.

Statistical analysis

Data are expressed as mean \pm SEM. Univariate correlations between lipid parameters and MMP activities as well as nitrotyrosine were analysed using the Pearson correlation coefficient. Because of the relatively small sample sizes in the current pilot study, bootstrapped confidence intervals were calculated, using 1000 samples with replacement, to make robust estimates of the correlation coefficients as described elsewhere [18,19]. Linear regression analysis was used to estimate the prediction of the selected variables. The effects of statin treatment on variables were analysed by unpaired two-tailed Student's *t*-test.

Results

Baseline characteristics of the 36 patients with coronary artery disease are summarized in Table 1.

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

We have determined whether serum nitrotyrosine correlates with MMP-2 and MMP-9 activities in patients with CAD. MMP-9 activity correlated positively with nitrotyrosine (Fig. 2a; $r = 0.535$, $P = 0.010$); however, there was no significant correlation between serum nitrotyrosine and MMP-2 activity (Fig. 2b).

Correlation between serum lipids and nitrotyrosine or MMPs activity

In patients with CAD, serum total cholesterol (Fig. 3a), LDL cholesterol (Fig. 3b) and triglyceride (Fig. 3c) 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 (Fig. 3d; $r = -0.455$, $P = 0.033$) with serum nitrotyrosine. Serum MMP-2 activity correlated positively with serum total cholesterol (Fig. 3e) and LDL cholesterol (Fig. 3f) levels ($r = 0.550$, $P = 0.015$ and $r = 0.448$, $P = 0.028$, respectively); however, neither serum triglyceride (Fig. 3g) nor HDL cholesterol (Fig. 3h) showed a significant correlation with MMP-2 activity. None of the serum lipid parameters correlated with MMP-9 activity in patients with CAD (Table 2).

Correlation between myocardial function and serum nitrotyrosine or MMPs activity

Serum nitrotyrosine level (Fig. 4a) and MMP-9 activity (Fig. 4c) correlated negatively with LVEF ($r = -0.548$, $P = 0.010$ and $r = -0.732$, $P = 0.0002$, respectively); however, MMP-2 activity did not correlate with LVEF (Fig. 4b) in patients with CAD. We

Table 1 Baseline characteristics of the 36 patients with coronary artery disease

Variables	Mean \pm SD or Occurrence (number of individuals)
Biographic and anamnestic variables	
Age (years)	67.4 \pm 10.5
Gender, m/f	19/17
Type 1 diabetes	0/36
Type 2 diabetes	5/36
Statin treatment	15/36
ACE inhibitor treatment	31/36
ARB treatment	1/36
Nitrate treatment	18/36
Hemodynamic variables	
Systolic blood pressure (mmHg)	141.7 \pm 11.1
Diastolic blood pressure (mmHg)	89.8 \pm 21.4
Heart rate (beats/min)	70.4 \pm 12.1
LVEF (%)	56.0 \pm 10.5
Coronary stenosis (%)	63.5 \pm 8.0
Occurrence of hypertension	29/36
Serum variables	
Total cholesterol (mmol/L)	4.6 \pm 0.9
LDL cholesterol (mmol/L)	2.5 \pm 0.8
HDL cholesterol (mmol/L)	1.3 \pm 0.4
Triglycerides (mmol/L)	1.8 \pm 0.8
3-nitrotyrosine (ng/mL)	17.4 \pm 11.3
Urea nitrogen (mmol/L)	5.9 \pm 2.4
Creatinine (μ mol/L)	86.3 \pm 27.5

did not find any correlation between serum lipid parameters and LVEF (data not shown).

Serum nitrotyrosine or MMPs activity in statin-treated patients

Fifteen of the 36 patients with CAD involved in this study were treated with different types of statins. In statin-treated patients, a significantly reduced serum nitrotyrosine level (Fig. 5a) was found (from 23.5 \pm 4.5 ng/mL to 13.6 \pm 5.1 ng/mL; $P < 0.05$) as compared to statin-naïve patients. MMP-2 and MMP-9 activities did not show any difference between statin-treated and statin-naïve patients (Fig. 5b,c).

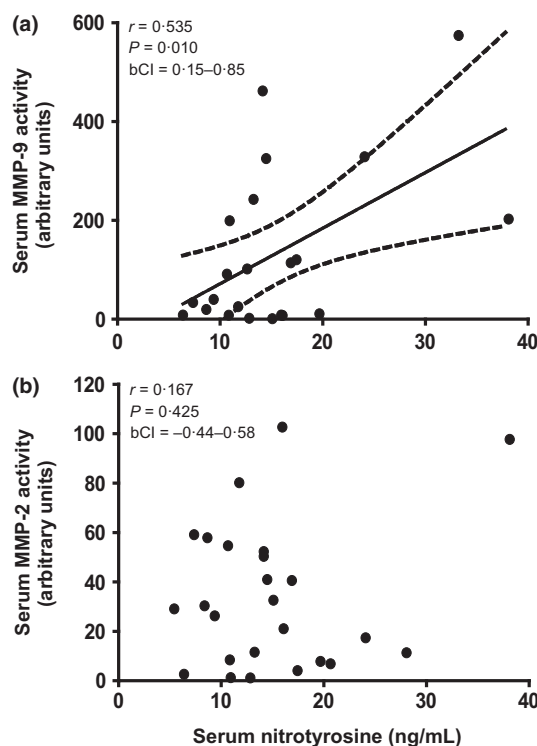


Figure 2 Correlation of serum nitrotyrosine content with MMP-2 and MMP-9 activities. Serum MMP-9 activity correlated positively with serum nitrotyrosine (a). Serum MMP-2 activity (b) did not correlate with serum nitrotyrosine. Continuous line: linear regression of data; dotted line: 95% confidence interval. r : Pearson's correlation coefficient, P : significance, bCI: bootstrapped confidence interval of correlation coefficient.

Correlation between renal function and serum nitrotyrosine or MMPs activity

In addition, we tested the correlation of renal function parameters with markers of peroxynitrite-MMP pathway and also with LVEF. Serum creatinine correlated positively with nitrotyrosine ($r = 0.422$, $P = 0.036$; Fig. 6a), but not with MMP-2 and MMP-9 activities (Table 3). Urea nitrogen level correlated positively with MMP-9 activity ($r = 0.423$, $P = 0.035$; Fig. 6b), but not with nitrotyrosine or MMP-2 activity (Table 3). Serum creatinine correlated negatively with LVEF ($r = -0.503$, $P = 0.009$; Fig. 6c); however, urea nitrogen did not correlate with LVEF (Table 3).

Discussion

In the present pilot study, 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

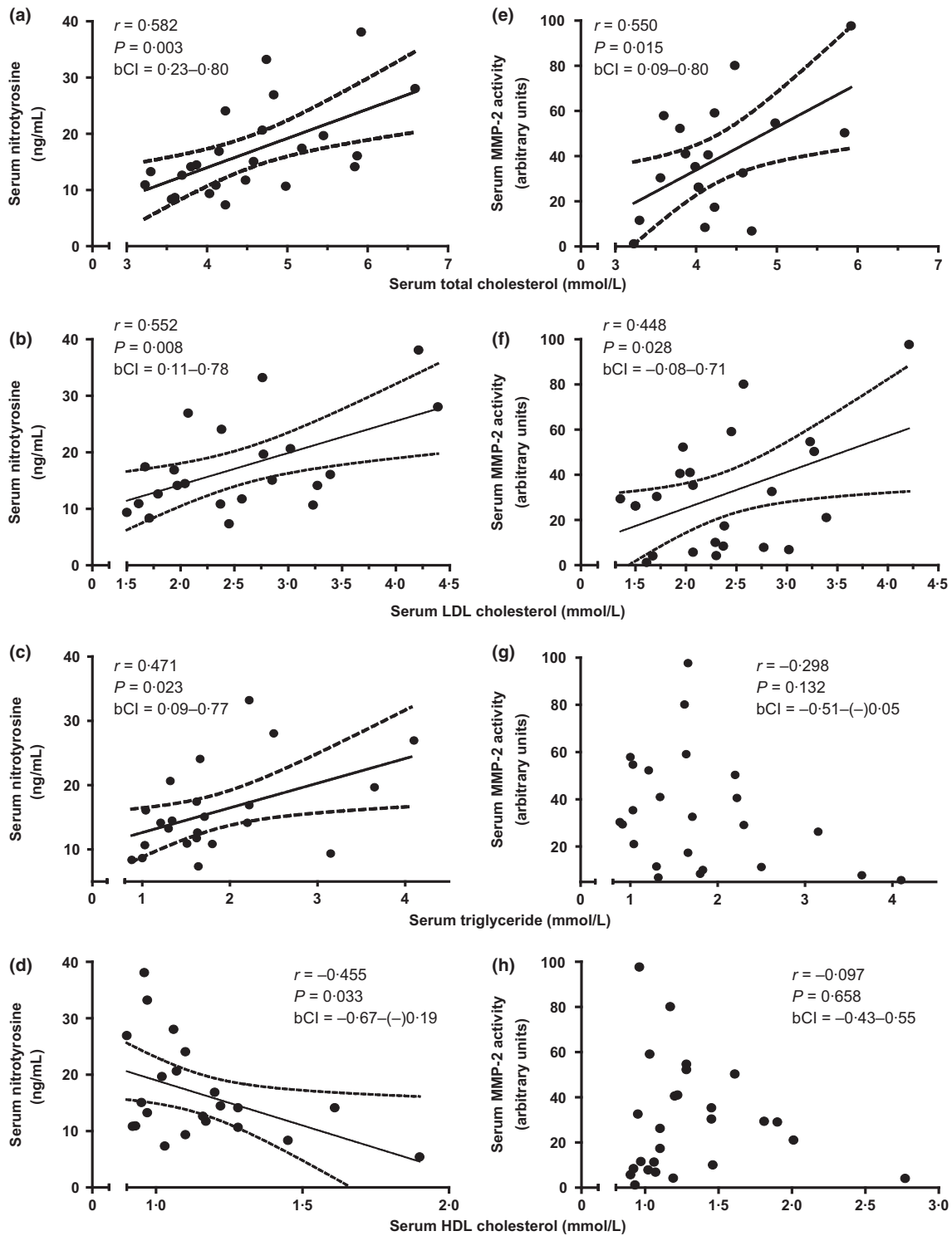


Figure 3 Correlation of serum nitrotyrosine and MMP-2 activities with serum lipids. Serum nitrotyrosine level correlated positively with total (a) and LDL cholesterol (b) as well as with triglyceride (c) and correlated negatively with HDL cholesterol (d). Serum MMP-2 activity correlated positively with serum total (e) and LDL cholesterol levels (f). Neither serum triglyceride (g) nor HDL cholesterol (h) showed a significant correlation with serum MMP-2 activity. Continuous line: linear regression of data; dotted line: 95% confidence interval. *r*: Pearson’s correlation coefficient, *P*: significance, bCI: bootstrapped confidence interval of correlation coefficient.

Table 2 Correlations between serum MMP-9 activity and lipid parameters

Variables		Pearson's correlation coefficient	Bootstrapped confidence intervals of correlation coefficient	P
MMP-9/	Total cholesterol	0.171	-0.27 to 0.49	0.436
MMP-9/	LDL cholesterol	0.020	-0.38 to 0.30	0.939
MMP-9/	HDL cholesterol	0.050	-0.31 to 0.59	0.840
MMP-9/	Triglyceride	0.024	-0.34 to 0.40	0.913

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.

Serum nitrotyrosine correlates with MMP activity

Here, we found a positive correlation of serum nitrotyrosine with MMP-9 activity, but not with MMP-2 activity. This suggests that peroxynitrite activates MMPs in humans as well. Previously, a positive correlation between nitrotyrosine content and MMP-9 expression was found in unstable atherosclerotic plaques derived from patients with ischaemic heart disease, which supports our present results [20]. However, the reason why we have not found a significant correlation between peroxynitrite and MMP-2 activity is not known. A plausible explanation is that MMP-2 activation and release can be less sensitively detected in the serum than that of MMP-9. While MMP-9 is an abundant enzyme in different cells and tissues including macrophages and leukocytes, MMP-2 is an intracellular enzyme occurring mainly in contractile tissues that can be activated and released; for example, due to acute myocardial ischaemia/reperfusion and infarction [8,21–24]. However, our CAD patient population did not have severe ischaemia at the time of tissue sampling.

Serum lipids correlate with nitrotyrosine and MMPs activity

Hyperlipidaemia is a well-characterized risk factor for cardiovascular diseases, and it has been also demonstrated that it contributes to elevated nitro-oxidative stress in humans [10,25]. We have previously shown in rats and in apoB-100 transgenic mice that hypercholesterolaemia increases nitro-oxidative stress, which leads to myocardial functional deterioration [26,27]. Here, we have also examined the correlation pattern of serum lipids with nitrotyrosine in patients with CAD and shown that nitrotyrosine correlated positively with triglyceride, total and LDL cholesterol levels, and negatively with HDL cholesterol. In a previous study [28], an increased serum

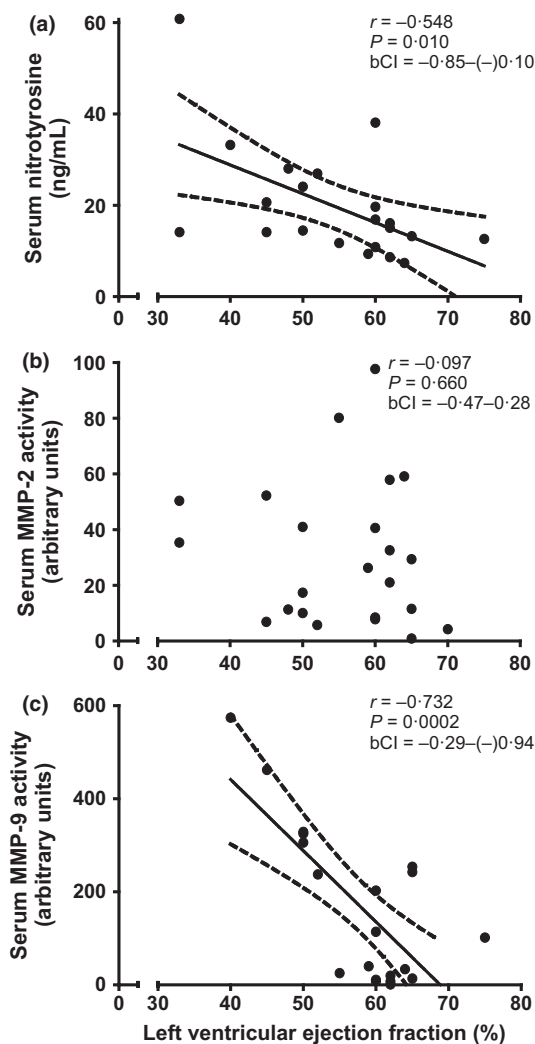


Figure 4 Correlation of left ventricular ejection fraction (LVEF) with serum nitrotyrosine, and serum MMP-2 and MMP-9 activities. Serum nitrotyrosine (a) and MMP-9 (c) activity correlated negatively with LVEF. Serum MMP-2 activity did not correlate with LVEF (b). Continuous line: linear regression of data; dotted line: 95% confidence interval. *r*: Pearson's correlation coefficient, *P*: significance, bCI: bootstrapped confidence interval of correlation coefficient.

nitrotyrosine level was reported in hypercholesterolaemic patients, wherein only LDL cholesterol showed significant positive correlation with nitrotyrosine. In patients with CAD, a minor correlation was found between only serum protein-bound nitrotyrosine and triglyceride [10]. A similar correlation was shown by others in patients with metabolic syndrome [29]. In our present study, we have measured serum-free nitrotyrosine, which seems to be a more sensitive marker for systemic peroxynitrite generation.

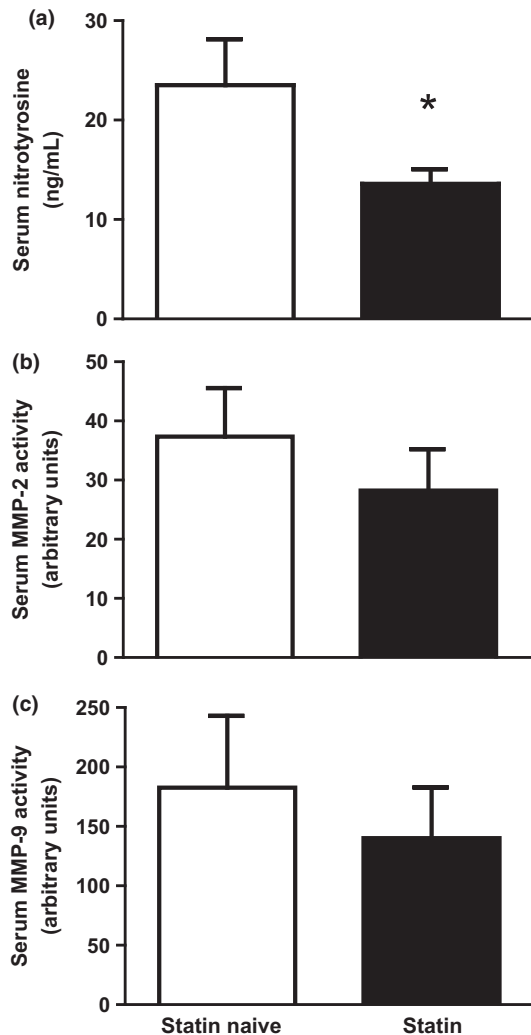


Figure 5 Serum nitrotyrosine levels (a) and serum MMP-2 (b) and MMP-9 (c) activities in statin-treated and statin-naive patients with CAD. Serum nitrotyrosine was significantly reduced in statin-treated patients with CAD as compared to statin-naive patients (* $P < 0.05$, data are shown as mean \pm SEM). MMP-2 and MMP-9 activities were not changed in statin-treated patients with CAD as compared to statin naive ones ($P = 0.409$ and $P = 0.564$, respectively).

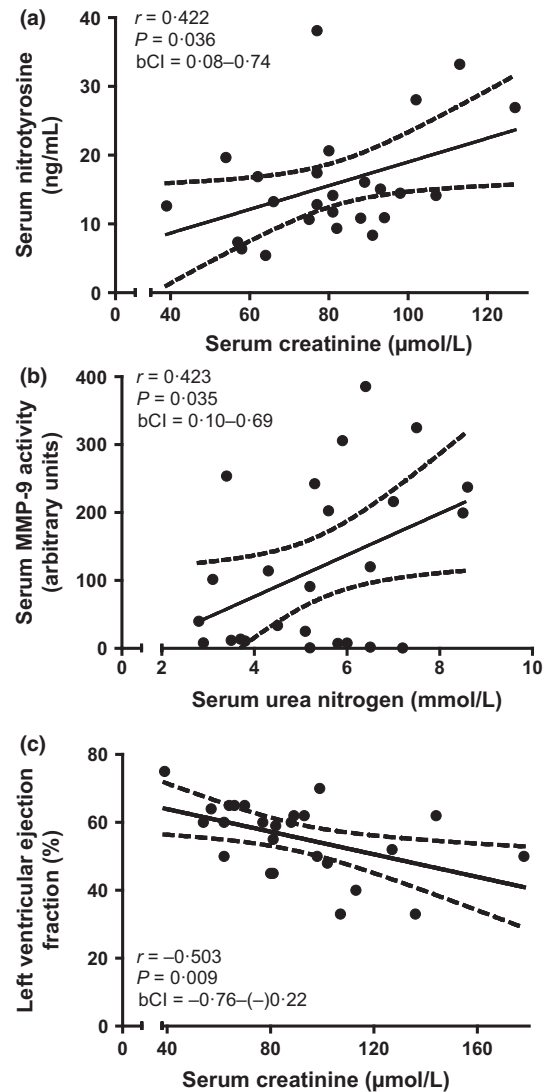


Figure 6 Correlation of renal function with serum nitrotyrosine, MMP activities and myocardial function. Serum nitrotyrosine correlated positively with serum creatinine (a). Serum MMP-9 activity correlated positively with urea nitrogen level (b). Continuous line: linear regression of data; dotted line: 95% confidence interval. r : Pearson's correlation coefficient, P : significance, bCI: bootstrapped confidence interval of correlation coefficient.

Here, we have found that serum MMP-2 activity correlated positively with total and LDL cholesterol levels. However, neither HDL cholesterol, nor triglyceride levels correlated with MMP-2 activity. According to our best knowledge, MMP-2 activity has been investigated by only one previous study in patients with CAD [30], in which a negative correlation between serum MMP-2 activity and HDL cholesterol was found; however, other correlations were not assessed. In another study [31], plasma MMP-2 protein level was shown

to correlate negatively with HDL cholesterol in patients with CAD. Noji *et al.* [32] measured plasma MMP-2 in patients with CAD several days before coronary intervention and interestingly found a positive correlation between MMP-2 protein level and HDL cholesterol; however, they did not assess MMP-2 activity. In our present study, serum MMP-9 activity did not show correlation with any of the serum

Table 3 Correlations between serum nitrotyrosine level, MMPs activities or LVEF, and renal function parameters

Variables		Pearson's correlation coefficient	Bootstrapped confidence intervals of correlation coefficient	P
MMP-2/	Creatinine	0.125	-0.20 to 0.45	0.504
MMP-9/	Creatinine	0.118	-0.30 to 0.48	0.550
NTyr/	BUN	0.105	-0.22 to 0.39	0.602
MMP-2/	BUN	0.071	-0.20 to 0.42	0.700
LVEF/	BUN	-0.250	-0.57 to 0.00	0.217

lipids. A similar finding was published previously in hypercholesterolaemic patients [33]. Two independent studies in patients with CAD and acute coronary syndrome showed that MMP-9 protein level positively correlated with LDL cholesterol [31,32]. Furthermore, correlation of plasma MMP-9 protein content with triglyceride level has been also shown [31]. However, in these studies, the authors did not measure MMP-9 activities, only MMP-9 protein immunoreactivity.

Myocardial function correlates with serum nitrotyrosine and MMPs activity

Here, we have demonstrated first time in the literature in patients with CAD that cardiac function characterized by LVEF showed a significant negative correlation with serum nitrotyrosine. This may suggest that the 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. This is partly in accordance with previous findings that both MMP-2 and MMP-9 protein levels correlate with LVEF or left ventricular end-diastolic volume index in cardiac patients with acute myocardial infarction or heart failure [34–36]. We have also examined here the correlation of serum cholesterol and triglyceride levels with LVEF; however, we did not observe any significant correlation among these data. In contrast, Wang *et al.* [37] described a positive correlation of LVEF with serum HDL cholesterol and a negative correlation with serum triglyceride in patients with angina.

Decreased serum nitrotyrosine in statin-treated patients

Most CAD patients are treated with various statins. Statins are well-characterized inhibitors of endogenous cholesterol syn-

thesis, and it has been also described that they attenuate nitro-oxidative stress [38] as assessed by plasma nitrotyrosine level in patients with CAD [10]. Accordingly, we found here a reduced serum nitrotyrosine level in statin-treated CAD patients as compared to statin naive ones.

Here, we have found that MMP-2 and MMP-9 activities were not significantly different in statin-treated patients with CAD as compared to statin naives, although a tendency of decrease was found. In contrast, It has been shown in 48 patients with CAD undergone coronary artery bypass surgery that protein levels and activities of MMP-2 and MMP-9 measured both in plasma and in pericardial fluid were reduced after 2-month pravastatin therapy as compared to untreated patients [39]. Other authors have also reported a reduction in plasma MMP-2 and/or MMP-9 protein levels after statin treatment of cardiac patients; however, in these studies, only MMP protein levels were measured [34–36,40].

Renal function correlates with serum nitrotyrosine and MMPs activity

It has been previously described that renal dysfunction may develop and contribute to mortality in patients with myocardial infarction [41]. It is also well known that ischaemic coronary disease is a leading cause of death in uraemic patients [42]. Although uraemic 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 nitrotyrosine as well as between urea nitrogen and MMP-9 activity in patients with CAD. These results suggest that both increased nitrotyrosine level and MMP-9 activity may have diagnostic values for the development of an incipient renal dysfunction after myocardial infarction even when the patients have creatinine and/or urea nitrogen levels near or within the normal range.

Limitations

The present manuscript provides data from a pilot study; therefore, the number of the enrolled patients is relatively low. However, bootstrap analysis of the correlation coefficients was performed, which is a widely accepted statistical method of making robust estimates of the coefficients in case of small sample sizes. The correlations we found were relatively weak, but statistically significant. This may show that there might be several other players in the interactions between the parameters we correlated in the present study. Indeed, MMP activity is determined by several confounding factors, such as level of the endogenous tissue inhibitors of MMPs (TIMPs), Zn²⁺ availability, phosphorylation status of

MMPs and redox state; for example, TIMPs may dissociate from MMPs during electrophoresis; therefore, TIMP-free or TIMP-bound MMPs in the serum cannot be assessed by zymography. Nevertheless, our results show that serum MMP-2 and MMP-9 activities as measured by zymography are valuable diagnostic markers.

Conclusions

This is the first demonstration that serum (i) nitrotyrosine correlates with MMP-9 activity, (ii) lipid parameters correlate with nitrotyrosine level and MMP-2 activity, (iii) myocardial function correlates with nitrotyrosine, MMP-9 activity and creatinine, and (iv) creatinine correlates with nitrotyrosine and urea nitrogen correlates with MMP-9 activity in patients with CAD. These findings suggest that nitrotyrosine and MMP-2 and MMP-9 activities may be of diagnostic values in cardiac and renal function of patients with CAD, and attenuation of nitrooxidative stress and MMP activities may provide therapeutic benefits in this patient population. Thus, to study markers of peroxynitrite–MMP pathway in large prospective studies may reveal their diagnostic avails.

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Conflict of interest

The authors declare that they have no conflict of interest.

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II.



Novel, selective EPO receptor ligands lacking erythropoietic activity reduce infarct size in acute myocardial infarction in rats



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ABSTRACT

Erythropoietin (EPO) has been shown to protect the heart against acute myocardial infarction in pre-clinical studies, however, EPO failed to reduce infarct size in clinical trials and showed significant safety problems. Here, we investigated cardioprotective effects of two selective non-erythropoietic EPO receptor ligand dimeric peptides (AF41676 and AF43136) lacking erythropoietic activity, EPO, and the prolonged half-life EPO analogue, darbeoetin in acute myocardial infarction (AMI) in rats. In a pilot study, EPO at 100 U/mL significantly decreased cell death compared to vehicle ($33.8 \pm 2.3\%$ vs. $40.3 \pm 1.5\%$, $p < 0.05$) in rat neonatal cardiomyocytes subjected to simulated ischemia/reperfusion. In further studies (studies 1–4), in vivo AMI was induced by 30 min coronary occlusion and 120 min reperfusion in male Wistar rats. Test compounds and positive controls for model validation (B-type natriuretic peptide, BNP or cyclosporine A, CsA) were administered iv. before the onset of reperfusion. Infarct size (IS) was measured by standard TTC staining. In study 1, 5000 U/kg EPO reduced infarct size significantly compared to vehicle ($45.3 \pm 4.8\%$ vs. $59.8 \pm 4.5\%$, $p < 0.05$). In study 2, darbeoetin showed a U-shaped dose-response curve with maximal infarct size-reducing effect at $5 \mu\text{g}/\text{kg}$ compared to the vehicle ($44.4 \pm 5.7\%$ vs. $65.9 \pm 2.7\%$, $p < 0.01$). In study 3, AF41676 showed a U-shaped dose-response curve, where $3 \text{ mg}/\text{kg}$ was the most effective dose compared to the vehicle ($24.1 \pm 3.9\%$ vs. $44.3 \pm 2.5\%$, $p < 0.001$). The positive control BNP significantly decreased infarct size in studies 1–3 by approximately 35%. In study 4, AF43136 at $10 \text{ mg}/\text{kg}$ decreased infarct size, similarly to the positive control CsA compared to the appropriate vehicle ($39.4 \pm 5.9\%$ vs. $58.1 \pm 5.4\%$ and $45.9 \pm 2.4\%$ vs. $63.8 \pm 4.1\%$, $p < 0.05$, respectively). This is the first demonstration that selective, non-erythropoietic EPO receptor ligand dimeric peptides AF41676 and

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AF43136 administered before reperfusion are able to reduce infarct size in a rat model of AMI. Therefore, non-erythropoietic EPO receptor peptide ligands may be promising cardioprotective agents.

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1. Introduction

Erythropoietin (EPO) and its analogue with prolonged half-life, darbepoetin alpha (Dpa) are the main components of anemia treatment in chronic kidney disease and in cancer patients with anemia [1]. However, EPO is a pleiotropic cytokine, as EPO receptors (EpoR) are expressed in several different tissues (e.g. bone marrow, skeletal muscle, kidney, pancreas, heart, etc. [for review see [2]]).

It is well known that EPO receptors are present in rat cardiomyocytes [3], and also in human atrial and ventricular cardiomyocytes, as well as in cardiac endothelial cells [4]. Stress (e.g. hypoxia) induces EPO receptors and beta-common receptors (also known as CD131, which provides increased ligand-binding affinity to the receptor complex) to form the heterocomplex, EpoR- β cR [5–7]. Then via this complex, EPO is able to reduce apoptosis and inflammation, and induce tissue repair [6]. Therefore, it is highly plausible that EPO has the capacity to protect the heart against acute or chronic myocardial ischemia [2]. EPO and Dpa have been shown to decrease myocardial infarct size, prevent myocyte loss and hypertrophy, improve cardiac contractility and ejection fraction, and increase capillary density after transient or permanent coronary artery occlusion in different animal models [8,9].

Based on the results of preclinical experiments, EPO and Dpa were evaluated for their effectiveness in the treatment of acute myocardial infarction (AMI) or heart failure in patients. Although, EPO improved left ventricular ejection fraction and NYHA functional class in patients with anemia and heart failure, Dpa had no effect on myocardial function in this population [10]. In most clinical trials, EPO or Dpa administered before or after percutaneous coronary intervention have failed to reduce infarct size in ST Segment Elevation Myocardial Infarction (STEMI) patients [2,11]. This lack of clinical effectiveness likely arises from the immediate hematopoietic activity of EPO analogues. Hematopoiesis is activated by association with homodimeric (i.e. erythropoietic) receptors before erythropoietic EPO analogues would exert their tissue-protective effects via heterocomplex receptors. Thereby, due to enhanced erythropoiesis, high-dose or long-term EPO or Dpa treatments may lead to thrombotic and/or thromboembolic adverse events (e.g. stent thrombosis, stroke, etc.), [2], and increased mortality in ischemic heart disease patients [12–14]. Another synthetic erythropoiesis-stimulating agent, peginesatide is a dimeric peptide without sequence homology to EPO. Peginesatide binds selectively and with high affinity to the EPO receptor [15]. It was indicated for the treatment of anemia until voluntarily withdrawal from the market shortly after its commercialization in 2013 due to 49 cases of anaphylaxis, including 7 fatalities [16]. Subsequently, an evaluation of the multi-use vials utilized, revealed large concentrations of subvisible particulates linked to hypersensitivity.

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- β cR heteroreceptors, but not to the “classical” homodimeric form of EpoR, thereby maintaining their protective effects without stimulating hematopoiesis [2]. In preclinical studies, CEPO or asialoEpo protect different tissues including myocardium by reducing apoptosis or inducing cardiac neovascularization with

less adverse effects on procoagulant/haemopoietic activity [17–20]. However, because the structure of asialoEpo and CEPO mimics the three dimensional structure of EPO, clinical evaluation of potential immunogenicity and long-term stability is warranted [6,21]. In contrast, a small peptide ligand may attenuate the aforementioned disadvantages (i.e. via reduction in the number of possible epitopes) and may serve as a suitable alternative compound to evoke cardioprotection by coupling to EPO receptors.

Based on the promising results in cardioprotection by using non-erythropoietic EPO analogues, the aim of the present study was to test the effects of two novel, selective EPO receptor ligand dimeric peptides, AF41676 and AF43136, which are structurally related to the peptide portion of peginesatide, but lacking erythropoietic activity, on infarct size in a rat model of AMI.

2. Materials and methods

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

2.1. Animals and test compounds

Male Wistar rats (225–370 g) purchased from Toxi-Coop Toxicological Research Center Zrt., Dunakeszi, Hungary and Charles-River (Budapest, Hungary) were housed in a room maintained at 12 h light-dark cycles and at a constant temperature of 22 ± 2 °C. The animals had free access to standard laboratory chow and drinking water.

Test compounds (including acetate vehicle) were synthesized and provided by Affymax except for Erythropoietin (Procrit[®], Amgen, Inc., Thousand Oaks, CA), Darbepoetin alpha (Aranesp[®], Amgen Europe b.v., ZK Breda, The Netherlands), B-type natriuretic peptide (BNP; Sigma-Aldrich, Budapest, Hungary) and Cyclosporine A (CsA; Sandimmun Neoral[®], Novartis Hungária, Budapest, Hungary). Acetate vehicle consisted of 40 mM acetate and 4.3% sorbitol, pH 5.0.

2.2. Structure of EPO receptor ligands

The two selective EPO receptor ligand dimeric peptides, AF41676 and AF43136, were synthesized as their acetate salts using a combination of solid phase peptide techniques to prepare the individual peptide chains followed by solution phase conjugation to the bifunctional linkers. The peptides were purified by reverse-phase preparative chromatography and characterized by mass spectrometry. Their erythropoietic activity has been significantly reduced by key amino acid substitutions. The amino acid sequence of AF41676 is (AcYACHYGPIITNaIVCQPPK)₂-IDA (SS: C3-C12) and the amino acid sequence of AF43136 is (AcLYLCRYGRVHNaIECQPLRK)₂-DIG (SS:C4-C13). 1Nal is 1-naphthylalanine. Peptide dimer linkers are iminodiacetic acid (IDA) and 1,1'-[oxybis(1-oxo-2,1-ethanediyloxy)]bis-2,5-pyrrolidinedione (DIG), and the paren-

Table 1
Study design.

Model	Protocol	n/group	Experimental groups			Endpoints
			negative controls	positive controls	test compounds	
In vitro SI/R	150 min SI/120 min R	7–8	acetate vehicle	BNP 10 ⁻⁸ M	EPO 1 U/mL 10 U/mL 100 U/mL 500 U/mL	cell viability
In vivo rat myocardial infarction	30 min ischemia/120 min reperfusion	9–11	acetate vehicle	BNP 10 nmol/kg bolus + 2 nmol/kg/min	EPO 5000 U/kg ip. iv.	infarct size, survival, hemodynamics
		8–11	acetate vehicle	BNP 10 nmol/kg bolus + 2 nmol/kg/min	Dpa 2.5 ug/kg 5 ug/kg 15 ug/kg 25 ug/kg	infarct size, survival, hemodynamics
		8–13	acetate vehicle	BNP 10 nmol/kg bolus + 2 nmol/kg/min	AF41676 0.3 mg/kg 1 mg/kg 3 mg/kg 10 mg/kg 30 mg/kg	infarct size, survival, hemodynamics
		8–10	saline, acetate vehicle	CsA 10 mg/kg	AF43136 10 mg/kg 30 mg/kg	infarct size, survival, hemodynamics

n: sample size/group; SI/R: simulated ischemia/simulated reperfusion; BNP: B-type natriuretic peptide; EPO: Erythropoietin; Dpa: darbepoetin alpha; CsA: cyclosporine A; iv: intravenous; ip: intraperitoneal.

theses indicate two cyclic peptide chains linked to their respective linkers via the epsilon amine on the side chain of lysine.

2.3. EPO receptor binding assay

The competition binding assay was carried out by incubating 100 pg of HuEPOr-Fc (R&D Systems) with serial dilutions of competitor and 20,000 cpm ¹²⁵I EPO, followed by incubation, addition of Protein G beads, washing, and counting, using conditions based on a method for the detection of anti-EPO antibodies [22]. The specificity of the binding properties of EPO, Dpa, AF41676, and AF43136 were evaluated by MDS Pharma Services (Taipei, Taiwan) in a screen of 66 diverse receptors, including both homo- and heterodimeric EPO receptors (LeadProfilingScreen).

2.4. Erythroid (BFU-E) colony assay

Erythroid colony assay was performed as described previously [23]. Briefly, human CD34⁺ cells (Cambrex, Walkersville, MD, USA) were resuspended at 15,000 cells/mL in IMDM with 10% FBS. EPO, Dpa, AF41676 and AF43136 were added to a methylcellulose-based colony assay media (MethoCult GF H4534, StemCell Technologies, Vancouver, BC, Canada) supplemented with rhSCF, rhGM-CSF, and rhIL-3. Cells were added to the methylcellulose and plated with subsequent incubation at 37 °C, in a 5% CO₂ incubator for 12–14 days. Colonies containing over 50 cells were counted by phase-contrast microscopy.

2.5. Investigation of erythropoietic activity

To evaluate erythropoiesis EPO, Dpa, AF41676 and AF43136 were examined for their potential to induce reticulocytosis in normocytic male Sprague-Dawley rats (n = 4). We administered a single intravenous (iv.) dose of EPO at 5000 U/kg, Dpa at 10 μg/kg, AF41676 at 10 mg/kg, AF43136 at 10 mg/kg and acetate vehicle (5 mL/kg) via the tail vein, respectively. Blood (0.5 mL) was collected for determination of reticulocyte number by retro-orbital puncture of methoxyflurane-anesthetized rats into EDTA-containing tubes at baseline (pre-dose) and 5 days following administration as described previously [23].

2.6. Study design

2.6.1. Investigation of direct cardio-cytoprotective effect by EPO

To determine the direct, non-erythropoietic effect of EPO on cardiomyocytes, 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 (Table 1).

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

In a further series of in vivo studies (studies 1–4, see below), animals were randomly assigned to the different experimental groups with a total number of 8–13 animals per group. Each test compound was administered before the onset of reperfusion (Table 1). During the individual studies, all-cause mortality was registered to compare the survival among the controls and the drug-treated groups.

Study 1: We have administered EPO at 5000 U/kg [used successfully for cardioprotection several times in the literature; for review see Ref. [24]] bolus injection 5 min before the onset of reperfusion intraperitoneally or intravenously 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. This dose was derived from a previous publication where 10 nM BNP was cardioprotective *ex vivo* [25], and due to its short half-life [26] a maintenance infusion (2 nmol/kg/min) was applied in our in vivo model. The vehicle was used as the negative control and comprised 40 mM acetate with 4.3% sorbitol, pH 5.0 and was administered iv. 5 min before the onset of reperfusion.

Study 2: To investigate the dose dependence of the EPO analogue, darbepoetin, Dpa was administered at the 15th min of ischemia, in 2.5, 5, 15 and 25 μg/kg boluses administered iv. The positive control BNP and the negative control acetate vehicle were administered identically to Study 1. The reason for Dpa administration 10 min earlier than EPO (i.e. at the 15th min of ischemia) was its prolonged half-life.

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 accordingly to Study 1.

Study 4: In an exploratory study, we tested AF43136, a slightly modified version of AF41676 (see above for structural differences) in two doses in order to monitor potential hypotensive effects, which have occurred previously with EPO analogues. 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 at a final volume load of 6 mL/kg due to the high concentration being administered (30 mg/kg). Saline and the positive control CsA (10 mg/kg, diluted in saline) were administered intravenously as a bolus infusion for 5 min starting at the 25th min of ischemia at a final volume load of 1 mL/kg. Acetate vehicle was administered same as test compounds at a final volume load of 6 mL/kg.

2.7. Simulated ischemia/reperfusion in cultured neonatal cardiac myocytes

Neonatal cardiomyocyte isolation and culture method was described previously in detail [27,28]. In brief, newborn Wistar rats were disinfected with 70% ethanol and sacrificed by cervical dislocation. Hearts were excised and transferred to cold, sterile phosphate buffered saline pH 7.2 (PBS). Ventricles were separated from atria, minced gently with sterile forceps and collected in 0.25% trypsin solution (Invitrogen, Life Technologies Hungary Ltd., Budapest, Hungary). At the end of digestion, cell suspension was centrifuged for 15 min at 2000 rpm at 4°C, supernatant was removed gently and the pellet was re-suspended in complete growth medium and plated onto 6-well plates to eliminate fibroblasts (pre-plating). Supernatants were collected in fresh Falcon tubes and cells were counted manually in a Burker chamber. Then cells of the supernatant were re-plated into 24-well fresh plates: 10^5 cells/well supplemented with 1 mL growth medium and incubated under normoxic conditions (37°C, in 95% air and 5% CO₂ gas mixture) in a standard CO₂ incubator. Growth medium was changed the following day. Cultures were used for treatment at the 3-day-old stage.

The simulated ischemic group was treated during the experimental protocol using a combination of hypoxic chamber and hypoxic solution [28] to simulate tissue ischemia. In treated groups, EPO (1, 10, 100, 500 U/mL) and BNP (10^{-8} M) or acetate vehicle were administered into the hypoxic solution and plates were kept in a hypoxic chamber (gassed with 95% N₂ and 5% CO₂ at 37°C) for 150 min. Following treatments, 120 min of simulated reperfusion was applied in growing medium (mixed with treating compounds) and the plates were kept in a normoxic incubator.

2.8. Cell viability measurement

At the end of the experiments, viability tests were performed in all groups with trypan blue staining after simulated reperfusion. Treating solutions were removed and cells were detached with trypsin. Single cell suspensions were collected into Eppendorf tubes and centrifuged at 2000 rpm for 15 min at 4°C. Supernatants were removed and the pellets were re-suspended in 50 µL PBS solution and mixed with 50 µL 0.4% trypan blue and incubated for 5 min. Cells were counted in a Burker chamber and the ratio of dead (blue) cells to total cell count was calculated and plotted.

2.9. Experimental model of in vivo myocardial infarction

Myocardial infarction in rats was described previously in detail [29]. Rats were anesthetized by intraperitoneal injection of pentobarbital (Euthasol, Produlab Pharma b.v., Raamsdonksveer, The Netherlands; 60 mg/kg bolus dose followed by 15–20 mg/kg when required during the experiment). The rats were weighed and stomach and chest area shaved. Body temperature was maintained using a constant-temperature heating pad. The trachea was intubated

with a cannula connected to a rodent ventilator. The animals were ventilated with room air (6.2 mL/kg, 70 ± 5 breath/min). Blood pressure, surface-lead ECG, and body core temperature were monitored throughout the experiments to ensure the stability of the preparation (Haemosys data acquisition system, Experimetria, Budapest, Hungary), while hemodynamic data were collected at baseline, 29th min of ischemia and 120th min of reperfusion. The right carotid artery was cannulated for measurement of blood pressure and the right femoral vein was cannulated for the administration of the test compounds. A thoracotomy was performed at the 5th intercostal space and the heart was exposed through the fifth intercostal space. The pericardium was incised and a 5-0 Prolene suture was placed around the left descending coronary artery.

The coronary artery was then occluded for 30 min by placing the ligature through a small piece of plastic tubing and pulling the snare tightly in place using a hemostat. After 30 min of coronary occlusion, reperfusion was initiated by releasing the snare and then continued for 120 min.

All-cause mortality was monitored throughout the entire study, i.e. from the anesthesia to the end of reperfusion. The cause of death was determined as follows: irreversible ventricular arrhythmias or hypotension.

2.10. Infarct size measurement

At the end of the 120 min reperfusion, the heart was excised and infarct size was determined [30]. Briefly, the left anterior descending coronary artery was re-occluded and the heart was perfused with 4 mL of 0.3% (w/v) Evans-blue dye (Sigma-Aldrich, Budapest, Hungary) with a constant 100 cmH₂O pressure into the aorta to delineate the area at risk zone. Stained hearts were rapidly frozen (at –20°C for at least 30 min), cut into 2 mm thick slices, and each slice was incubated at 37 °C in 1.5 mL of 1% (w/v) 2,3,5-triphenyltetrazolium-chloride (TTC, Merck Biosciences, Budapest, Hungary) dissolved in 50 mM phosphate buffer (pH 7.4) for 10 min. Slices were then transferred into 10% formalin solution for 5–10 min, rinsed, and then scanned between glass plates. The differently stained areas of the images were quantified by digital planimetry (Infarctsize™ 2.5, Pharmahungary, Szeged, Hungary). Evaluation of all images was carried out in a blinded manner by an experienced investigator throughout the study. Area at risk zone (AAR) was expressed as the percentage of total ventricular area (LV), and infarct size (IS) was expressed as a percentage of the AAR.

2.11. Statistical analysis

Infarct size was expressed as a percentage of the AAR as mean \pm standard error of the mean (S.E.M). Relative changes in infarct size due to treatments were also presented as a percentage of the respective untreated control groups. Differences among means of IS/AAR (%) were analyzed by One-way analysis of variance (ANOVA) followed by Fisher LSD post hoc test to compare each group to the control when appropriate. To analyze the reticulocyte stimulation test, One-way ANOVA was used followed by Tukey post hoc test, where means of reticulocyte number/L of EPO and Dpa were compared to the vehicle, while means of the AF test compounds were compared to EPO. Statistical significance was accepted at $p < 0.05$. Mortality was analyzed with Chi-square test followed by Fisher's exact test. CsA group was compared to the saline control group by Student's *t*-test.

Table 2

Data for erythroid colony assay and EPO receptor binding assay of the test compounds.

Test compounds	Binding (EC ₅₀ , pM)	BFU-E (pM)
EPO	7	2
Dpa	12	14
AF41676	1240	34680
AF43136	27	2623

BFU-E: Erythroid blood forming unit; EC₅₀: Half maximal effective concentration; EPO: Erythropoietin; Dpa: darbepoetin alpha.

Table 3

Data for reticulocyte stimulation test measured 5 days after administration of the test compounds.

Test compounds	Reticulocytes (10 ⁹ /L)
Acetate vehicle	373 ± 24.3
EPO (5000 U/kg)	762 ± 146*
Dpa (10 μg/kg)	1056 ± 104*
AF41676 (10 mg/kg)	284 ± 51.3#
AF43136 (10 mg/kg)	286 ± 57.0#

Data are expressed as mean ± SD.

* p < 0.05 vs. acetate vehicle.

p < 0.05 vs. EPO.

3. Results

3.1. EPO receptor binding and erythroid colony formation abilities for EPO, Dpa and AF peptides

EPO, Dpa and AF43136 bound to the EPO receptor with high affinity (with AF41676 exhibiting modest affinity), AF41676 and AF43136 did not show in vitro erythropoietic activity (Table 2). EPO and Dpa differentiated CD34⁺ cells into erythroid precursors with high efficiency (EC₅₀ = 2 to 14 pM), whereas AF41676 and AF43136 required vastly greater amounts (Table 2) (approximately 3–4 orders of magnitude).

3.2. The effects of EPO, Dpa and AF peptides on erythropoiesis

Reticulocytosis, a predictive marker of erythropoiesis, was significantly stimulated 5 days following administration of EPO at 5000 U/kg and Dpa at 10 μg/kg, but not by administration of AF41676 and AF43136 compared to acetate vehicle (Table 3). For more details, see Data supplement Fig. S1 in the online version, at <http://dx.doi.org/10.1016/j.phrs.2016.08.013>.

3.3. The effects of EPO on cell death

Erythropoietin showed a U-shaped response curve on cell viability in cultured neonatal rat cardiomyocytes subjected to simulated ischemia/reperfusion (Fig. 1B). EPO at 100 U/mL significantly decreased the ratio of dead cells (by approx. 16%) compared to the vehicle control group. The positive control BNP reduced cell death compared to the controls by approx. 30% (Fig. 1B).

3.4. The effects of EPO on infarct size and hemodynamics (Study 1)

Different EPO dosing regimens were evaluated. EPO at 5000 U/kg administered intravenously, before the onset of reperfusion decreased infarct size significantly compared to the vehicle by approximately 24%, while intraperitoneal administration was less effective (approximately 11% non-significant decrease) (Fig. 2B, Table 4). The positive control BNP (10 nmol/kg + 2 nmol/kg infusion for 15 min) decreased infarct size significantly compared to the vehicle by approx. 47% (Fig. 2B, Table 4). There was no dif-

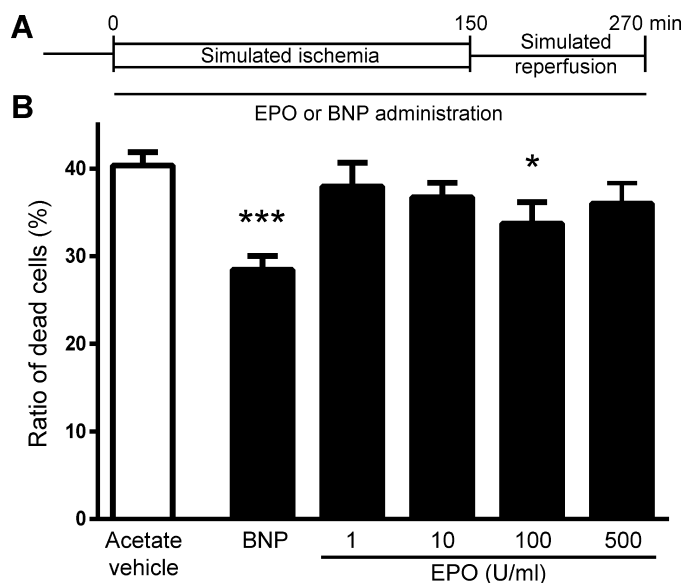


Fig. 1. Panel A: Experimental protocol for the pilot study. Panel B: Dose-response relationships for EPO and the effect of BNP on cell death in cultured neonatal rat cardiac myocytes subjected to 150 min simulated ischemia followed by 120 min simulated reperfusion. Data are expressed as mean ± S.E.M., *p < 0.05, ***p < 0.001 vs. simulated ischemia/reperfusion (SI/R) control; n = 7–8 wells.

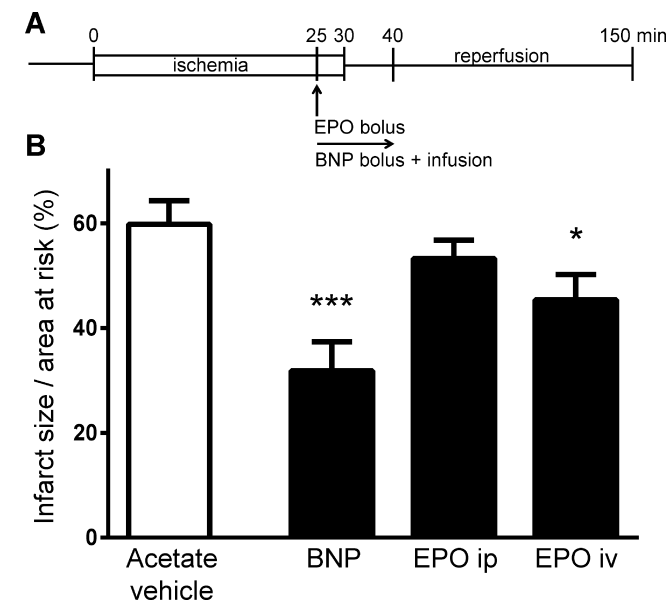


Fig. 2. Panel A: Experimental protocol for Study 1. Panel B: The effects of BNP and EPO on infarct size in rats subjected to in vivo 30-min coronary occlusion followed by 120 min reperfusion. EPO was administered either intraperitoneally (EPO ip) or intravenously (EPO iv) for rats. Data are expressed as means ± S.E.M., *p < 0.05, ***p < 0.001 vs. vehicle control; n = 8–9.

ference in area at risks among the groups (Table 4). BNP reduced mean arterial blood pressure (MABP) significantly during ischemia compared to the vehicle, whereas EPO did not show any significant effect on MABP or heart rate (HR). (Table 4). There was no significant difference in survival among the groups (data not shown).

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

Different doses of darbepoetin were administered intravenously at the 15th min of ischemia. Dose-response relationship of darbe-

Table 4
Hemodynamic parameters, area at risk and infarct size data for study 1.

	n	AAR/LV (%)	IS/AAR (%)	mean arterial blood pressure (mmHg)			heart rate (beats/min)		
				BL	ISCH	REP	BL	ISCH	REP
Vehicle (ac)	9	34.3 ± 2.7	59.8 ± 4.2	142 ± 7	119 ± 7	107 ± 6	404 ± 10	399 ± 14	400 ± 14
BNP	8	38.8 ± 5.5	31.8 ± 5.2***	124 ± 10	89 ± 5*	91 ± 6	401 ± 14	405 ± 6	413 ± 11
Epo ip	8	33.9 ± 2.2	53.3 ± 3.3	151 ± 7	118 ± 5	108 ± 9	418 ± 20	410 ± 18	399 ± 15
Epo iv	8	37.3 ± 3.7	45.3 ± 4.5*	126 ± 11	115 ± 8	111 ± 9	427 ± 6	396 ± 13	403 ± 12

Data are expressed as means ± S.E.M., *p < 0.05, ***p < 0.001 vs. vehicle. AAR/LV and IS/AAR were measured at the end of 120 min reperfusion, hemodynamic data were collected at baseline, 29th min of ischemia and 120th min of reperfusion. BNP: B-type natriuretic peptide; Epo: Erythropoietin; ip: intraperitoneal; iv: intravenous; AAR/LV: area at risk/left ventricle. IS/AAR: infarct size/area at risk; BL: baseline; ISCH: 29th min of ischemia; REP: 120th min of reperfusion.

Table 5
Hemodynamic parameters, area at risk and infarct size data for study 2.

	n	AAR/LV (%)	IS/AAR (%)	mean arterial blood pressure (mmHg)			heart rate (beats/min)		
				BL	ISCH	REP	BL	ISCH	REP
Vehicle (ac)	8	41.5 ± 3.3	65.9 ± 2.7	115 ± 4	95 ± 7	75 ± 3	411 ± 11	422 ± 13	389 ± 10
BNP	7	51.6 ± 1.5	44.2 ± 3.7*	107 ± 5	71 ± 4*	75 ± 4	386 ± 15	389 ± 18	386 ± 14
Dpa 2.5 µg/kg	8	51.5 ± 3.0	59.3 ± 6.3	112 ± 3	91 ± 5	80 ± 4	408 ± 13	437 ± 11	387 ± 17
Dpa 5 µg/kg	8	43.3 ± 5.3	44.7 ± 5.7*	123 ± 2	62 ± 4*	76 ± 6	430 ± 3	378 ± 9	398 ± 16
Dpa 15 µg/kg	7	41.3 ± 4.1	61.9 ± 3.3	126 ± 11	80 ± 5	91 ± 4*	368 ± 14*	418 ± 8	416 ± 8
Dpa 25 µg/kg	7	49.9 ± 2.5	75.1 ± 3.9	127 ± 1	104 ± 4	74 ± 4	396 ± 14	420 ± 11	406 ± 14

Data are expressed as means ± S.E.M., *p < 0.05 vs. vehicle AAR/LV and IS/AAR were measured at the end of 120 min reperfusion, hemodynamic data were collected at baseline, 29th min of ischemia and 120th min of reperfusion. BNP: B-type natriuretic peptide; Dpa: darbepoetin alfa; AAR/LV: area at risk/left ventricle. IS/AAR: infarct size/area at risk; BL: baseline; ISCH: 29th min of ischemia; REP: 120th min of reperfusion.

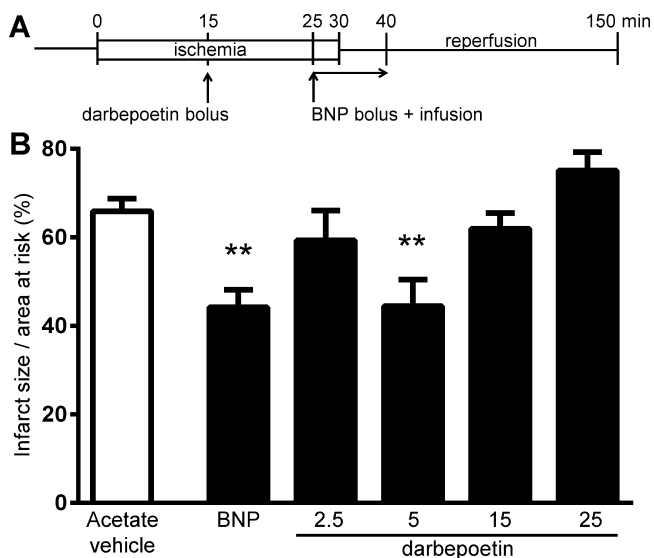


Fig. 3. Panel A: Experimental protocol for Study 2. Panel B: Dose–response relationships for darbepoetin and the effect of BNP on infarct size in rats subjected to in vivo 30-min coronary occlusion followed by 120 min reperfusion. Data are expressed as means ± S.E.M., **p < 0.01 vs. vehicle control; n = 7–8.

poetin showed a U-shaped curve with maximal infarct size limiting effect at 5 µg/kg compared to the vehicle (approx. 33% reduction, Fig. 3B, Table 5). The infarct size-limiting effect of darbepoetin was comparable to that of the positive control BNP (approx. 33% for both, Fig. 3B, Table 5). There was no significant difference in the area at risks among groups (Table 5). 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. Neither Dpa nor BNP had any effect on HR (Table 5). There was no significant difference in survival among the groups (data not shown).

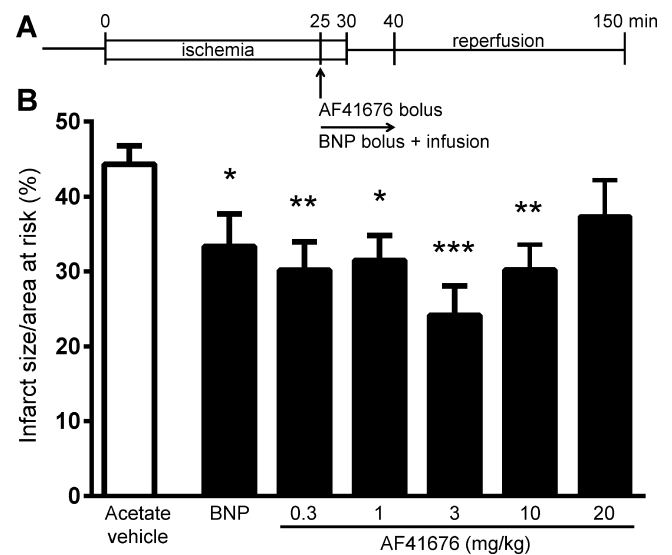


Fig. 4. Panel A: Experimental protocol for Study 3. Panel B: Dose–response curve for AF41676 on infarct size in rats subjected to in vivo 30-min coronary occlusion followed by 120 min reperfusion. Data are expressed as means ± S.E.M., *p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle control; n = 7–8.

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

Dose-dependent efficacy of AF41676 was investigated by five different intravenously-administered doses in a bolus injection 5 min before the onset of reperfusion. On the U-shaped dose-effect curve, AF41676 at 0.3, 1, 3 and 10 mg/kg decreased infarct size significantly, of which the 3 mg/kg dose showing the most potent infarct size limiting effect compared to the vehicle (approx. 46% reduction, Fig. 4B, Table 6). BNP also reduced infarct size significantly compared to the vehicle (approx. 25% reduction; Fig. 4B, Table 6). There was no significant difference in the area at risks among the groups (Table 6). BNP as well as 10 and 20 mg/kg AF41676 reduced MABP during ischemia compared to the vehicle (Table 6). AF41676 at 20 mg/kg reduced HR at the 29th min of

Table 6
Hemodynamic parameters, area at risk and infarct size data for study 3.

	n	AAR/LV (%)	IS/AAR (%)	mean arterial blood pressure (mmHg)			heart rate (beats/min)		
				BL	ISCH	REP	BL	ISCH	REP
Vehicle (acetate)	8	33.0 ± 3.4	44.3 ± 2.5	118 ± 9	116 ± 11	100 ± 9	418 ± 14	427 ± 16	402 ± 10
BNP	8	34.9 ± 2.4	33.4 ± 3.0*	103 ± 9	73 ± 6*	98 ± 6	396 ± 16	405 ± 10	490 ± 18*
AF41676 0.3 mg/kg	8	43.3 ± 4.0	30.2 ± 3.3**	109 ± 6	111 ± 4	94 ± 3	429 ± 5	450 ± 4	423 ± 8
AF41676 1 mg/kg	8	36.7 ± 4.0	31.5 ± 3.9*	114 ± 9	104 ± 6	80 ± 8	427 ± 8	433 ± 11	410 ± 9
AF41676 3 mg/kg	8	44.5 ± 2.6	24.1 ± 3.4***	125 ± 5	116 ± 10	104 ± 5	408 ± 17	418 ± 11	411 ± 4
AF41676 10 mg/kg	8	34.1 ± 3.5	30.2 ± 4.8**	122 ± 5	75 ± 8*	75 ± 8*	443 ± 6	393 ± 17	413 ± 17
AF41676 20 mg/kg	7	40.9 ± 2.4	37.4 ± 2.5	125 ± 8	84 ± 1*	83 ± 9	413 ± 11	361 ± 26*	438 ± 16

Data are expressed as means ± S.E.M., *p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle. AAR/LV and IS/AAR were measured at the end of 120 min reperfusion, hemodynamic data were collected at baseline, 29th min of ischemia and 120th min of reperfusion. BNP: B-type natriuretic peptide; AAR/LV: area at risk/left ventricle. IS/AAR: infarct size/area at risk; BL: baseline; ISCH: 29th min of ischemia; REP: 120th min of reperfusion.

ischemia and at 10 mg/kg reduced MABP at the end of reperfusion (Table 6). In spite of the transient hypotensive effect of the 10 and 20 mg/kg doses of AF41676, there was no significant difference in survival among the groups (data not shown).

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

AF43136 was administered in 10 and 30 mg/kg doses iv. as a bolus infusion for 5 min starting at the 25th min of ischemia at a final volume load of 6 mL/kg. AF43136 at 10 mg/kg dose reduced infarct size significantly by approximately 30% compared to the acetate vehicle control, however, infarct size data of 30 mg/kg AF43136 is equivocal due to the high mortality ratio and the consequent small sample size at the end of the experiments (n = 2; Fig. 5B, Table 7). The positive control CsA used in study 4 decreased infarct size significantly by approximately 23% compared to the saline control (Fig. 5B, Table 7). There was no difference in the area at risks among the groups (Table 7). AF43136 at 30 mg/kg decreased mean arterial blood pressure significantly during ischemia compared to the acetate vehicle, but it did not reduce blood pressure at 10 mg/kg (Table 7). In addition, AF43136 at 10 mg/kg increased mean arterial blood pressure by the end of 120 min reperfusion compared to the acetate vehicle (Table 7). AF43136 at 30 mg/kg reduced survival rate significantly compared to acetate vehicle (Fig. 5C).

4. Discussion

Here, we have shown 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. This is the first demonstration that these EPO receptor ligand peptides are cardioprotective in a rat model of AMI, and their effects are comparable with that of EPO and Dpa.

In the present study, we demonstrated that EPO, independently of its erythropoietic activity, protected cultured neonatal cardiac myocytes against simulated ischemia/reperfusion. Our findings confirm previous studies that EPO has cytoprotective- [31] and anti-apoptotic effects on cardiac myocytes [32].

In *in vivo* coronary occlusion/reperfusion experiments, we found that iv., but not ip. administration of the same dose of EPO before the onset of reperfusion decreased infarct size in rats, although it has been previously demonstrated that EPO reduces infarct size by both ip. [32,33] or iv. [34] administration. This discrepancy could be due to pharmacokinetic differences or different duration of treatments [35]. We also report the first time in the literature the dose-response relationship of Dpa administered before the onset of reperfusion. Although, the infarct size-limiting dose of Dpa administered before reperfusion varies widely (from 2.5 [36] to 30 µg/kg [37] in other studies), our results point out that a lower dose range

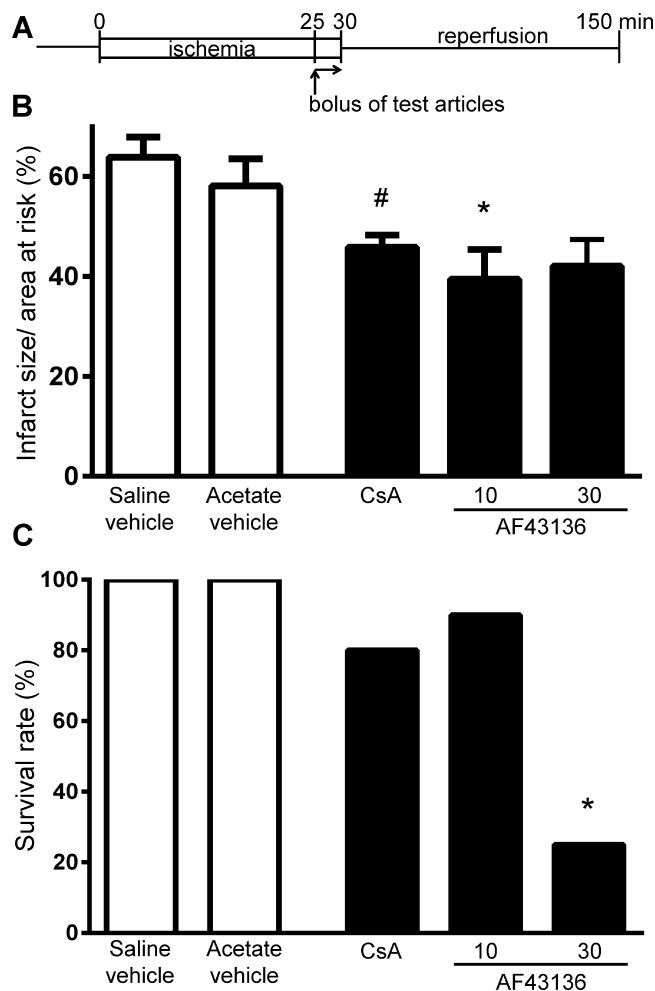


Fig. 5. Panel A: Experimental protocol for Study 4.

Panel B: The effect of 2 doses of AF43136 and cyclosporine A (CsA) on infarct size in rats subjected to *in vivo* 30-min coronary occlusion followed by 120 min reperfusion. Data are expressed as means ± S.E.M., *p < 0.05 vs. vehicle control, #p < 0.05 vs. saline control; n = 7–8. Infarct size data of 30 mg/kg AF43136 contains only 2 samples because of high mortality rate (see in Panel C).

Panel C: The effect of 2 doses of AF43136 and cyclosporine A (CsA) on survival. Data are expressed as means ± S.E.M., *p < 0.01 vs. vehicle control.

of Dpa is sufficient to reduce infarct size in a rat model of AMI with maximal effect at 5 µg/kg.

Noteworthy, in the present study, B-type natriuretic peptide (BNP) and cyclosporine A (CsA) administration before reperfusion served as appropriate positive controls to reduce infarct size. Previous studies have shown that BNP treatment maintained dur-

Table 7
Hemodynamic parameters, area at risk and infarct size data for study 4.

	n	AAR/LV (%)	IS/AAR (%)	mean arterial blood pressure (mmHg)			heart rate (beats/min)		
				BL	ISCH	REP	BL	ISCH	REP
Vehicle (saline)	8	27.9 ± 2.6	63.8 ± 4.1	110 ± 5	91 ± 4	72 ± 3	416 ± 9	432 ± 23	440 ± 21
Vehicle (acetate)	8	30.6 ± 3.1	58.1 ± 5.4	106 ± 4	90 ± 6	67 ± 4	433 ± 15	427 ± 20	415 ± 17
CsA	7	34.5 ± 2.5	45.9 ± 2.4 [†]	99 ± 6	82 ± 6	76 ± 5	445 ± 12	425 ± 22	434 ± 12
AF43136 10 mg/kg	8	28.2 ± 5.5	39.4 ± 5.9 [†]	111 ± 6	105 ± 3	88 ± 5 [*]	394 ± 11	413 ± 12	426 ± 13
AF43136 30 mg/kg	8	20.3 ± 4.8 [§]	42.1 ± 5.4 [§]	96 ± 6	47 ± 9 [†]	54 ± 18 [§]	438 ± 15	383 ± 19	437 ± 15 [§]

Data are expressed as means ± S.E.M., [†]p < 0.05 vs. vehicle; [§]n = 2 due to high mortality. AAR/LV and IS/AAR were measured at the end of 120 min reperfusion, hemodynamic data were collected at baseline, 29th min of ischemia and 120th min of reperfusion. CsA: cyclosporine A; AAR/LV: area at risk/left ventricle. IS/AAR: infarct size/area at risk; BL: baseline; ISCH: 29th min of ischemia; REP: 120th min of reperfusion.

ing the entire reperfusion reduces myocardial infarct size [38,39], however, there is no evidence about shorter, targeted BNP administration for reducing reperfusion injury in *in vivo* AMI models. The cardioprotective capabilities of CsA against reperfusion injury, especially in rats, are still inconsistent. Several reports support our data [40,41] (for review see [42]), but others have shown that CsA is not effective in reducing myocardial infarct size [42,43]; the reason for that is not exactly understood.

The major finding of our present study is that two novel selective non-erythropoietic EPO receptor ligand dimeric peptides, AF41676 and AF43136, reduce infarct size significantly when administered before reperfusion in an *in vivo* rat model of AMI. AF41676 was effective in reducing infarct size at a wide dose-range (0.3–10 mg/kg). Although, high doses (10 and 20 mg/kg) of AF41676 decreased mean arterial blood pressure, they did not influence survival, and we did not register any severe side effects during the experiments. In contrast, AF43136 reduced infarct size, but it was lethal in higher doses due to a rapid decline in mean arterial blood pressure during its administration. As analogs of the peptide portion of peginesatide, AF41676 and AF43136 were initially prepared as an attempt to define non-erythropoietic agents. These analogs exhibit reliable binding to the extracellular portion of the EPO receptor, however, do not stimulate erythropoiesis suggesting that they may act as cell protectants.

It has been previously demonstrated that the modified EPO analogues, CEPO and asialoEpo, preserve the tissue protective effects of EPO, such as infarct size reduction, prevention of cardiac hypertrophy and improvement of cardiac function [7,17–20], without any erythropoietic safety problems [2]. However, these EPO analogues are unfavorable due to their potential for high antigenicity, inadequate long-term stability and relatively high costs of production [6,21]. Therefore, previously, a small EPO analogue, pyroglutamate helix B surface peptide (pHBSP or ARA 290) consisting of 11 amino acids of the beta-chain of EPO, was generated to substitute EPO or its large peptide analogues to achieve cardioprotection [44] without inducing erythropoiesis. pHBSP is a small fragment of EPO, and one would assume that when excised out of the cytokine, it would only randomly adopt the appropriate conformation and thus be a very poor binder to the receptor. So far, only one study has demonstrated that pHBSP reduces infarct size in acute coronary ligation in rats [45]. 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 (EPOR). The two AF peptides are structurally related to the peptide portion of peginesatide and therefore expected to bind EPOR tightly. These features support our findings, that non-erythropoietic EPO analogue dimeric peptides may be promising cardioprotective agents against acute myocardial infarction.

However, there are some limitations in the present study. Direct translational conclusion to human cardioprotection cannot be drawn from these preclinical results. Several previous preclinical

studies showed significant cardioprotection after treatments with EPO or its derivatives (e.g. darbepoetin; 8–9), however, in clinical trials they failed to improve any hard endpoints (i.e. survival, cardiac function, or infarct size) in AMI patients [2,46]. The reason for the failure of translation is not known, however, the possible mechanisms for the lack of EPO-induced cardioprotection in humans has been extensively reviewed elsewhere [2]. Despite the negative results of EPO-cardioprotection in humans, modulation of EPO receptor stimulation by novel receptor ligands should be a target of continued investigations as a promising cardioprotective strategy. Indeed, others have developed several non-erythropoietic EPO-analogues (e.g. CEPO, asialoEPO, ARA290) to preserve EPO's cytoprotective effects without erythropoietic activity [2,17–20,47]. Their common potential target is to interact selectively with the innate repair receptor of EPO, which mediates tissue protection [48]. Recent human data showed the safety of ARA290 in patients with type 2 diabetes [48] or Friedreich's ataxia [49]. Moreover, the protective effect of ARA290 has also been shown in a porcine model of renal ischemia/reperfusion injury [50], and by improving the regeneration capacity of corneal small nerve fibers in sarcoidosis patients [51].

5. Conclusions

Our study demonstrates for the first time that selective non-erythropoietic EPO receptor ligand dimeric peptides AF41676 and AF43136 administered before the onset of reperfusion are able to reduce infarct size in a rat model of acute coronary occlusion/reperfusion. Therefore, non-erythropoietic EPO receptor ligands may be potential cardioprotective agents in acute myocardial infarction. Further safety and translational studies will be needed to ensure the safety and efficacy of these agents as viable clinical treatments.

Conflict of interest

The authors declare that they have no conflict of interest. The study was supported financially by Affymax, however, data were not influenced by either Pharmahungary or Affymax, thus they have also no conflict of interest regarding the present manuscript.

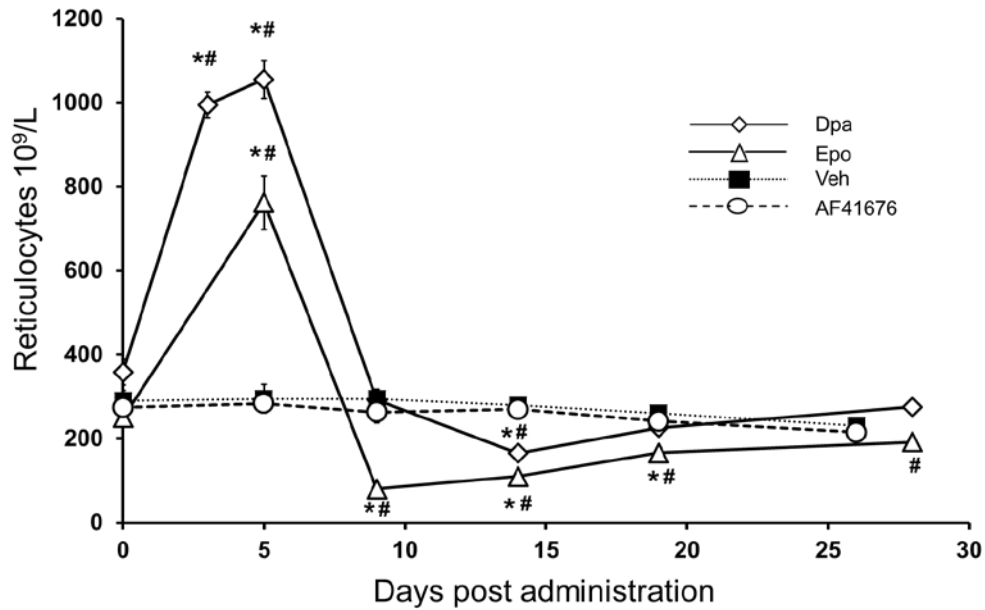
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Supplementary material



Supplementary Figure 1: The effects of 5,000 Units/kg EPO, 10 µg/kg Dpa, 10 mg/kg AF41676, and acetate vehicle on reticulocytes number following administration of single iv. boluses. Data are expressed as mean±SD, *p<0.05 vs. vehicle, #p<0.05 vs. baseline analyzed by repeated measures ANOVA, n=4 rats per time point/ group.