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Nitric oxide-related cardioprotection during the development of myocardial ischaemia and athletic heart

Doctoral Thesis

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Abbreviations

AAR	- Area at risk
ADMA	- Asymmetric dimethylarginine
ANOVA	- Analysis of variance
ANP	- atrial type natriuretic peptide
AP-1	- Activator protein 1
Arg	- Arginine
Asp	- Aspartic acid
ATP	- Adenosine triphosphate
AV	- Atrioventricular
Bad	- Bcl-2-associated death promoter
BH2	- Dihydropteridin
BH4	- Tetrahydrobiopterin (2-amino-6-(1,2-dihydroxypropyl)-5,6,7,8-tetrahydro-1H-pteridin-4-one)
BNP	- Brain type natriuretic peptide
b-TFE	- balanced turbo field echo
cAMP	- Cyclic adenosine monophosphate
Cav1.2	- Calcium channel, voltage-dependent, L type, alpha 1C subunit
cGMP	- Cyclic guanosine monophosphate
CHD	- Congenital heart disease
cMRI	- Cardiac magnetic resonance imaging
CNBD	- Cyclic nucleotide binding domain
CNG	- Cyclic nucleotide-gated ion channels
cNOS	- Constitutive nitric oxide synthase (also known as eNOS, NOS3)
CO	- Carbon monoxide
CO	- Cardiac output
CON	- control, untreated mice
CSE	- Cystathionine-γ-lyase
DDAH	- Dimethylarginine dimethylaminohydrolases
DHFR	- Dihydrofolate reductase
DNA	- Deoxyribonucleic acid
dP/dtmax	- Maximum rates of systolic pressure rise

dP/dtmax	- Minimum rates of systolic pressure decline
Ea	- Arterial elastance
Ea/Ees ratio	- ventricular-arterial coupling
ECMO	- Extracorporeal membrane oxygenation
ECG	- Electrocardiogram
eCSC	- Endogenous cardiac stem and progenitor cells
EDRF	- Endothelium-derived relaxing factor
Ees	- ventricular elastance
EF	- Ejection fraction
ELISA	- enzyme-linked immunosorbent assay
eNOS	- Endothelial nitric oxide synthase (also known as cNOS, NOS3)
ERK	- Extracellular signal-regulated kinase
ETC	- Mitochondrial electron transport chain
ETT-TUKEB	- Hungarian Scientific Council National Ethics Committee for Scientific Research
FDA	- US Food and Drug Administration
FS	- Fractional shortening
GAF domain	- This name derives from the first proteins it was found in: c**G**MP-specific phosphodiesterases, **a**denylyl cyclases and **F**hlA
Glu	- Glutamic acid
GSK3β	- Glycogen synthase kinase 3β
GTP	- Guanosine triphosphate
H-NOX	- Heme-nitric oxide and oxygen binding
H_2S	- Hydrogen sulfide
HCN	- Hyperpolarization-activated cyclic nucleotide-gated ion channels
HIF-1α	- Hypoxia-inducible factor-1α
HSP70	- 70 kilodalton heat shock proteins
IA	- Infarcted area
I/R	- Ischemia reperfusion
ICAM-1	- Intercellular Adhesion Molecule 1

I_f	- funny current
IGF-1	- Insulin-like growth factor 1
IL-1	- Interleukin 1
iNO	- Inhaled nitric oxide
iNOS	- Inducible nitric oxide synthase
iNO+TAD	- Combination of inhaled nitric oxide and tadalafil
IP	- Intraperitoneal
IP3	- Inositol 1,4,5-trisphosphate
IRAG	- Inositol 1,4,5-trisphosphate (IP3) receptor-associated PKG substrate
iv	- intra venous
IVSd	- interventricular septum thickness at end-diastole
IVSs	- interventricular septum thickness at end-systole
JAK	- Janus-activated kinase
JNK1	- c-Jun NH_2-terminal kinase 1
k-t BLAST	- Broad-use Linear Acquisition Speed-up Technique
LAD	- Left anterior descending artery
L-NMMA	- NG-monomethyl-L-arginine
LTCC	- L-type Ca^{2+} channels
LV	- Left ventricle
LVAD	- Left ventricular assist device
LVEDVi	- Left ventricular end-diastolic volume index
LVEF	- Left ventricular ejection fraction
LVESVi	- Left ventricular end-systolic ventricular index
LVH	- Left ventricular hypertrophy
LVIDd	- Left ventricular dimensions at end-diastole
LVIDs	- Left ventricular dimensions at end-systole
LVMi	- Left ventricular mass index
LVPWd	- Posterior wall thickness at end-diastole
LVPWs	- Posterior wall thickness at end-systole
LVSVi	- Left ventricular stroke volume index
MAPK	- Mitogen-activated protein kinases

MEKK1	- Mitogen-activated protein kinase kinase
MI	- Myocardial infarction
MIP-1 /-2	- Macrophage Inflammatory Proteins
mitoK_{ATP}	- Mitochondrial ATP-sensitive K^+ channel
mitochondrial PTP	- Mitochondrial Permeability Transition Pore
MPO	- myeloperoxidase
NADPH	- Nicotinamide adenine dinucleotide phosphate
NF-κB	- nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	- Nuclear factor of activated T cells
NIH	- National Institutes of Health
nNOS	- Neural nitric oxide synthase
NO	- Nitrogen oxide
NO_2	- Nitrogen dioxide
NOHLA	- Nω-hydroxy-L-arginine
NOMI	- Nitric oxide for inhalation to reduce reperfusion injury in STEMI
NOS	- Nitric oxide synthase
NOS1	- nitric oxide synthase 1 (also known as nNOS)
NOS2	- nitric oxide synthase 2 (also known as iNOS)
NOS3	- nitric oxide synthase 3 (also known as cNOS, eNOS)
Nox	-NADPH oxidase
p53	- cellular tumor antigen p53
p70S6K	- extracellular regulated kinase system with downstream p70 ribosomal protein S6 kinase
PAP	- Pulmonary artery pressure
PAS	- Protein fold named for its association with the Per, ARNT, and Sim proteins
PDE	- Phosphodiesterase
pGC	- Particulate guanylate cyclase
PI3K(p110α)	- Phosphoinositide 3-kinase (p110α)
PIP3	- Phosphatidylinositol 3,4,5-trisphosphate
PKA	- cAMP dependent protein kinase

PKG	- cGMP dependent proteinkinase
PPARα	- peroxisome proliferator-activated receptor alpha
PPHN	- Persistent pulmonary hypertension of the new-born
ppm	- Parts-per-million, 10−6
PRSW	- Preload-recruited stroke work
PV	- pressure-volume
PVR	- Pulmonary vascular resistance
RBBB	- Right Bundle Branch Block
RhoA	- Ras homolog gene family member A
RISK	- Reperfusion Injury Salvage Kinase
ROS	- Reactive oxygen species
RV	- Right ventricle
RVEDVi	- Right ventricular end-diastolic volume index
RVEF	- Right ventricular ejection fraction
RVESVi	- Right ventricular end-systolic volume index
RVMi	- Right ventricular mass index
RVSVi	- Right ventricular stroke volume index
RyR	- Ryanodine receptor
SADMA	- Symmetric dimethylarginine
SAFE	- Survival Activating Factor Enhancement
SAP	- Systemic arterial pressure
SEK1	- Stress-activated protein/ERK kinase 1
SEM	- Standard error of mean
SERCA	- Sarco/endoplasmic reticulum Ca^{2+}-ATPase
sGC	- Soluble guanylate cyclase
STAT	- Signal transducer and activator of transcription
SV	- Stroke volume
SW	- Stroke work
T	- isovolumic relaxation
T2w-SPIR	- Triggered blood suppressed T2-weighted spectral inversion recovery
TAD	- Tadalafil

TNF-α	- Tumor necrosis factor α
TnI	- Troponin I
TTC	- Triphenyl tetrazolium chloride
TTE	- Transthoracic echocardiography
VASP	- Vasodilator-Stimulated Phosphoprotein
VO2	- Maximal oxygen uptake
VCAM-1	- Vascular cell adhesion protein 1
XO	- Xanthine oxidase
XOR	- Xanthine oxidoreductase
YC-1	- 3-[5′-hydroxymethyl-2′-furyl]-1-benzylindazole

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1. Introduction

1.1. Nitric oxide (NO) signaling in cardiovascular physiology and pathophysiology

1.1.1. Identification of NO

Nitrogen oxide (NO) is not only an important intermediate in chemical industry, but an important signaling molecule as well. This free radical is a byproduct of combustion and is naturally produced during electrical discharges of lightning in thunderstorms (National Center for Biotechnology Information and U. S. National Library of Medicine, n.d.).

It was first described in the 18th century by Joseph Priestly – an English polymath – in the first volume of his collection of his most important scientific texts: Experiments and Observations on Different Kinds of Air (Johnson et al., 1775). It took almost three centuries for this "nitrous air" to amaze the scientific world. Search for the mysterious endothelium-derived relaxing factor (EDRF) led to the recognition that despite being a gaseous molecule and a free radical NO is a key vertebrate biological messenger (Ignarro et al., 1987; Palmer et al., 1987). It became Molecule of the year in 1992 and in 1998 Robert F. Furchgott, Louis C. Ignarro and Ferid Murad were awarded the Nobel Prize in Physiology or Medicine for their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system ("The Nobel Prize in Physiology or Medicine 1998," n.d.).

1.1.2. General chemical and biological properties of NO

Nitric oxide is an inorganic, odorless, colorless and only slightly water-soluble gas in radical form *in statu nascendi*, with rapid dimerization capacity. It is non-flammable, but it will accelerate combustion and increase the risk of fire and explosion in combustible and flammable materials (Bhatraju et al., 2015; Bloch et al., 2007; National Center for Biotechnology Information and U. S. National Library of Medicine, n.d.). As a byproduct of combustion NO may reach significant concentrations in ambient or indoor air. When exposed to oxygen NO is readily and rapidly oxidized to nitrogen dioxide (National Center for Biotechnologyr Information and U. S. National Library of Medicine, n.d.).

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$$2\ NO + O_2 \rightarrow 2\ NO_2$$

1.1.3. General behaviour of NO in biological systems: toxicity and safety issues

Nitric oxide has a half-life of seconds in biological fluids and is highly diffusible (Lei et al., 2013). It can get oxidized to nitrate and nitrite, react with superoxide to form peroxynitrite, nitrosylate thiol containing amino acids and react with heme containing proteins (Bhatraju et al., 2015; Bryan and Loscalzo, 2011; National Center for Biotechnology Information and U. S. National Library of Medicine, n.d.).

Under normal circumstances absorption occurs through the eyes and lungs. (Nitric oxide levels can reach a concentration up to 1000ppm in tobacco smoke (Bryan and Loscalzo, 2011)!) After reaching the lower respiratory tract NO diffuses through the alveoli into the microvasculature and reacts with the hemoglobin in the erythrocytes. Methemoglobin is formed, which does not bind oxygen and impairs oxygen delivery (Bloch et al., 2007). Methemoglobin is rapidly reduced to ferrous hemoglobin and in the end nitrite and nitrate will be formed. The greater part of the nitrate, and thus the inhaled nitric oxide, is excreted into the urine and eliminated from the body (Kapil et al., 2010).

In the presence of moisture and oxygen nitric and nitrous acid are formed and corrosive conditions develop, making NO a skin, eye and a mucous membrane irritant (National Center for Biotechnology Information and U. S. National Library of Medicine, n.d.). Clinical experience states that prolonged exposure to NO will result in rebound pulmonary hypertension after weaning of exposure (Bloch et al., 2007; Bryan and Loscalzo, 2011).

When exposed to oxygen NO will form the pulmonary irritant nitrogen dioxide (NO_2). The amount of NO_2 formed depends on gas concentrations and the time reacting gasses are mixed (National Center for Biotechnology Information and U. S. National Library of Medicine, n.d.).

1.1.4. Natural sources and protective effects of nitric oxide

Nitric oxide is a known byproduct in almost all type of organisms. It is endogenously synthetized from L-arginine, oxygen and NADPH by various nitric oxide synthase enzymes (NOS) (Knowles and Moncada, 1994). NO production changes with

altitude changes (acute and chronic) and has heavy influence on altitude adaptation (Beall et al., 2012).

An NOS independent way, the reduction of inorganic nitrate (nitrate → nitrite → nitric oxide) can also elevate nitric oxide levels. After consumption of nitrate rich vegetables facultative anaerobe bacteria reduce it to nitrite, which is then swallowed and absorbed (Kapil et al., 2010; Lundberg et al., 2011). Some enzymes, e.g. xanthine oxidase may contribute to the endogenous conversion of nitrate to nitrite (Lei et al., 2013; Zweier and Talukder, 2006). Nitrate elevation can lead to protection on both molecular (Table 1.) and systematic levels resulting among others in cardo-protection, blood pressure reduction, beneficial modulation of hypercholesterolemia and increased exercise tolerance (Kapil et al., 2010; Lundberg et al., 2011; Phillips et al., 2009).

Table 1. Chemical and molecular protective effects of nitric oxide.(Phillips et al., 2009)

Effect as	**Description**
1. Antioxidant	Free radical scavenger, inhibits cell respiration, restores antioxidant enzyme levels
2. Anticytokines	Suppresses TNF-α and IL-1
3. Anti-adhesion molecules	Inhibits selectins, VCAM-1 and ICAM-1
4. Antiapoptotic	Downregulates gene p53, inhibits human caspases, induces expression of HSP70
5. Antichemokines	Downregulates MIP-1 and MIP-2
6. Antidetrimental signaling	Regulates MAPKs, promotes preconditioning, produces elevated levels of cGMP
7. Antinuclear proteins	Inhibits NF-κB and AP-1

1.1.5. Nitric oxide synthesis

Arginine derived NO synthesis has been best studied in mammals and three isoforms of the NOS enzyme were identified (Knowles and Moncada, 1994). NOS oxidizes the guanidine group of L-arginine in a process that consumes five electrons and

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results in the formation of NO with stoichiometric formation of L-citrulline. The process involves the oxidation of NADPH and the reduction of molecular oxygen. The transformation occurs at a catalytic site adjacent to a specific binding site of L-arginine. (Figure 1.)

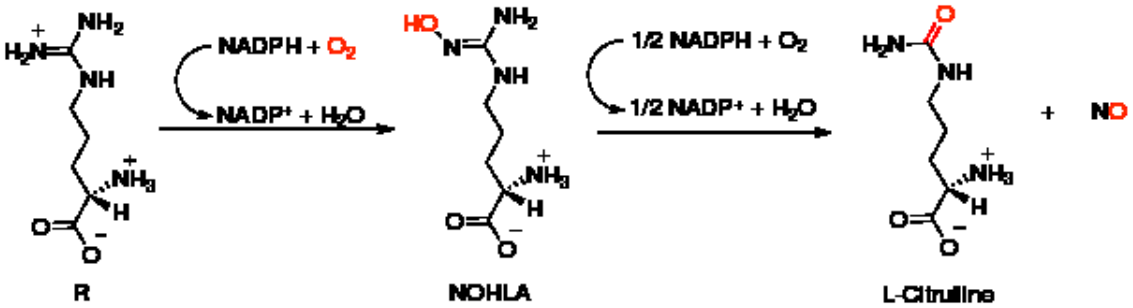

Figure 1. NO synthesis. Oxidation of L-Arg to L-citrulline occurs via two successive mono-oxygenation reactions producing Nω-hydroxy-L-arginine (NOHLA) as an intermediate. 2 mol of O2 and 1.5 mol of NADPH are consumed per mole of NO formed. (Based on the work of Prof. Moncada)[8]

1.1.5.1. Endothelial nitric oxide synthase (eNOS)

This isoform is also known as nitric oxide synthase 3 (NOS3) or constitutive NOS (cNOS) and is functionally similar to the nNOS, as both are clearly Ca^{2+}- Calmodulin dependent. In humans it is encoded by the NOS3 gene and is partially membrane associated. Endothelial NOS generates NO in the blood vessels and is involved with vascular tone and platelet regulation (Knowles and Moncada, 1994). Huang et al. proved the elementary protective role of eNOS against hypertension and maintaining baseline bloodpressures (Huang et al., 1995).

In myocytes eNOS binds with Caveolin-1 and Caveolin-3 and is localized in caveolae and both increased Ca^{2+} concentration and phosphorylation can activate it. NOS3 has his own unique localization and signaling pathway, which among others will influence myocyte contraction and action potential development (Tang et al., 2014).

Several mutations of the eNOS gene are known that could display significant differences under different clinical conditions. The three most widely studied NOS-3 polymorphisms in humans include the G894CT (Glu298Asp) in exon 7, the 4a/5b 27-

basepair variable number of tandem repeats in intron 4, and the T-786C variant in the promoter region (Pereira et al., 2007). These polymorphisms are associated with a variety of clinical conditions, e.g. atherosclerosis, hypertension, heart failure and adaptation to physical exercise (Lembo et al., 2001; Matsa et al., 2013; Pereira et al., 2007, p. 3; Wolfarth et al., 2008).

Cardiomyocyte specific NOS3 overexpression preserved LV function and endothelial NOS3 overexpression improved survival and decreased pulmonary edema after myocardial infarction (Janssens et al., 2004; Jones et al., 2003). Potential therapeutic benefits of NOS3 overexpression and increased NO bioavailability were proven in gene transfer models. In chronic ischemia clinically relevant neovascularization was induced and it attenuated acute ischemia reperfusion injury and ischemic remodeling (Kupatt et al., 2007; Szelid et al., 2009). Overexpression of NOS3 in cardiomyocytes was also suggested to be protective against arrhythmias by restoring sympatho-vagal balance (Massion et al., 2004).

1.1.5.2. Neuronal nitric oxide synthase (nNOS)

Neuronal nitric oxide was the first isoform to be purified and cloned, is also known as NOS1 and is encoded in humans by the NOS1 gene. It is Ca^{2+}- Calmodulin dependent and is constitutively expressed at high activity in the central and peripheral nervous system and in the human skeletal muscle as well. nNOS is responsible for the largest proportion of constitutive NOS activity in humans (Knowles and Moncada, 1994). In cardiomyocytes NOS1 is predominantly localized in the sarcoplasmic reticulum, signals through the cGMP independent pathway and acts as a major regulator of excitation contraction coupling and myocyte Ca^{2+} handling. NOS1 is necessary for the positive force-frequency response and β-adrenergic induced positive inotropy as well (Tang et al., 2014).

1.1.5.3. Inducible nitric oxide synthase (iNOS)

Also known as type II NOS (NOS2), this isoform was identified in activated macrophages. From the first studies on it was evident, that this one differs from the others: it is Ca^{2+}-independent (Knowles and Moncada, 1994). It is expressed in response to

inflammatory stimuli and the NO produced is important in protection against pathogens (Francis et al., 2010, p. -).

1.1.6. NOS uncoupling: superoxide production

NOS enzymatic activity depends on substrate and cofactor availability and on the rate of electron transfer (Thomas et al., 2008). Substrate or cofactor shortage result in uncoupling: electrons removed from NADPH and normally travelling through the enzyme are diverted from L-arginine to a molecular oxygen and superoxide is produced (Rochette et al., 2013; Roe and Ren, 2012). The resulting oxidative stress is accompanied by reduced NO bioavailability (Roe and Ren, 2012; Schäfer et al., 2004).

Substrate (arginine) availability is influenced by competition with arginase, arginine uptake and methylation, and proteolysis to form symmetric and asymmetric dimethylarginine (SADMA and ADMA) (Rochette et al., 2013; Thomas et al., 2008).

The critical regulator of NOS function is one of its cofactors, tetrahydrobiopterin (BH4). BH4 is either synthetized from GTP or regenerated from dihydrobiopterin (BH2) and its loss either by oxidation or reduced expression of the recycling enzyme dihydrofolate reductase (DHFR) is the most prominent cause of NOS uncoupling (Roe and Ren, 2012). Intracellular ROS will accelerate BH4 oxidation and in turn the reduced BH4 form will trigger further uncoupling. In addition paradox eNOS expression during oxidative stress further amplifies ROS production (Kim and Park, 2010; Rochette et al., 2013).

Oxidized BH4 and NOS uncoupling contribute e.g. to vascular dysfunction, hypertension, ischemia/reperfusion injury, diabetes and other cardiovascular diseases (Rochette et al., 2013).

1.1.7. Endogenous NOS inhibitors

Symmetric and asymmetric methyl-arginines - e.g. ADMA, SDMA or NG-monomethyl-L-arginine (L-NMMA) - inhibit NO synthesis in vivo by competing with L-arginine at the active site of NO synthases (Leone et al., 1992). ADMA and SDMA are the major circulating forms of methylarginines in humans. (Leiper and Vallance, 1999).

ADMA is continuously formed during intracellular protein turnover and its concentration is very sensitive to changes in expression or activity of its degrading enzymes, the dimethylarginine dimethyl-amino-hydrolases (DDAH) (Rochette et al.,

2013). ROS increase may reduce DDAH activity and the resulting ADMA generation works as a second ROS-dependent, amplifying, feed-forward cellular loop that further stimulates ROS production (Rochette et al., 2013). Increasing bioactive NO availability can prevent ROS formation and is able to restore normal levels of ADMA (Carlström et al., 2011).

SDMA does not have the same biological activity as ADMA and is not hydrolyzed by DDAH, but it can interrupt the transportation of arginine and other cationic amino acids as well (Huang et al., 2004; Leiper and Vallance, 1999).

1.1.8. Nitric Oxide signaling

Nitric oxide exerts its effects either via the cGMP dependent, or via the cGMP independent signaling pathway. Balance between these two pathways depends upon redox status that influences net NO chemistry and NO and cGMP synthetic capacity by NOS and soluble guanylate cyclase (sGC) (Kass et al., 2007b).

1.1.8.1 cGMP dependent NO signaling

1.1.8.1.1. Cyclic guanosine 3',5'-monophosphate

cGMP is a second messenger signaling molecule and is generated from guanosine triphosphate (GTP) by two distinct enzymes: the cytoplasmic heterodimeric haemoprotein sGC activated by NO and carbon monoxide (CO), and the transmembrane receptor particulate guanylate cyclases (pGC), activated upon binding of atrial and brain type natriuretic peptides (ANP and BNP), respectively (Burley et al., 2007; Garcia-Dorado et al., 2009; Tsai and Kass, 2009). Since the discussed experimental work focuses on the role of the NO mediated signaling, the second, receptor linked GC class will not be discussed in detail.

Direct targets of cGMP and participants of cGMP signaling are PKGs, cyclic nucleotide-gated ion channels (CNG), cGMP hydrolyzing phosphodiesterases (PDEs) and PDEs that contain allosteric cGMP binding sites. cGMP affinity among its targets varies and is modulated by phosphorylation or other modifications (Biel, 2009; Francis et al., 2010; Tsai and Kass, 2009).

1.1.8.1.2. Soluble guanylate cyclase (sGC)

sGC consists of two homologous subunits and each subunit consists of a heme-nitric oxide and oxygen binding (H-NOX), PAS (protein fold named for its association with the Per, ARNT, and Sim proteins), coiled-coil, and catalytic domain (Derbyshire and Marletta, 2012). Out of the two known active sGC isoforms (sGCα1β1 and sGCα2β1) sGCα1β1 is the principal isoform found in the heart and vasculature. NO mediated cardioprotection is lost without the α1 subunit and without the β1 subunit functional heterodimers are not formed (Derbyshire and Marletta, 2012; Nagasaka et al., 2008).

NO binding to the soluble guanylate cyclase (sGC) can increases its activation 100-200 fold (others mention 200-400 fold increase) (Francis et al., 2010; Tsai and Kass, 2009). Two different states of sGC activation – basal and high activity – make sure that only an acute increase of NO levels, and the binding of a second NO to the enzyme is capable of fully activating the sGC (Cary et al., 2006; Tsai and Kass, 2009).

sGC response to its ligands diminishes upon repeated exposure. S-nitrosation induced desensitization is rapid and has major influence on therapeutic applications (Derbyshire and Marletta, 2012).

Since sGC is soluble, intracellular cGMP pool formation depends on the site of sGC activation and restriction of cGMP diffusion by PDE. This functional compartmentalization accounts for different intracellular effects (Burley et al., 2007; Francis et al., 2010).

1.1.8.1.3. Protein kinase G (PKG)

PKG was first identified in lobster tail muscle and so far two genes and families are known (PKGI and PKGII) (Burley et al., 2007). The members of the PKGI family (PKGIα and PKGIβ) are more commonly involved with NO mediated cGMP signaling. PKGIα transduces NOS / sGC signaling in the cardiovascular system and it requires one tenth of cGMP concentration for activation than does PKGIβ (Burley et al., 2007; Francis et al., 2010; Tsai and Kass, 2009).

Oxidative processes can provide a NO / cGMP independent mechanism for PKGIα activation and auto-phosphorylation can change isozyme affinities and functions and further fine tune their regulatory role (Francis et al., 2010).

1.1.8.1.3.1. PKG regulation of calcium levels and signaling

A modest NO availability will result in lower intracellular Ca^{2+} levels and interact with smooth muscle contraction, myocyte contractility, β-adrenergic response and attenuate cardiac hypertrophy (Francis et al., 2010).

Modulation of L-type calcium channels (LTCC): LTCC phosphorylation is restricted to the caveolar microdomain by PDE5 and appears to be gender dependent. This regulatory pathway is believed to blunt contractile response to β-adrenergic stimulation, and attenuate calcineurin activation to prohibit NFAT (nuclear factor of activated T cells) translocation and hypertrophy (Tang et al., 2014).

In addition direct phosphorylation of the Cav1.2 channel (Calcium channel, voltage-dependent, L type, alpha 1C subunit) also reduces Cav1.2. current and limits contractility (Yang et al., 2007).

Troponin, regulating cardiac muscle contraction: troponin I phosphorylation reduces myofilament Ca^{2+} sensitivity (Layland et al., 2002).

Phosphorylation of G-protein-activated phospholipase-Cβ3: phosphorylation will inhibit this enzyme and inhibit agonist stimulated intracellular calcium release (Xia et al., 2001).

Phosphorylation of inositol 1,4,5-trisphosphate (IP3) receptor-associated PKG substrate (IRAG): PKGIβ converts IRAG into an inhibitor of IP3-receptor activity and suppresses calcium release (Francis et al., 2010).

Phospholamban phosphorylation: canceling its inhibition of the sarcoplasmic reticulum calcium/ATPase pump will increase calcium sequestration. Phospholamban phosphorylation can also occur via a cGMP independent pathway, namely PKA up-regulation with S-nitrosylation (Francis et al., 2010; Tang et al., 2014).

Ryanodine receptor (RyR) phosphorylation: phosphorylation will influence the function of the cardiac sarcoplasmic reticulum Ca^{2+} release channel (Burley et al., 2007; Zhang et al., 2005).

1.1.8.1.3.2. PKGI-mediated inactivation of the Ras homolog gene family member A (RhoA)

Rho kinase regulates actin cytoskeleton organization, stress fiber formation and vascular smooth muscle contraction and is inactivated and blocked by serine phosphorylation (Ellerbroek et al., 2003; Murthy et al., 2003; Surma et al., 2011). RhoA

kinase inhibitors have demonstrated beneficial effects in the treatment of various cardiovascular diseases including arterial hypertension, atherosclerosis, I/R injuries, hypertrophic remodeling, cardiac dysfunction and heart failure (Surma et al., 2011).

1.1.8.1.3.3. Gene regulation

PKGI activity influences the expression of smooth muscle specific contractile proteins, proteins in the NO sGC signaling pathway, proteins regulating smooth muscle migration and differentiation, proteins influencing neuronal plasticity and learning and proteins in pathological conditions (Alzheimer's and schizophrenia) (Francis et al., 2010).

1.1.8.1.3.4. Phosphorylation of Vasodilator-Stimulated Phosphoprotein

Vasodilator-Stimulated Phosphoprotein (VASP) is phosphorylated by both protein kinase G and protein kinase A (PKA). VASP signaling influences fibroblast motility, neutrophil migration, neuronal migration and endothelial function and could be potentiating metastasis formation (Krause et al., 2003).

1.1.8.1.3.5. Phosphodiesterase 5 regulation

PKGI mediated activation of the cGMP specific PDE5 is a powerful negative feedback on the NO / sGC / cGMP signaling pathway (Das et al., 2015a; Kass et al., 2007b).

1.1.8.1.3.6. Cytoprotection

Cytoprotection was observed in myocytes, neurons and glia and epithelial cells.

Opening of mitochondrial K+/ATP channels: cGMP can activate PKG localized at the cytosolic surface of the mitochondrial outer membrane. This may lead to the phosphorylation of a target protein that carries the cardio-protective signal to a protein kinase C isozyme (PKC-ε) residing in the intermembrane space of mitochondria and increase opening of mitochondrial K+/ATP channels (mitoK_{ATP}) (Costa et al., 2005; Francis et al., 2010).

MitoK_{ATP} independent anti-apoptotic pathway: Activation of extracellular signal-regulated kinase (ERK) and ERK dependent inactivation of glycogen synthase kinase 3β (GSK3β) by PKG phosphorylation also attenuates ischemia reperfusion injury and prevents cardio-myocyte apoptosis (Das et al., 2009; Francis et al., 2010).

Blockade of p38 mitogen-activated protein kinase is also reported to reduce cell damage, although these protective effects were not observed after sildenafil induced cGMP increase (Das et al., 2009; Francis et al., 2010).

It was also suggested that the Bcl-2-associated death promoter (Bad) is likely to be an important downstream substrate of PKG-I, and its phosphorylation may contribute to the cGMP mediated survival in neural cells (Johlfs and Fiscus, 2010).

1.1.8.1.3.7. Pro-apoptotic effects

PKGI activity in colon cancer cells promotes apoptosis by phosphorylation and activation of the mitogen-activated protein kinase kinase (MEKK1), activation of the stress-activated protein/ERK kinase 1 (SEK1), and activation of the c-Jun NH_2-terminal kinase 1 (JNK1) pathway (Deguchi et al., 2004; Soh et al., 2001). It also suppresses growth and promotes apoptosis in some endothelial cells and human colon tumor HT29 cells (Francis et al., 2010).

1.1.8.1.4. Cyclic nucleotide regulated ion channels

Cyclic nucleotide regulated ion channels belong to the superfamily of voltage gated cation channels and are directly regulated by the binding of cAMP or cGMP. Channel activation is directly coupled to extracellular cation influx and plasma membrane depolarization. Two families can be distinguished: cyclic nucleotide gated (CNG) channels and hyperpolarization-activated cyclic nucleotide gated channels (HCN) (Biel, 2009; Roubille and Tardif, 2013). CNG channels prefer cGMP over cAMP, while HCN channels have 10 fold higher affinity for cAMP (Biel, 2009; Podda and Grassi, 2013).

1.1.8.1.4.2. Cyclic nucleotide-gated channels (CNG):

These channels play a key role in visual and olfactory signal transduction, but their role in other cell types remains unclear. CNG channels pass monovalent cations without discrimination. CNGs do not desensitize or inactivate after prolonged exposure to their ligands and they are influenced by divalent ion currents and several other factors (Biel, 2009; Podda and Grassi, 2013).

1.1.8.1.4.3. Hyperpolarization-activated cyclic nucleotide-gated ion channels (HCN):

All four known HCN channel isoforms were identified in the myocardium and have a preference for K^+. They activate on hyperpolarisation, which was the reason why the

current resulting from their activation was named funny (I_f). Regulation of these channels is complex, but modulation through their cyclic nucleotide binding domain region appears to be a common mechanism. Selective blockade of these channels is getting more and more attention in the treatment of heart failure patients (Roubille and Tardif, 2013).

1.1.8.1.5. cGMP catabolism - phosphodiesterases

Phosphodiesterases are catabolic enzymes in charge of the fate of cGMPs and cAMPs. This 21 gene family of proteins was grouped into 11 different isozymes (Table 2.) and 48 isoforms. Due to all these variants any organism is estimated to have more than fifty cGMP hydrolyzing enzymes at the same time. Cyclic nucleotide affinity varies, PDE5 / PDE6 / PDE9 are highly cGMP specific, PDE1 / PDE2 / PDE 11 have dual substrate affinity, and PDE3 / PDE 10 are cGMP sensitive but cAMP selective. Phosphodiesterases can be found in all tissues, but their distribution varies (Das et al., 2015a; Francis et al., 2010; Kass et al., 2007b). cGMP-phosphodiesterases with known activity in the cardiovascular system are:

- PDE1 is a Ca^{2+}/calmodulin dependent enzyme,
- PDE2, a cGMP-stimulated cAMP esterase that can also hydrolyze cGMP,
- PDE3 although it is cAMP specific, it is inhibited by cGMP
- PDE5 the first identified selective cGMP esterase
- PDE9A could work as a “housekeeping” PDE to maintain low intracellular cGMP levels and controls nitric oxide independent cGMP and hypertrophic heart disease (Francis et al., 2010; Lee et al., 2015, p. 9).

A large number of factors influence the catalytic function from PDEs, including processes that affect protein expression and breakdown, enzyme localization and substrate binding. For most PDEs their catalytic rate is high, thus absolute PDE concentration within a cell is low. In addition the Km value of different PDEs is also in a broad range of concentrations. This diversity together with enzyme and substrate compartmentalization enables phosphodiesterases to take part in a large variety of signaling processes (Francis et al., 2010).

Under pathologic conditions PDE regulation contributes to the progression of several diseases. PDE3 down regulation influences heart failure progression, PDE1 influences smooth muscle hypertrophy, sustained stimulation of PDE1 and PDE5 results in NO tolerance, PDE1 and PDE5 induction can be behind exacerbated hypertension and

PDE5 up-regulation is part of pulmonary hypertension and congestive heart failure (Kass et al., 2007b).

1.1.8.1.5.1. PDE regulation

Phosphodiesterases can undergo complex post-translational modifications. Among others, activation by Ca2+/calmodulin binding, or allosteric binding of cGMP may mediate PDE and protein interactions. (Das et al., 2015a).

Allosteric cGMP binding to tandem regulatory GAF domains (its name derives from the first proteins it was found in: c**G**MP-specific phosphodiesterases, **a**denylyl cyclases and **F**hlA) can act as an auto feedback mechanism (increasing catabolic activity for cGMP) or cross-regulation mechanism (activating catabolism of cAMP). Five of the known PDEs are equipped with a GAF domain: PDE2, PDE5, PDE6, PDE10 and PDE11. Binding affinities for the allosteric sites are much higher than for the catalytic sites, thus before reaching the catalytic site cGMP first induces a more active catalytic state and sustains an activated cGMP breakdown for a significant time. In addition ligand occupation of the catalytic site, phosphorylation of the regulatory domain, oxidation/reduction and other processes enhance GAF binding affinity. Allosteric binding of cGMP to a phosphodiesterase might as well serve as an intracellular storage and protection till later release (Francis et al., 2010; Kass et al., 2007b).

Four of the PDEs (PDE1, PDE3, PDE4, PDE5) contain phosphorylation sites for various kinases and PDE phosphorylation by PKG is another mechanism to induce positive feedback. Phosphorylation of PDE5 by PKG may serve to increase its cGMP affinity and catalytic activity and represents an alternative mode of regulatory feedback inhibition to normalize cGMP levels (Das et al., 2015a; Kass et al., 2007b).

Another type of modulation can happen at the catalytic site. In the case of PDE3 catalytic site affinity is similar for both cyclic nucleotides, but a higher V_{max} for cAMP confers specificity, while cGMP becomes a competitive inhibitor of PDE3 hydrolysis (Kass et al., 2007b).

In the case of PDE1 an auto-inhibitory domain is known to maintain low activity in the absence of Ca^{2+}, and neighboring calmodulin binding domains were found to reactivate the enzyme in the presence of Ca^{2+}-Calmodulin (Kass et al., 2007b).

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Table 2. PDE isozymes, their specificity and selective inhibitors *(Kass et al., 2007b).*

PDE isoenzyme	***Substrate***	***Tissue expression***	***Specific inhibitors***
1	Ca2+/calmodulin	Heart, brain, lung, smooth muscle	KS-505a IC 86340
2	dual specificity	Adrenal gland, heart, lung, liver, platelets	EHNA BAY 60-7550
3	cGMP inhibited, cAMP selective	Heart, lung, liver, platelets, adipose tissue, inflammatory cells	Cilostamide, enoxamone, milrinone
4	cAMP specific	Sertoli cells, kidney, brain, liver, lung, inflammatory cells	Rolipram, roflumilast, cilomilast
5	cGMP specific	Lung, platelets, vascular smooth muscle, heart	Sildenafil, tadalafil, vardenafil
6	cGMP specific	Photoreceptor	Dipyridamole
7	cAMP specific, high affinity	Skeletal muscle, heart, kidney, brain, pancreas, T lymphocytes	BRL-50481
8	cAMP selective	Testes, eye, liver, skeletal muscle, heart, kidney, ovary, brain, T lymphocytes	None
9	cGMP specific	Kidney, liver, lung, brain, ?heart	BAY 73-6691
10	cGMP sensitive, cAMP selective	Testes, brain	None
11	cGMP sensitive, dual specificity	Skeletal muscle, prostate, kidney, liver, pituitary and salivary glands, testes	None

1.1.8.1.5.2. PDE5 expression and regulation

One PDE5 coding gene (PDE5A) and three gene expression variants are known. It is assumed that different promoters for the PDE5 isoforms allow physiologically relevant differential control of PDE5 gene expression and provide a long-term feedback mechanism for PDE5 activation (Kass et al., 2007a). High PDE5 expression rate in smooth muscle cells make determination of exact localization in vascularized tissues difficult. Intracellular localization is different among cellular compartments and seems to be at least partially dependent on the presence of eNOS (Kass et al., 2007a).

PDE5 has two highly homologous GAF domains (A and B), but high affinity cGMP binding occurs only to the GAF-A domain. This domain is very similar to the PDE2 GAF-B and PDE6 GAF A domains and stimulates substrate catalysis to its ten-fold. Although the enzyme is largely inactive in the absence of GAF ligand binding, under usual circumstances it is very likely to be occupied by cGMP and maintain full enzyme activity. Binding site phosphorylation either by PKG or PKA enhances cGMP binding affinity and stabilizes the increased catabolic activity. This is the way, by which sGC activators can promote a feed-forward enzyme activation and limit cGMP rise. The very same processes ensure easy and lasting binding of PDE5 inhibitors (Das et al., 2015a; Kass et al., 2007a).

1.1.8.2. cGMP independent pathway

NO directly regulates protein function via posttranslational modification of S-nitrosylation (addition of a nitrosyl group to a free thiol on a cysteine residue of the target protein). S-nitrosylation is a redox based signal and it not only takes part in mammalian signaling, but can be used against the invasion of microbes or cancer cells as well (Stamler et al., 2001; Tang et al., 2014).

Nitrosylation and nitrosative chemistry can lead to secondary oxidative modifications and production of reactive nitrogen species. These reactive nitrogen species can interact with reactive oxygen species, which can either impair its signaling and increase cytotoxicity (irreversibly oxidizing and nitrating proteins) or open up new signaling pathways. NO may as well promote cytotoxicity by mobilization of free iron or by the production of other oxidants, radicals and reactive aldehydes (Stamler et al., 2001).

Recently it was proven that one of the main reperfusion injury quenching mechanisms of NO signaling is the reversible S-nitrosation of a mitochondrial protein

that will decelerate the mitochondrial electron transport chain (ETC) and inhibit extensive reactive oxygen species production during reperfusion (Schumacker, 2013).

NOS1 produced NO takes advantage of the cGMP independent signaling pathway and modulates myocyte Ca^{2+} handling. Co-localization of NOS1 with the SR Ca^{2+} release channel, ryanodine receptor and xanthine oxidoreductase (XOR) on the sarcoplasmic reticulum enables their S-nitrosylation (Tang et al., 2014).

1.2. Ischemia-reperfusion injury

In case of an acute cardiac ischemic event, besides restoration of blood flow in ischemic areas, reduction of pre- and post-procedural damage is indispensable (Garcia-Dorado et al., 2009). Revascularization of the culprit lesion is accompanied by ischemia-reperfusion (I/R) injury, which is responsible for about 40-50% percent of myocardial damage suffered (Figure 2.) (Yellon and Hausenloy, 2007).

Ischemia reperfusion can cause four types of cardiac dysfunction: the mechanical dysfunction known as myocardial stunning, the disruption of microvascular circulation in the reperfused tissue (“no reflow”), reperfusion arrhythmias and lethal reperfusion injury (Yellon and Hausenloy, 2007).

During lethal reperfusion injury myocardial cell death occurs mainly as necrosis: histological analysis of reperfused infarcts showed composed areas of contraction band necrosis and ultrastructure showed sarcolemmal rupture and massive calcium deposits. Contraction band necrosis, when myocytes show characteristic disruption of their architecture and disorganization of their sarcomeres, is usually referred to as hypercontraction or round-up (Garcia-Dorado et al., 2009).

1.2.1. Biochemical and metabolic changes during ischemia reperfusion

Ischemia-reperfusion subjects the myocardium to several biochemical and metabolic changes. During prolonged ischemia intra- and extracellular acidosis, energy depletion, Na^{+} and $Ca2^{+}$ overload and hyper-osmolality develops. Upon reperfusion mitochondrial re-energization, generation of reactive oxygen species (ROS), intracellular Ca2+ overload, rapid restoration of physiologic pH, and inflammation interact and induce cell death.

1.2.1.1. The ischemic cascade

A switch from aerobic to anaerobic metabolism result in lactic acid accumulation. The resulting acidosis together with the fall in ATP concentration and consequent failure of ATP dependent processes triggers an uncontrolled rise in intracellular ion levels. ROS production increases and disrupts sarcolemmal and mitochondrial function, facilitating cell death (McAlindon et al., 2015).

1.2.1.2. pH normalization

The rapid wash-out of lactic acid and the activation of the sodium-hydrogen exchanger and the sodium–bicarbonate symporter results in quick pH restoration and may lead to potassium ion overload, which in turn results in a reversed Na^{+}/Ca^{2+} exchanger activity and additional calcium influx (Bond et al., 1991; Garcia-Dorado et al., 2009; Yellon and Hausenloy, 2007). After restoration of pH mitochondrial ROS formation is initiated and mitochondrial PTP (Permeability Transition Pore) channels activate (Kim et al., 2006).

1.2.1.3. ROS generation

Xanthine oxidase (XO), the mitochondrial electron transport chain, NADPH oxidase (Nox) and NOS uncoupling are the known sources of ROS (Zweier and Talukder, 2006). Nox is thought to be the major source of O_2^{-} and H_2O_2 in the heart and it is upregulated during ischemia reperfusion injury. Nox is a double edged sword as minimal activity is needed to maintain peroxisome proliferator-activated receptor alpha (PPARα) and hypoxia-inducible factor-1α (HIF-1α) conferred cardioprotection. The consumption of NAD(P)+/NAD(P)H by Nox is an alternative regulatory mechanism of redox state and thus mitochondrial electron transport (Chen and Zweier, 2014; Matsushima et al., 2014).

ROS induce mitochondrial PTP opening, attract neutrophils, mediate sarcoplasmic reticulum dysfunction and contribute to intracellular $Ca2^{+}$ overload, damage the cell membrane by lipid peroxidation, induce enzyme denaturation, and cause direct oxidative damage to DNA.(Yellon and Hausenloy, 2007; Zweier and Talukder, 2006).

1.2.1.4. Calcium oscillation

Parallel restoration of respiration to pH normalization allows mitochondrial repolarization and ATP synthesis. This favors calcium uptake into the mitochondria and sarcoplasmic reticulum and despite the higher calcium influx cytosolic calcium levels fall. Increased calcium concentration in the sarcoplasmic reticulum interacts with the ryanodine receptor and calcium oscillations (release/reuptake) propagate across the cell (Garcia-Dorado et al., 2009). This can lead to hyper-contraction and disorganization of the cell structure, but to damage the sarcolemmal membrane the proteolysis of the subsarcolemmal cytoskeleton by calpains (calcium dependent and calcium activated proteases) is necessary as well(Garcia-Dorado et al., 2009; Inserte et al., 2004).

1.2.2.5. Targeting the mitochondria

The opening of the mitochondrial PTP channel during the first few minutes of reperfusion collapses mitochondrial membrane potential and uncouples oxidative phosphorylation: ATP depletion and cell death occur (Yellon and Hausenloy, 2007).

Direct or PI3K / AKT / NOS / GC mediated PKG and PKC activation will induce the mitochondrial ATP-dependent K channel (K_{ATP}) and ROS formation resulting in p38

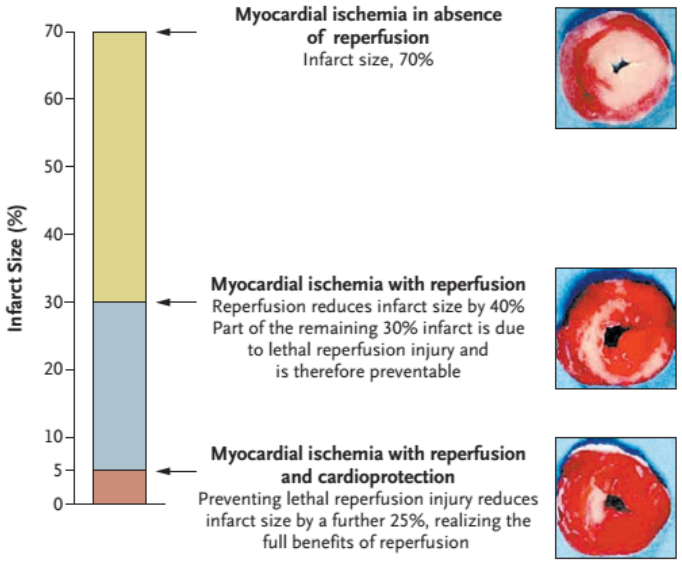

Figure 2. Components of final myocardial injury. This scheme taken from Yellon and Hausenloy depicts the contribution of both ischemia and reperfusion injury to final myocardial injury.[52]

mitogen-activated kinase and PKC activation and priming of mitochondrial PTP for opening (Heusch et al., 2008).

Two endogenous cardioprotective pathways, the Reperfusion Injury Salvage Kinase (RISK) and the Survival Activating Factor Enhancement (SAFE) pathways, are known to inhibit mitochondrial PTP, activate mitochondrial connexin-43 channels and open mitochondrial ATP dependent potassium channels (Hausenloy et al., 2013).

Activation of sarcolemmal G-protein–coupled receptors or of receptors for growth factors result in activation of the RISK program. The RISK pathway includes the pro-survival kinase cascades MEK1/2-Erk1/2 and involves PI3K/Akt/NO signaling, p70S6K (extracellular regulated kinase system with downstream p70 ribosomal protein S6 kinase) and GSK3β (glycogen synthase kinase 3β) activation (Heusch et al., 2008).

The SAFE pathway is made up by the TNF-α (tumor necrosis factor-α) receptor and STAT3 (Hausenloy et al., 2013). After activation of gp130 (sarcolemmal glycoprotein 130) receptors or TNF α receptors, the janus-activated kinase (JAK) and signal transducer and activator of transcription (STAT) pathway is activated with projection to the nucleus and possibly to mitochondria (Heusch et al., 2008). As an upstream regulator of TNF-α NO influences this pathway as well (Thielmann et al., 2002).

1.2.2.6. Neutrophil involvement in lethal myocardial reperfusion injury

Neutrophil response to reperfusion is triggered by cytokines, the complement system, ROS and various lipid mediators. Compared to ischemia only, reperfusion accelerates neutrophil infiltration and focuses their accumulation in the sub-endocardium (Chatelain et al., 1987; Vinten-Johansen, 2004). Initial, rapid neutrophil adhesion to the endothelium is accompanied by a reduced endothelial function (Vinten-Johansen, 2004). For the first 6 hours cells stay in the intravascular space and after the 6th hour start to migrate to the parenchyma (Zhao et al., 2000).

Neutrophils can form aggregates with platelets, plug capillaries and mechanically stop blood flow (Engler et al., 1983; Hataishi et al., 2006; Wong et al., 2013). Together with platelets and damaged endothelial cells they release vasoconstrictors and contribute microvascular constriction (Niccoli et al., 2009; Wong et al., 2013). Both processes contribute actively to the “No reflow” phenomenon.

Neutrophils can induce endothelial dysfunction and by impairing NO generation contract arteries (Vinten-Johansen, 2004). Endothelial dysfunction is nearly restored after 72 hours following reperfusion (Zhao et al., 2000).

Neutrophils release more than 20 different enzymes which primary target is the extracellular matrix. While these enzymes have a relatively long half-life and catalyze specific reactions, ROS produced by neutrophil is responsible for fast, aspecific and widespread destruction (Vinten-Johansen, 2004).

The reduction of neutrophil infiltration is associated with reduced infarct size and attenuated adverse cardiac remodeling while after the 3-7th post-ischemic day this could possibly assist repair mechanisms (Carbone et al., 2013; Huang et al., 2007). Limitation of neutrophil recruitment is one of main cardio-protective mechanisms attributed to inhaled NO (Hataishi et al., 2006).

1.2.2.7. NOS uncoupling

Nitric oxide signaling, previously described in detail, can interact with lethal myocardial injury on several levels. Ischemia reperfusion will induce NOS uncoupling, reduce NO bioavailability and produce ROS. Most probably due to the decreased GC activity under acidic conditions cGMP levels will drop and the activity of the cGMP dependent pathway will decrease. The very same pathway that directly effects contractility, calcium handling, activates protective cascades, regulates mitochondrial PTP, inhibits platelet activation, reduces endothelial adhesion protein expression, increases endothelial permeability and induces vasorelaxation (Garcia-Dorado et al., 2009). In addition the cGMP independent pathway reversibly S-nitrosylates one of the electron transport chain components (a subunit of mitochondrial complex I) and thus slows it down (Schumacker, 2013). A study with selective sGC/NO/cGMP signaling inhibition even suggested, that nitrosylation might play a more important role in cardioprotection (Sun et al., 2013).

1.3. Modulation of the nitric oxide mediated signaling pathway – therapeutic applications

Nitric oxide generating drugs have long been used by physicians and are still in the first line of medical therapies (e.g. nitro-glycerine) while other fields of use are severely

limited by systemic vasodilatation. To avoid this unwanted side effect novel NO donor compounds and agents to sensitize or to directly activate NO signaling were developed and are being tested (Bryan and Loscalzo, 2011).

1.3.1 Inhaled nitric oxide

Before 1990 NO was considered poison. Zapol and Frostel in their publication from 1991 proved that inhalation of low concentrations of nitric oxide (up to 80 ppm) is safe and effectively relaxes pulmonary vascular smooth muscle, without systemic vasodilatation (Frostell et al., 1991). Short gas transit time and low concentrations ensure that during inhaled NO administration NO_2 concentrations remain under 5 ppm and measuring met-hemoglobin levels establishes adequate therapy-monitoring (Bhatraju et al., 2015; Frostell et al., 1991) (Figure 3.).

1.3.1.1. Taking advantage of pulmonary effects

Nitric oxide/oxygen blends are used in critical care to promote capillary and pulmonary dilation to treat a variety of diseases. These are often last-resort gas mixtures before the use of extracorporeal membrane oxygenation (ECMO) (Bryan and Loscalzo, 2011).

Nitric oxide inhalation is an approved therapy for persistent pulmonary hypertension of the new-born (PPHN), for chronic obstructive pulmonary disease, in patients with pulmonary hypertension and undergoing cardiac surgery, in patients undergoing left ventricular assist device (LVAD) implantation in patients with right ventricular infarction or pulmonary embolism (Adhikari et al., 2014; Argenziano et al., 1998; Bhatraju et al., 2015; Bloch et al., 2007; Bryan and Loscalzo, 2011).

1.3.1.2. Extrapulmonary effects of inhaled NO

Identifying inhaled NO related platelet inhibition broke the paradigm that its effects are limited to the lungs (Frostell et al., 1991; Högman et al., 1993). A number of extra-pulmonary effects were described ranging from reducing neointima formation and inflammatory modulation in malaria to reduction of ischemia-reperfusion injury and protection of the central nervous system. Cardiovascular applications were studied and

verified as well (Bergmark et al., 2012; Bryan and Loscalzo, 2011; Charriaut-Marlangue et al., 2013; Mathru et al., 2007; Phillips et al., 2009).

1.3.1.2.3. Cardiopulmonary resuscitation

In mice it was proven that NO inhalation has protective effects on the outcomes of cardiopulmonary resuscitation. The neuro-protective effects largely depend on sGC-mediated mechanisms, although the role of sGC independent and NO metabolite mediated arteriole dilatation could not be excluded (Ichinose, 2013).

1.3.1.2.6. Myocardial ischemia-reperfusion injury:

Several experiments were conducted to prove the effectiveness of inhaled NO therapy in attenuating myocardial reperfusion injury (Bhatraju et al., 2015; Bloch et al., 2007). Pre-clinical studies agree on the infarct size limitative and cardiac function preserving effects of this therapy (Hataishi et al., 2006; Liu et al., 2007; Nagasaka et al., 2008; Neye et al., 2012). A larger scale international, randomized, double-blind clinical study was conducted recently, the NOMI trial – as this work strongly connects to it - will be discussed separately.

1.3.1.3. Theories behind extrapulmonary effects

Although therapeutic systemic applications are known and were proven by several groups, the question remains: how can NO, with its short half-life in biological fluids, reach the periphery and elicit its effects?

First extrapulmonary effects were attributed to changes in platelet and leukocyte function and only after discarding this turned the scientific interest towards stable NO metabolites. Nitric oxide can react with heme in hemoglobin forming nitrosyl hemoglobin and can nitrosylate the cysteine in the hemoglobin to form SNO-hemoglobin. This reaction will occur in the presence of high oxygen concentrations and NO will be released under low oxygen concentrations (Bryan and Loscalzo, 2011; McMahon and Doctor, 2006).

Nitrite and nitrate are also candidates for the cardio protective effects, as they could be major stable reservoirs of NO. Nitrite was proven to dramatically reduce infarct size in a canine model, and was suggested as potent adjunctive therapy in myocardial reperfusion injury (Gonzalez et al., 2008).

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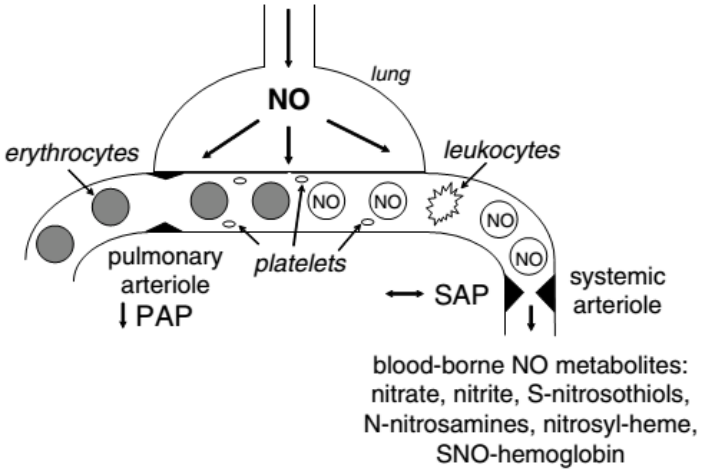


Figure 3. Schematic image of an alveolar capillary unit. NO dilates pulmonary arterioles and reduces pulmonary artery pressure (PAP). Inhaled NO does not dilate systemic arterioles or alter systemic arterial pressure (SAP) under normal conditions.[6]

Nitric oxide also reacts with a variety of plasma components - proteins, peptides and lipids – and forms intravascular agents capable of endocrine vasodilatation: S-nitrosthiols, N-nitrosamines, iron-nitrosyls and nitrated lipids (Bryan and Loscalzo, 2011; Gonzalez et al., 2008).

1.3.2. Other inhaled drugs to modify NO signaling

Inhalation is an effective way to administer various NO donor agents: nitroglycerin relieves angina pectoris, and sodium nitroprusside may improve oxygenisation in hypoxic neonates and reduce PVP and PVR in children with congenital heart disease (CHD). O-nitrosoethanol (an S-nitrosothiol) was reported to improve oxygenation and systemic hemodynamics in hypoxemic newborns. Nitrite can also be converted to NO by a variety of enzymes (e.g. hemoglobin and xanthine oxidoreductase) (Bryan and Loscalzo, 2011). PDE5 inhibitors (e.g. sildenafil) and sGC stimulators potentiate the pulmonary vasodilator effects of NO (Evgenov et al., 2007; Ichinose et al., 2001).

1.3.3. Phosphodiesterase 5 inhibition

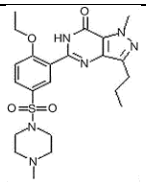
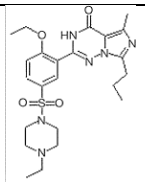
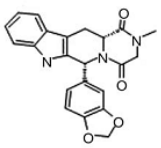
After the commercial success of Viagra it took almost a decade for PDE5 inhibition to make its comeback into more direct cardiovascular applications. The recognition that it is responsible for ~22-43% of cytosolic cGMP hydrolytic activity and that under

pathologic conditions its expression is upregulated 2 to 6 fold opened new frontiers in cardiovascular research (Das et al., 2015a; Kass et al., 2007a).

PDE5 inhibitors provide protection from ischemia-reperfusion injury and preserve left ventricular function, improve survival rate, reduce apoptosis in the infarct border zone and reduce heart failure progression (Das et al., 2015a; Salloum et al., 2008). Extracardial uses include dampening of systemic inflammation in diabetic patients, protection of stem cells from I/R injury, reducing I/R injury in skin island flaps, neuroprotection and anticancer effects (Das et al., 2015a; Gulati and Singh, 2014a, 2014b; Kayiran et al., 2013). The most studied compounds are sildenafil, vardenafil and tadalafil. (Table 3.)

PDE5 inhibitors interact with gaso-transmitters (e.g. NO and H_2S), as it was seen after blocking the H_2S producing enzyme: cardio-protective effects of tadalafil were abolished (Das et al., 2015a).

Table 3. PDE5 inhibitors for cardiovascular use. Data on metabolism and pharmacokinetics in humans was taken from http://toxnet.nlm.nih.gov/ (US National Library of Medicine, n.d.).

	Sildenafil citrate	Vardenafil	Tadalafil
Molecular structure(Kukreja et al., 2004)			
Bioavailability	40%	14.5%	N/A
Tmax	1h	40min	2h
T1/2	4h	4h	17.5h
Metabolism	CYP3A4, CYP2C9	CYP3A4, CYP3A5 CYP2C	CYP3A4
Active metabolite	Yes (20% of activity)	Yes (7% of activity)	No
Elimination	Feces	Feces 91-95%, urine 2-6%	Feces 61%, urine 36%

Recently some researchers questioned the role of PDE5 in the cardiac cGMP-PKG signaling, as they failed to detect PDE5 in tissue lysates (mouse, canine and human) and to increase hypertrophy by creating cardiac specific PDE5 knock out animals (Degen et al., 2015; Lukowski et al., 2010).

1.3.3.1. Protection against ischemia-reperfusion injury

Both sildenafil and vardenafil were effective not only when administered prior ischemia, but when given at the time of reperfusion as well. Preclinical data on I/R injury and ischemic cardiomyopathy showed beneficial effects of PDE5 inhibition on collagen deposition and verified functional therapeutic benefits with both echocardiography and invasive pressure volume analysis of the left ventricle (Pokreisz et al., 2009; Salloum et al., 2014; Sesti et al., 2007).

To exert cardio-protection these agents influence mitochondrial K_{ATP} and mitochondrial Ca^{2+} channels, and the cGMP/PKG pathway also confers protection. Beside PKG activation enhanced eNOS and iNOS expression and an increased Bcl-2/Bax ratio also mediate cardioprotection (Chau et al., 2011; Salloum et al., 2014, 2008). Tadalafil also attenuated ischemia-reperfusion injury via PKG dependent generation of hydrogen sulfide (Salloum et al., 2009). In vitro studies proved that these anti-necrotic and apoptotic effects are independent from the vasculature and hemodynamics (Das et al., 2015a).

Myocardial infarct size reduction was also observed in diabetic models, which are known to be refractory to some cardio-protective modalities. Chronic tadalafil treatment improves cardiac function, NO bioavailability and also promotes antioxidant like effects (suppresses ROS production, NADPH oxidase activity, lipid peroxidation and oxidizes glutathione) (Das et al., 2015a; Koka et al., 2014, 2013).

1.3.3.2. Protection against ischemic and doxorubicin induced cardiomyopathy

Sildenafil and tadalafil attenuated ischemic cardiomyopathy and slowed down heart failure progression. Beneficial effects are associated among others with the activation of Rho kinases and the production of hydrogen sulfide (Das et al., 2015a; Salloum et al., 2014, 2009).

Both sildenafil and tadalafil improved left ventricular function in doxorubicin induced cardiomyopathy, without interfering with the chemotherapeutic benefits (Das et al., 2015a).

1.3.3.4. Protection against cardiac hypertrophy:

PDE5 inhibition restores cGMP signaling and blunts the Akt-PI3K, the calcinerurin / NFAT and ERK 1 / 2 signaling pathways, both known to contribute to cardiac hypertrophy (Bueno et al., 2000; Molkentin et al., 1998; Takimoto et al., 2005). Sildenafil treatment is known to suppress pressure overload induced cardiac hypertrophy and recently it was shown that its cardioprotective effect is estrogen dependent (Sasaki et al., 2014; Takimoto et al., 2005). Marked PDE5 expression was found in the left ventricle of patients with end-stage ischemic or dilated cardiomyopathy and blunted cGMP response in PDE5 overexpressing animals lead a more marked impairment of LV systolic and diastolic function (Pokreisz et al., 2009).

After the first unsuccessful trials preclinical data were reevaluated and the presence of PDE5 in cardiomyocytes questioned. These authors link the anti-hypertrophic effects to myofibroblasts and other cell types. In addition it was suggested that PDE5 inhibitor mediated cardioprotective effects are linked to the inhibition of PDE1C by the large compound concentrations used in the preclinical studies (Lukowski et al., 2014).

1.3.4. sGC stimulators and activators

Nitric oxide is not the only ligand capable to induce sGC activation. Nitrosoalkanes, alkyl isocyanides and CO can bind to the sGC heme, albeit they only weakly activate it (Derbyshire and Marletta, 2012; Priviero and Webb, 2010). The search for sGC modulating drugs and resulted in identification of activators and stimulators of sGC (Derbyshire and Marletta, 2012).

1.3.4.1 sGC activators

Activators act independent of NO and of the prosthetic heme group as well. The most well-known activator of sGC is Cinaciguat (BAY 58-2667) and can activate regulatory sites on both α- and β- subunits of the enzyme. During oxidative stress, when the enzyme is exposed to superoxide and peroxynitrite, elevation in the oxidation induced

heme-free sGC levels increases Cinaciguat potency (Mitrovic et al., 2011; Schmidt et al., 2009). In addition this compound also stabilizes sGC, prolongs its half-life and can reverse the oxidation induced enzyme degradation (Derbyshire and Marletta, 2012; Schmidt et al., 2009). A number of clinical studies investigated Cinaciguat in heart failure and found beneficial effects on cardiac output and pulmonary pressure with accompanied hypotension (Mitrovic et al., 2011).

Another very promising agent is Ataciguat, which has already proven beneficial effects in post-MI heart failure and improved chronic heart failure without lowering blood pressure (Fraccarollo et al., 2014).

1.3.4.2. sGC stimulators

The sGC stimulators are heme dependent and potentiate the effects of NO and CO. YC-1 was the first stimulating agent to be described, but was less potent than NO donors in inducing vasorelaxation. The search for more potent drugs led to the synthesis of pyrazolopyridinylpyrimidines derivatives: BAY 41-2272, BAY 41-8543, AND BAY 63-2521 (Priviero and Webb, 2010). Clinical studies evaluating Riociguat (BAY 63-2521, trade name Adempas) in pulmonary hypertension and heart failure revealed positive effects and good tolerability. This is the first of its class to get FDA approval (chronic thromboembolic pulmonary hypertension and pulmonary arterial hypertension) (Derbyshire and Marletta, 2012; Mitrovic et al., 2011).

Under pathologic conditions with increased oxidative stress sGC oxidation and degradation diminishes the effect of stimulator molecules (Priviero and Webb, 2010).

1.4. The role of NOS in physical conditioning

1.4.1. Structural changes in the left and right ventricles among athletes

Intensive physical conditioning is associated with development of enlarged myocardial mass, resulting in both left- and right ventricular (LV, RV respectively) hypertrophy and increased stroke volumes at rest. This phenomenon is usually referred to as athlete's heart (Prior and Gerche, 2012; Scharf et al., 2010; Scharhag et al., 2002). Despite different workload between sport disciplines (strength, endurance and combined strength and endurance) LV mass increases in a similar fashion in all athletes (Prior and Gerche, 2012). (Although data are somewhat conflicting (Baggish and Wood, 2011).)

However in endurance athletes the load on the right ventricle can be disproportionately higher than the load on the left ventricle (LA Gerche et al., 2011; Perseghin et al., 2007). Little is known about athletes engaged in static exercise, although one study reported RV volume increase in sprinters (Prior and Gerche, 2012).

1.4.2. Functional changes in the left and right ventricles among athletes

Compared to pathological remodeling athletes heart is known to have preserved or even enhanced left ventricular function. When comparing ejection fraction, athletes have values close to general public (with the exception of endurance athletes with dilated hearts: their ejection fraction can be as low as ~40%), suggesting that less vigorous contractions are sufficient to maintain circulation. There is a shift in the focus of interest towards strain and strain rate measurements to assess both systolic and diastolic function. These studies on diastolic filling suggested that cardiac remodeling in athletes heart is not associated with diastolic dysfunction and that athletes may be able to utilize the Frank-Starling mechanism more effectively (Baggish and Wood, 2011; Prior and Gerche, 2012).

As part of a balanced, biventricular enlargement right ventricular function shows similar changes: ejection fraction is comparable with untrained individuals, however RV deformation is reduced. Studying RV function under exercise demands could be the key to unveil more characteristic differences in athlete's heart (Baggish and Wood, 2011; Prior and Gerche, 2012).

Rigorous training also results in electrical remodeling and distinct ECG changes. Sinus bradycardia, first degree AV block, incomplete RBBB, early repolarization and voltage criteria for LVH may be considered normal, while other abnormalities such as T wave inversion, complete bundle branch blocks, and left atrial enlargement on ECG are not regarded as training related changes and further evaluation is recommended (Corrado et al., 2010; Prior and Gerche, 2012).

Positive correlation was observed with myocardial mass of both ventricles and with maximal oxygen uptake - a marker of maximal work capacity (Scharhag et al., 2002). Recently total heart volume was introduced as an independent predictor of maximal work capacity in both athletes and non-athletic individuals (Steding et al., 2010).

1.4.3. Mechanisms of athletic cardiac remodelling

In athlete's heart myocardial mass increases with minimal extracellular expansion and is mainly mediated by trophic hormones. Insulin-like growth factor 1 (IGF1) elevation positively correlates with LV mass index and LV end diastolic dimension index (Weeks and McMullen, 2011). IGF1 receptor stimulation leads to phosphoinositide 3-kinase p110α [PI3K(p110α)] activation. PI3K(p110α) is a lipid kinase that is responsible for phosphatidylinositol 3,4,5-trisphosphate (PIP3) formation. PIP3 acts as a second messenger to cause downstream signaling events, such as phosphorylation and activation of Akt, a serine/threonine kinase that plays a central role in cardiac myocyte growth and survival via its effects on protein synthesis and apoptosis. PI3K(p110α) and Akt are critical for normal heart growth, and growth in response to exercise training (Ellison et al., 2012; Weeks and McMullen, 2011). Myocardial IGF-1 overexpression also increases the survival and number of endogenous cardiac stem and progenitor cells (eCSC) and prevents myocyte attrition during ageing (Ellison et al., 2012).

Exercise training is one of the most powerful regulators of vascular eNOS activity (Indolfi et al., 2002). eNOS signaling is involved with length-dependent increase in cardiac contraction force, regulates mobilization, recruitment, migration and differentiation of cardiac and vascular progenitor cells and at least in part induces c-kitpos eCSC activation and ensuing vascular/endothelial differentiation, as well as homing of circulating bone marrow-derived progenitor cells and their differentiation into vascular lineages (Ellison et al., 2012).

Modulation of SERCA2a and phospholamban activity explain faster Ca^{2+} transient decay rate after exercise training. Akt and NOS signaling (as discussed in section 1.1.8.1.3.1.) have also been shown to modulate LTCC stability and phospholamban-SERCA interaction, which could influence cardiomyocyte Ca^{2+} entry, handling and contractility (Ellison et al., 2012).

1.4.4. Possible interactions of NO and NOS3 polymorphisms with athletic cardiac remodelling

Nitric oxide (NO) influences exercise performance through effects on skeletal muscles and cardiac function as well (Matter et al., 1999; Stamler and Meissner, 2001).

Genetic variants of the main cardiac NO source, the endothelial NO synthase may have an impact on NO bioavailability and NOS signaling.

The 894 G/T variant of the *NOS3* gene predicts a Glu to Asp amino acid substitution at codon 298 in the mature protein and results in decreased NO production *in vitro* (Tesauro et al., 2000). This genetic variant also associates with sport-related cardiac adaptation, since non-athletic carriers of the Asp allelic variant developed higher stroke volumes and heart rates during sub-maximal exercise following a long-term endurance training (Hand et al., 2006). The genotype distribution of this variant shows no difference between elite endurance athletes and sedentary controls (Wolfarth et al., 2008). However, elite male triathletes of the South African Ironman Triathlon, with the NOS 298 Glu/Glu genotype had better athletic performance with significantly lower finishing times than individuals carrying the Asp allelic variant (Saunders et al., 2006).

1.4.5 Exercise induced right ventricular dysfunction

Compensatory mechanisms – reduction of vascular resistance and increase of compliance – are limited in the lesser circulation (La Gerche et al., 2014). Extreme exercise will result in a disproportionate load on the right ventricle and may promote pro-arrhythmic remodeling in athletes. This remodeling, termed by some as exercise induced arrhythmogenic right ventricular cardiomyopathy, might be the underlying cause for a higher risk of major arrhythmias in endurance athletes (Gerche et al., 2012; Heidbüchel et al., 2003; Heidbüchel and La Gerche, 2012; LA Gerche et al., 2011; La Gerche et al., 2010).

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2. Objective: to identify new aspects of NOS signaling under physiologic and pathophysiologic conditions

Nitric oxide signaling is a fundamental part of cardiovascular regulation in both physiologic and pathophysiologic processes. My work addresses two distinct and special aspects of NO mediated cardioprotection: reduction of ischemia reperfusion injury and adverse ischemic remodelling, and athletic adaptation of the human heart.

2.1 Murine experiment on potentiation of NO derived cardioprotection

A murine ischemia-reperfusion study was conducted to assess the potential benefit of concomitant cGMP generation using inhaled NO (iNO) and inhibition of its degradation by PDE5 inhibition using tadalafil (TAD). My objectives were to:

- Establish a reproducible murine I/R model.
- Evaluate the effect of iNO and TAD therapies on functional and structural changes after ischemia reperfusion injury.

2.2. Clinical study on the role of NOS3 polymorphisms in physiologic adaptation in elite athletes

Effects of genetically determined changes of NO bioavailability on exercise induced cardiac adaptation were studied in elite athletes with single gene approach. My objectives were:

- To establish a DNA extraction and single nucleotide polymorphism (SNP) analysis protocol for local screening programs.
- To assess the influence of the Glu298Asp polymorphism in NOS3 gene on athletic adaptation

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3. Methods

3.1. Murine experiment on potentiation of the NO mediated cardioprotection with PDE5 inhibition

3.1.1. Drugs used in the experiments

Tadalafil (commercial name Cialis): tadalafil activity is unaffected by food and has a relatively short time to onset of action (16 to 17 minutes). Maximal plasma concentration is reached within 2.0 hours and it has an elimination half-life of 17.5 hours. Tadalafil is a highly selective inhibitor of PDE with 10 000-fold selectivity for PDE-5 over PDE-1 to PDE-4 and approximately 700-fold selectivity for PDE-5 over PDE-6 (Salloum et al., 2009). As it is not water soluble, a 30% cyclodextrane solution was used as medium. Solution concentration was 0.4ug/ul.

Inhaled nitric oxide (iNO): The INOVent gas delivery system is an older type clinical equipment currently applied in the experimental animal laboratory at the Catholic University Leuven for nitric oxide gas delivery to intubated animals in range from mice to pigs via a dedicated ventilator. Two cylinders containing nitric oxide balanced in nitrogen are attached to the delivery device with gas-tight specific tubing with US-type connectors. Gas content is 10L / cylinder (Product code [according to manufacturer]: 660.011.01, Pressure: 155 bar [corresponds to 1535 L of gas at 1 bar pressure], manufacturer: Ino Therapeutics LLC, Hampton, NJ 08827, United States)

3.1.2. Induction of I/R and experimental design

Animal experiments were approved by the Ethics Committee for Animal Experimentation (KU Leuven) and were performed in accordance with the Guide for Care and Use of Laboratory Animals (NIH). C57Bl6J mice (Charles River Laboratories, Chatillon-sur-Chalaronne, France) were housed in temperature and light-cycle controlled facilities and had access to rodent chow and water ad libitum. Age matched (8-10 weeks old), adult, male mice (20-35g) were anesthetized using sodium pentobarbital (40-60 mg/kg, IP Nembutal, Sanofi Synthelabo, Belgium) supplemented with morphine hydrochloride (0.5-1 mg/kg, SC, Stellorphine, Sterop Laboratories, Brussels, Belgium) and ventilated with room air using 250 µL tidal volume at 150 strokes/min (Miniventilator, Hugo Sachs Elektronik, Germany). Depth of anesthesia was controlled

by pedal withdrawal reflex during the entire procedure. Following a left sided thoracotomy, ischemia was induced by 60 min transient ligation of the left anterior descending artery (LAD) using 7-0 silk suture (Ethicon, Johnson&Johnson, Brussels, Belgium). After one hour, the ligation was released and blood flow restored. Wounds were closed using 6-0 Ticron suture (Sherwood Davis&Geck, Quebeck, Canada) and animals were allowed to recover in temperature-controlled cages. Post-operative pain suppression (buprenorphine, 100 μg/kg IP, Temgesic, Schering-Plough, Hull, UK) was administered during the first two post-operative days.

Mice were randomized after I/R into four treatment groups and followed for four weeks (4w): untreated CON (CON, n4w=17), inhaled nitric oxide (iNO, n4w=17), tadalafil (TAD, n4w=16) and combination treatment with iNO and TAD (iNO+TAD,

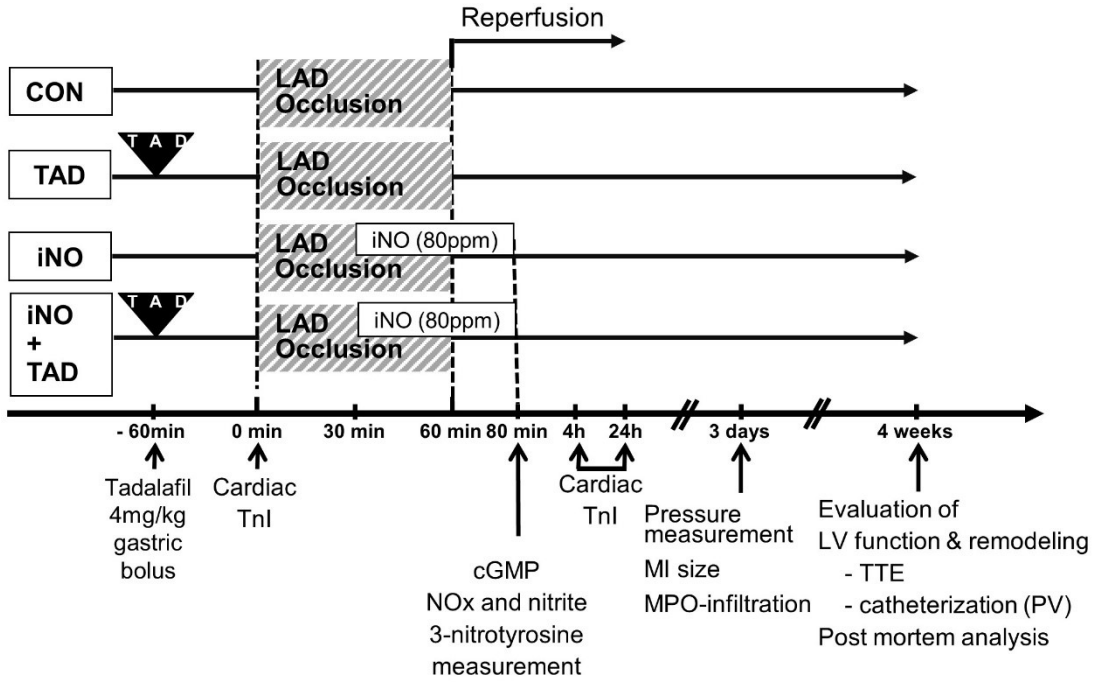

Figure 4 Experimental design. Four different study groups were established: ischemia-reperfusion without additional treatment (CON), with inhaled nitric oxide (iNO), with gastric tadalafil administration (TAD, 4 mg/kg single bolus), or with combined treatment (iNO+TAD). Subgroups of mice were euthanized immediately after NO inhalation (80 min), at three days for analysis of myocardial infarct size (IS) and myeloperoxidase-positive (MPO) cell infiltration and at four weeks after LAD occlusion for transthoracic echocardiography (TTE) and pressure volume (PV) catheterization.

n4w=15, Figure 4). An additional subset of mice in these 4 groups was studied after 3 days to evaluate infarct size and determine inflammatory cell infiltration. Nitric oxide (Ino Therapeutics LLC, Hampton, NJ, 80 ppm in room air) was administered through an intratracheal tube during mechanical ventilation and was started 30 minutes prior to and continued for 20 minutes after reperfusion. Dose and timing of NO inhalation was based on previously reported results (Hataishi et al., 2006). Tadalafil (Kemprotec Ltd., Cumbria, UK) dissolved in 30% solution of 2-hydroxypropyl-β-cyclodextrin (#C0926, Sigma-Aldrich) was administered via gastric gavage (4mg/kg) one hour prior to I/R, which was determined based on previously reported interspecies dose extrapolations. (Ahmad et al., 2009; Salloum et al., 2009; Koka et al., 2010) To determine acute cardiac cGMP responses to myocardial ischemia during different treatment regimens, an additional subset of animals (n=6-8 per treatment arm) was euthanized 20 min after reperfusion (Figure 4).

3.1.3. Echocardiography

Transthoracic echocardiography (TTE) was performed using a MS 400 transducer (18-38 MHz) connected to a Vevo 2100 scanner (Visualsonics Inc., Toronto, Canada) in anesthetized (2% isoflurane in oxygen, Ecuphar, Oostkamp, Belgium), temperature-controlled mice. Recordings were evaluated using the Vevo dedicated cardiac software and LV dimensions at end-diastole (LVIDd) and end-systole (LVIDs), interventricular septum and posterior wall thickness at end-diastole and end-systole (IVSd, IVSs, LVPWd, LVPWs) were measured, wall-thickening and fractional shortening (FS) were calculated.

3.1.4 Invasive hemodynamic measurements

Invasive blood pressure measurement was performed in all animals at day 3 prior to vital staining of the myocardium. In mice followed for 4 weeks, invasive pressure-conductance hemodynamic recordings were performed using urethane, etomidate and morphine hydrochloride (1000, 1 and 0.5-1 mg/g body weight, IP) anesthesia supplemented with pancuronium bromide (PAVULON, 2 mg/kg IP) neuromuscular blockade. Mechanical ventilation was performed using room air at tidal volume 7 µL/g BW (MiniVent, Hugo Sachs Elektronik, Germany) and fluid homeostasis was supported by 80-100 µL/30g infusion of 15% albumin in physiologic saline and body temperature

was maintained at 37ºC using an infrared lamp connected to a T-thermocouple rectal probe (Hugo Sachs, Germany). A 1.0-F pressure-conductance catheter (PVR1035, Millar Instruments, TX) was inserted in the LV via the right carotid artery and steady-state LV pressure-volumes were recorded after 10 min stabilization. To obtain occlusion loops with progressively lowering preload, the inferior vena cava was compressed between liver and diaphragm with a cotton swab without opening the abdomen. Parallel conductance, attributable to cardiac muscle and connective tissues was recorded after infinitesimal volume (5-6 µL 15% NaCl solution) IV injection and deducted from the total volume. After all measurements were completed, blood was withdrawn from the inferior vena cava using a 24G heparinized needle and used for cuvette calibration. Recorded measurements were evaluated using the P-V module of the Chart software version 7.5 (ADInstruments, UK). All data were inferred from the average of measurements with breath holding during the expiratory phase. Each measurement represents at least 10 successive baseline loops. Indices of systolic and diastolic function were calculated including stroke volume (SV), cardiac output (CO), ejection fraction (EF), stroke work (SW), preload-recruited stroke work (PRSW), arterial elastance (Ea), ventricular elastance (Ees), ventricular-arterial coupling (Ea/Ees ratio), maximum and minimum rates of systolic pressure rise or decline (dP/dtmax and dP/dtmin) and the time constant of isovolumic relaxation (τ) according to Weiss' method.

3.1.5. Measurement of infarct size and myocardial necrosis

Three days after I/R, a subset of mice was re-anesthetized and the initial left thoracotomy was reopened. To delineate the perfused area, blue tissue marking dye (24111 Marking Dye for Tissue, Polysciences Inc., Warrington, PA) was injected via the right carotid artery following repeated ligature of the LAD. Saturated potassium chloride was injected and hearts were excised and embedded into low-gelling temperature agarose blocks (Agarose Type VII-A, #0701, Sigma). Specimens were cut into 500-µm thick slices using a vibratome (VT1000S, Leica Microsystems, Diegem, Belgium) and stained with triphenyl tetrazolium chloride (TTC) for 5 minutes at 37ºC. White colored infarcted versus blue colored non-ischemic and red colored risk area (AAR) were identified on digital images (Canon EOS 5D camera, EF 100mm f/2.8 Macro USM lens) and analyzed by planimetry.

To determine myocardial necrosis, circulating troponin I (TnI) was measured in plasma retrieved from mixed tail blood samples from sedated animals before I/R (Baseline) and 4, and 24 hours after I/R. Plasma TnI was measured using ELISA (#2010-1-HSP, Life Diagnostics, West Chester, PA) according to the manufacturer's instructions.

3.1.6. Measurement of tissue and circulating cGMP levels

Blood samples were collected using isoflurane anesthesia into 3-isobutyl-1-methylxanthine-containing tubes (150 μM final concentration) and plasma fractions were archived. Following euthanasia by cervical dislocation, LVs were pulverized using liquid nitrogen and extracted using 500 μL 6% trichloroacetic acid followed by 3 isovolumic extractions of water-saturated diethyl ether. Aquatic fractions were lyophilized and used for cGMP measurements. Plasmatic and cardiac cGMP levels were determined using Biotrack cGMP enzyme immunoassay (RPN 226, GE Healthcare, Belgium) according to the manufacturer's instructions.

3.1.7. Measurement of nitric oxide-derived oxidative end products

In blood samples collected 20 min after I/R, plasma was separated and supplemented with N-ethylmaleimide (8 mM final concentration) to protect thiol-groups and stored frozen at -80 oC until analysis. Nitrites (NO2-) were reduced using the triiodide reagent, while nitrites, nitrates and S-nitroso compounds expressed as NOx were converted using vanadium (III)-chloride to NO, followed by ozone-based chemiluminescence measurement in line with the Sievers Model 280i analyzer (GE Analytical Instruments, Boulder, CO) as described previously. (Yang et al., 2003; MacArthur et al., 2007) Cardiac tissue collected 20 min after reperfusion was homogenized under liquid nitrogen and extracted in T-PER reagent (#78510, ThermoFisher Scientific). Protein concentration was measured by bicinchoninic acid assay (BCA assay, #23227, ThermoFisher Scientific) and adjusted to 5 mg/mL. 3-Nitrotyrosine content was determined in cardiac extracts and plasma using OxiSelect™ Nitrotyrosine ELISA Kit (Cell Biolabs Inc, San Diego, CA).

3.1.8. Histological and immune-histochemical determination of collagen deposition and myeloperoxidase-positive cell infiltration

To assess mononuclear cell infiltration 3 days after I/R, myeloperoxidase (MPO) staining was performed using rabbit anti-human MPO antibody (#A0398, Dako, Belgium). Mosaic scans of MPO-stained LV sections were used to count the number of cells infiltrating the LV septal and LV free wall at three different planes distal to the site of LAD ligation. Collagen deposition was measured in a semi-quantitative manner on Sirius red-stained myocardial sections at three different planes and related to LV tissue area. Analysis was performed using color thresholding: red color attributable to interstitial collagen was quantified, related to LV area and expressed as the average relative collagen percentage for each animal. Mosaic images were scanned using the automated Mozaix function of the AxioVision 4.6 software on an AxioVert 200 microscope (Carl Zeiss, N.V., Zaventem, Belgium) and different myocardial planes were evaluated using the ImageJ software (ver. 1.47s, NIH, Bethesda, MD).

3.1.9. Statistical analysis

All data are expressed as mean ± SEM. Differences between groups were determined using one-way ANOVA with Bonferroni's post-hoc test. For time-dependent follow-up of cTnI, two-way ANOVA with Dunnett's test for multiple comparisons versus untreated CON was applied. Non-Gaussian distributed MPO-cell infiltration data were compared using Kruskal-Wallis method with Dunn's post-hoc test. Probability value of $p<0.05$ for all tests was considered statically significant. Statistical analysis was performed using GraphPad Prism 6 software (ver. 6.04, GraphPad Inc., La Jolla, CA).

3.2. Clinical study on the role of NOS3 polymorphisms in physiologic adaptation in elite athletes

3.2.1. Selection of candidate individuals and study protocol

Hungarian athletes were screened and selected on the basis of event/sport participation, high level qualification and recent international representation. Inclusion criteria consisted of at least 10 years of national and 3 years of international qualifications (world championships and / or Olympic Games). (Figure 5.) Athletes with VO2

maximum greater than 50ml/kg/min during cardiopulmonary stress test using a bicycle ergometer were referred to cardiac magnetic resonance (cMRI). Eight players were excluded due to low VO2 maximum (<50ml/kg/min) and eleven athletes did not complete the cardiac magnetic resonance examination (cMRI) due to intolerance (Figure 5.). Sport disciplines with mixed exertion load, including speed, strength and endurance components were selected, therefore water polo players (n=48), kayakers (n=21), canoeists (n=19), rowers (n=22) and swimmers (n=16) were involved. Majority of the water polo players were Olympic level athletes (36/48), while in the kayaker group 10/21, in the canoeist group 11/19, in the rower group 12/22 and in the swimmer group 13/16 Olympic level athletes – among them 39 gold medalists - were screened. Training protocol of all examined sportsmen contained mainly strength training. Age and sex matched individuals (n=162) were screened for the control group. Since lower VO2 maximum consumption level (<50ml/kg/min) was part of the inclusion criteria in this group, control individuals with higher than 50ml/kg/min VO2 maximum (n=3) were excluded from the study and four volunteers who could not tolerate cMRI (n=4) were not able to complete the study protocol.

In this study, all athletes belong to the same ethnic group, are subjected to similar environmental factors including dietary habits, smoking status (only 6/126 athletes were active smokers during the study), duration of elite athletic status and timing (during season) of the examinations.

3.2.2. Screening protocol

Stress test was performed in both athletes and non-athletes using a bicycle ergometer with an “all-out” protocol. Athletes with a VO2 maximum greater than 50ml/kg/min and controls with VO2 maximum lower than 50ml/kg/min were referred for cardiac magnetic resonance imaging. Blood samples for DNA isolation were collected at the first visit. Athletes were all tested and measured during their competitive season period in two consecutive years. Participation, including cardiac magnetic resonance imaging and blood sampling for DNA extraction was voluntary and part of a prospective athletic screening program. Written informed consent was collected from all participants and approval of the Hungarian Scientific Council National Ethics Committee for Scientific Research (ETT-TUKEB 13687-1/2011) was provided before data and samples

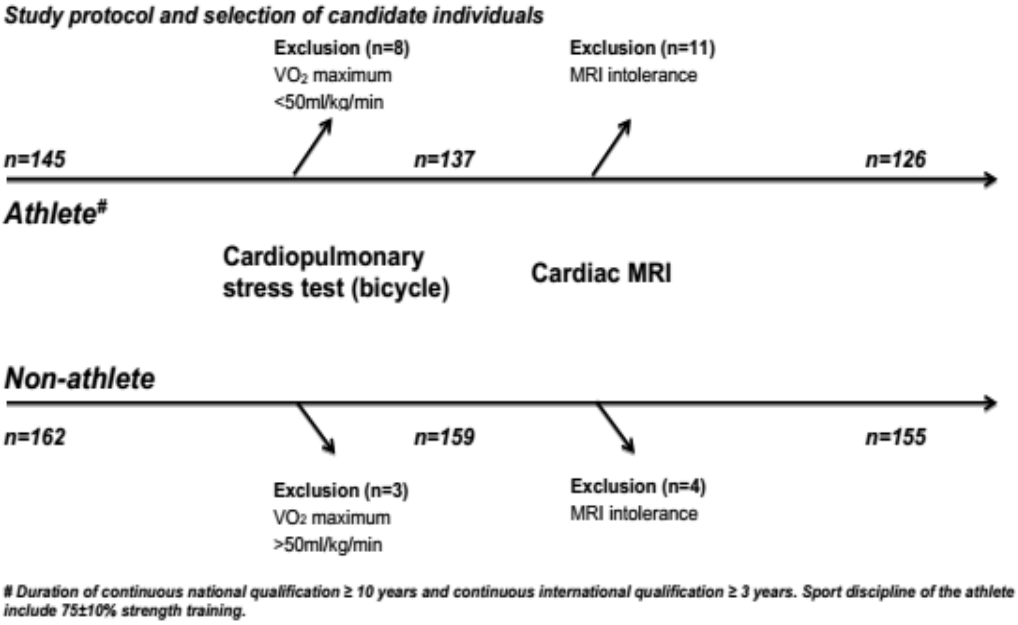

Figure 5. Study protocol and selection of candidate individuals. Top level Hungarian athletes (n=145) and healthy control individuals were screened. Athletes above and controls under a VO2 maximum greater than 50ml/kg/min were referred to cardiac magnetic resonance (cMRI). Eight athletes were excluded due to low VO2 maximum (<50ml/kg/min) and eleven athletes did not complete the cardiac magnetic resonance examination (cMRI) due cMRI intolerance. Control individuals with higher than 50ml/kg/min VO2 maximum (n=3) were excluded and four volunteers could not tolerate cMRI (n=4).

were used in this study. The Hungarian Scientific Council National Ethics Committee for Scientific Research (ETT-TUKEB) is a supreme authority of the Semmelweis University Ethics Committee, which received and approved the national level decision.

3.2.3. Cardiopulmonary stress test

A continuous ramp test to exhaustion was performed on an electromagnetically braked bicycle ergometer (Ergoline Ergometrics 900, Bitz, Germany). The initial exercise load was 50 W and increased in a linear ramp pattern with 25 W every 60 seconds. Athletes and non-athlete individuals were asked to continuously pedal until exhaustion, maintaining constant revolutions-per-minute at 40-50 rpm. Gas exchange parameters and ventilatory variables were recorded breath-by-breath (PowerCube, Ganshorn Medizin, Niederlauer, Germany). Vital parameters and blood lactate levels were measured before

and after the stress test. To quantify perceived exertion during physical activity the rating of perceived exertion was collected and exhaustion was defined by completing the BORG scale. The Borg scale ranges from 6 to 20, where 6 means “no exertion at all” and 20 means “maximal exertion”. Numbers, that best describe their level of exertion, are chosen by tested individuals and would be expected to coincide with their heart rate (e.g. BORG 10 = 100 beats/minute)

3.2.3. Cardiac magnetic resonance imaging (cMRI)

Cardiac MRI scans were performed on a Philips Achieva 1.5T magnet. (Philips Healthcare, Eindhoven, The Nederlands). The imaging instrument had Dual Nova HP gradients (maximum strength: 33/66 mT/m, slew rate: 180/90 mT/m/ms) and running software version R2.5.3 and recently R2.6.3. (Philips Healthcare, Eindhoven, The Nederlands) Five element cardiac coil was used for signal reception. The MR protocol included retrospectively gated balanced steady-state free precession cine movies in three long axis orientations (two-chamber, four-chamber and left-ventricular outflow tract views) and short axis slices covering both ventricles. Slice thickness was 8mm, while inter-slice gap was set to zero. Each cardiac cycle was divided into 25 to 30 phases. Triggered blood suppressed T2-weighted spectral inversion recovery (T2w-SPIR) sequence was used for edema detection. Delayed enhancement images were recorded in the same views as the cine movies to assess abnormal contrast uptake. While administering the Gadovist contrast (0.125mmol/kg IV) k-t BLAST (Broad-use Linear Acquisition Speed-up Technique) balanced turbo field echo (b-TFE) sequence was used to capture rest perfusion datasets of the three long axis slices. Respiratory motions were corrected using breath-holds in end-expiration. In selected cases coronary origins were depicted using a fat-suppressed 3-dimensional b-TFE sequence utilizing respiratory navigator with prospective motion correction. Medis QMASS MR 7.1 and 7.2 (Medis medical imaging systems bv, Leiden, The Netherlands) were used for evaluation. Endocardial and epicardial contours were traced manually for both the left and the right ventricles and volumetric measures (including papillary muscles), ejection fractions, maximal end-diastolic wall thickness and maximal end-diastolic wall thickness, left ventricular end diastolic volume index ratios were determined (LA Gerche et al., 2011). Body height and weight were measured and archived in SI units. Body surface area was

calculated using the Mosteller formula based on the subject's weight and height (BSA (m2) = (height (cm) x weight (kg)/3600)½).

3.2.4. DNA extraction and genotyping

Genomic DNA was isolated from whole peripheral blood with a protease based technique (Flexigene DNA System, Qiagen, Hilden, Germany). Samples (1 ml) were added to a lysis buffer and were thoroughly mixed and centrifuged. After discarding the supernatant, samples were denaturized, DNA was ethanol precipitated and reconstituted in the provided buffer. Samples were stored at -80 °C. Estimation of the DNA yield and quality control was done by spectrophotometry and determination of the 260/280 absorption ratio (Nanodrop-2000, Thermo Scientific, Wilmington, USA). Genotyping of The Glu298Asp single nucleotide polymorphism (dbSNP: rs1799983, OMIM: +163729) was done with RT-qPCR (StepOne Plus, Applied Biosystems). Pre-designed primers were provided by Applied Biosystems (kit number: C___3219460_20) and the reaction was performed according to the manufacturer's protocol. For each run parallel samples with positive controls were used. Genetic analysis was performed blinded to patient data, with the provided software. Results are presented according to the National Heart, Lung, and Blood Institute recommendations on reporting genetic results in research studies (Bookman et al., 2006).

3.2.5. Statistical analysis

Data are presented as mean ± SD for continuous variables, or n (%) for categorical variables. Comparisons between two groups were performed using Student's t-test for continuous variables (MR parameters), chi-square test for categorical data (genotype, gender and athletic status). Analysis of variance (ANOVA) indicated that genotype and athletic status may influence right ventricular indices (post-hoc test: Tukey HSD). Linear regression was used to explore whether gender and genotype are independent predictors for changes in right ventricular stroke volume index (RVSVi) and right ventricular mass index (RVMi). Multivariate analysis was performed on groups based on genotype and athletic status (Aspartate carriers + non-athletes; Non-aspartate carriers + non-athletes; Aspartate carriers + athletes and Non-aspartate carriers + non-athletes). P values less than 0.05 were considered significant. Calculations were performed using the SPSS 22.0 program package (IBM Corporation).

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4. Results

4.1. Murine experiment on potentiation of NO mediated cardioprotection with PDE5 inhibition

4.1.1. Combined therapy with iNO and TAD confers synergistic myocardial protection after I/R

To investigate the respective treatment effect on cardiac myocyte necrosis markers, we measured peak troponin I release at 4h and at 24h after reperfusion. After 4h, peak plasma TnI levels were significantly reduced by iNO (n=9; 17.6±5.1 ng/l) and TAD (n=7; 17.3±5.0 ng/l) compared to untreated CON animals (n=9; 24.6±5.3 ng/l). This effect was further amplified in the combined iNO-TAD treatment arm (n=9; 11.4±2.4 ng/l), resulting in greater than 50% reduction in peak TnI levels 4h after reperfusion (Figure 6.).

To evaluate the effect of pharmacologic intervention on myocardial inflammatory cell infiltration and tissue damage, we determined the number of myeloperoxidase-positive cells and the extent of infarcted area relative to risk area three days after I/R. The

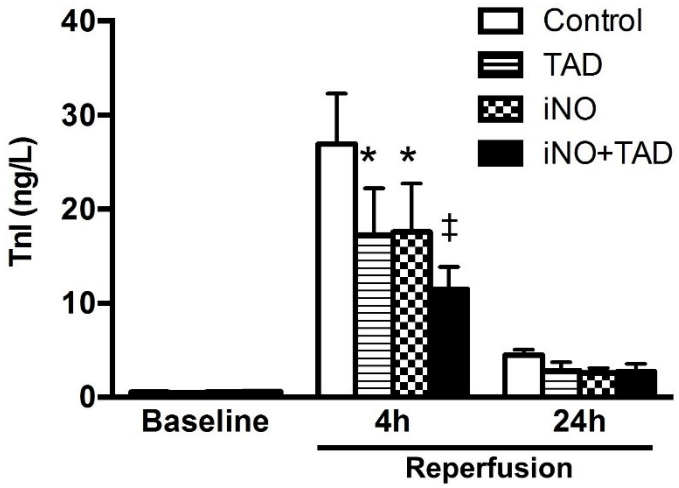

Figure 6. Troponin I (TnI) plasma levels after I/R. Cardiac TnI release increased significantly compared to baseline after the LAD-ligation and peaked at 4h after reperfusion followed by a marked decline within 24 hours. TAD (n=7), iNO (n=9) and iNO+TAD (n=9) treatments all significantly reduced TnI levels compared to non-treated CON (n=9) with most prominent effect achieved in iNO+TAD group. * $P<0.05$, ǂ $P<0.001$ vs CON.

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risk area (AAR) encompassed 57% of the LV area in all groups and did not compromise hemodynamic status or early survival (Table 4.).

Table 4. Invasive LV blood pressure measurements 3 days after I/R.

Treatment	N=	HR (BPM)	LVPmax (mmHg)	LV dP/dt_{max} (mmHg/s)	LV dP/dt_{min} (mmHg/s)
CON	19	612±10	84±3	8584±638	-6727±509
TAD	14	620±17	82±4	9083±946	-6259±521
iNO	11	609±11	81±3	8322±636	6148±312
iNO+TAD	13	625±1	84±3	9038±1087	6512±625

HR- Heart rate in beats per minute (BPM); LVP_{max} – left ventricular maximal pressure, dP/dt_{max} and dP/dt_{min}- peak rate of systolic pressure rise and decline. Measurements were conducted in the left ventricle. All data are presented as mean ± SEM.

The TTC-stained non-viable area within the AAR was significantly smaller in TAD (n=8), iNO (n=5) and iNO-TAD (n=5) animals (27±4%, 22±3% and 24±4%, respectively, versus 43±2% in non-treated CON (n=7) mice, $P<0.05$ for all). Limited discriminatory power of TTC stains and small sample size did not allow further differentiation between treatment groups (Figure 7).

To evaluate the effect of iNO and TAD during I/R on subsequent long term LV structural and functional remodeling, we measured LV dimensions and fractional shortening after 4 weeks using transthoracic echocardiography and performed pressure-volume analysis (Figure 8.)

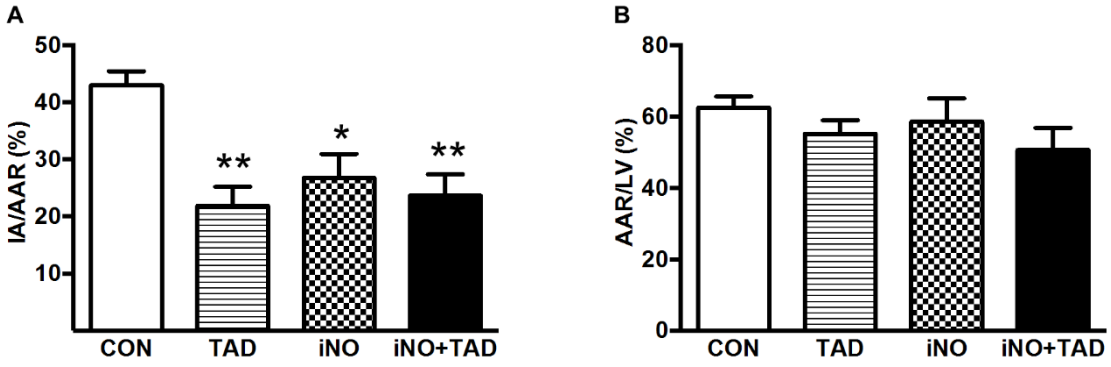

Figure 7. Planimetric analysis of TTC-stained heart sections. All 3 treatment strategies significantly reduced infarcted area (IA) expressed relative to the area at risk (AAR) when compared to non-treated CON animals (panel A). Area at risk expressed as percentage of LV area (AAR/LV area) was comparable in all groups (Panel B; CON n=7, TAD n=8, iNO n=5, iNO+TAD n=5). * $P<0.05$, ** $P<0.01$ vs CON

Pressure-volume analysis showed a significantly greater stroke volume in mice treated with iNO+TAD, resulting in a proportionately higher cardiac output at comparable heart rates between groups (Table 6.). Mice who inhaled NO had an intermediate response with a lesser increase in SV and CO, but higher LV end-systolic pressure and stroke work. Increased preload-recruitable stroke work, a load-independent parameter of contractility, failed to reach statistical significance in iNO+TAD, suggesting that increased stroke volumes may also be accounted for by altered loading conditions (e.g. lower arterial elastance, an index of ventricular afterload) independent of better preserved inotropy. Ventricular elastance, Ees, which defines the end-systolic pressure-volume relation and LV end-systolic stiffness and is considered a useful marker of acute changes in contractile function (Pacher et al., 2008), did not differ appreciably between groups in the chronic post-infarction phase 4 weeks after reperfusion and remained in what is considered the normal range in mice. This is consistent with the absence of overt systolic heart failure in the present I/R model. Consequently, ventricular-vascular coupling indexed by the Ea/Ees ratio did not show major differences between groups. Diastolic function parameters, including LVEDP, dP/dtmin and isovolumic relaxation time index (τ) were comparable between CON and treated groups (Table 6.) and consistent with comparable interstitial collagen deposition pattern (Figure 9.).

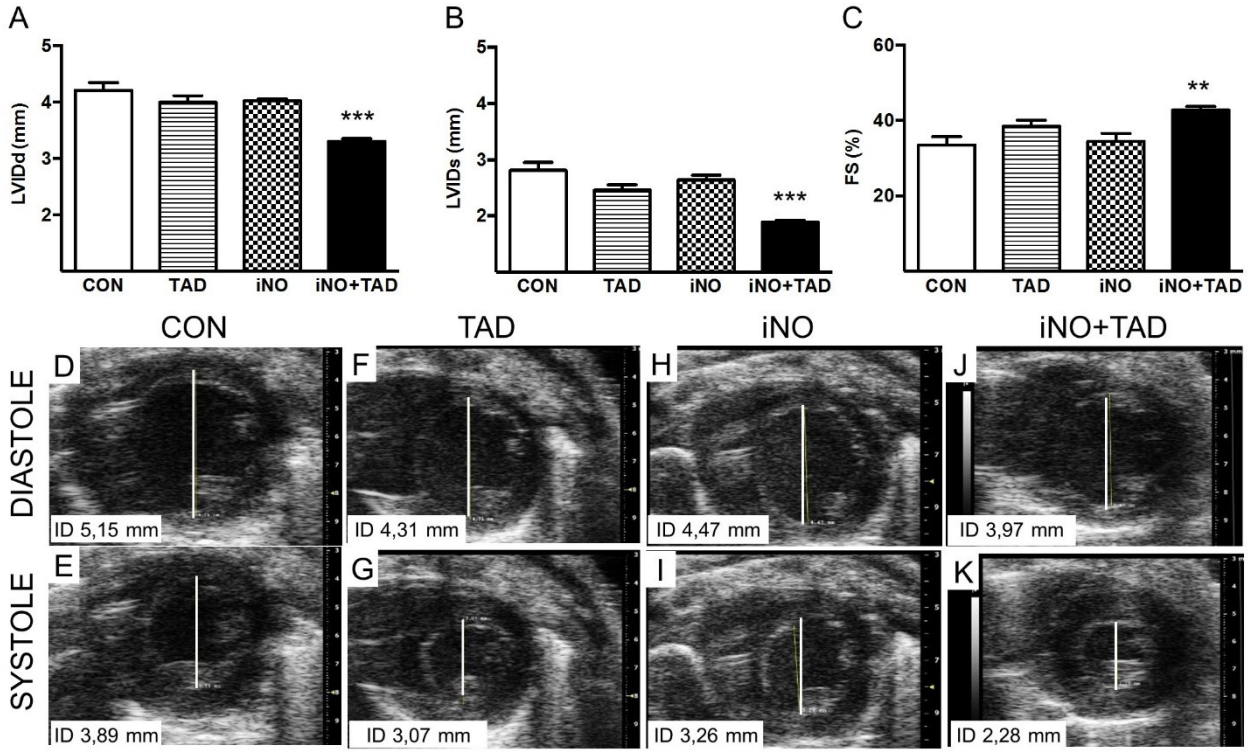

Figure 8. Echocardiographic measurement of cardiac remodelling and function. After 4 weeks, LV internal diameters at end-diastole and end-systole (LVIDd; LVIDs), reflecting the extent of LV dilatation and changes in contractile function, were attenuated in iNO+TAD (combined treatment, n=13) when compared to CON (untreated mice, n=11) or TAD- (Tadalafil, n=12) and iNO- (inhaled Nitric Oxide, n=10) treated mice (Panel A and Panel B). Mice receiving iNO+TAD treatment had a better preserved fractional shortening (FS), an estimate of global LV function, than non-treated CON or single treatment groups (Panel C). Representative B-mode mid-ventricular images are shown of LVID at end-diastole (Panels D-J) and at end-systole (Panels E-K). *** $P<0.001$ vs CON, iNO and TAD and ** $P<0.01$ vs CON and iNO.

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Table 5. TTE measurement of LV dimensions and regional function 4 weeks after ischemia-reperfusion.

Treatment	N (mm)	IVSd (mm)	IVSs (mm)	WT_{IVS} (%)	LVPWd (mm)	LVPWs (mm)	WT_{PW} (%)	HR (BPM)
CON	**11**	1.07±0.05	1.31±0.004	24±6	1.05±0.03	1.23±0.004	18±4	395±16
TAD	**12**	**0.93±0.02** **	1.30±0.01	40±3	0.99±0.01	1.23±0.005	25±1	452±17
iNO	**10**	**0.90±0.03** **	1.32±0.002	**48±5** **	**0.94±0.03** *	**1.27±0.015** **	**35±5** **	442±14
iNO+TAD	**13**	**0.87±0.02** **	1.32±0.007	**52±3** ***	**0.93±0.02** **	1.23±0.003	**32±2** *	450±19

HR- Heart rate in beats per minute (BPM); IVSd and IVSs – diastolic and systolic interventricular septal thickness, LVPWd and LVPWs – diastolic and systolic left ventricular posterior wall thickness, WT_{IVS} and WT_{PW} – percentage of interventricular septal and posterior wall thickening. All data are presented as mean±SEM. CON = untreated mice, TAD = Tadalafil, iNO = inhaled nitric oxide, iNO+TAD = combination treatment. * P<0.05; ** P<0.01; ** P<0.001 versus CON

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Table 6. Pressure-volume analysis of cardiac function at 4 weeks.

	CON (n=11)	**TAD** (n=13)	**iNO** (n=12)	**iNO-TAD** (n=13)
HR (BPM)	601±12	604±14	603±15	609±13
LVESP (mmHg)	78±3	85±4	**89±2***	82±3
LVEDP (mmHg)	2.4±0.6	2.1±0.3	3.5±0.7	2.3±0.5
SV (μL)	10.2±0,9	11.0±1.1	13.6±1.1	**14.9±1.2***
CO (μL/min)	6129±566	6637±713	8277±707	**9156±773***
EF (%)	50.6±4	54±4	54±4	59±3
SW (mmHg x μL)	771±85	932±91	**1205±97***	1134±119
PRSW	69±8	71±6	75±10	94±8
Ea (mmHg/μL)	7.8±1.1	7.0±0.5	6.3±0.7	5.7±0.3
Ees (mmHg/μL)	10.1±2.8	10.3±1.5	6.7±1.1	9.1±2.2
Ea / Ees	1.01±0.16	0.79±0.09	1.10±0.14	0.83±0.13
dP/dt_{max} (mmHg/s)	8795±946	9956±646	11899±879	10617±969
dP/dt_{min} (mmHg/s)	-7498±538	-8248±586	-8930±822	-7837±396
τ (ms)	5.2±0,2	5.2±0.2	5.0±0.3	5.1±0.2

HR- Heart rate in beats per minute (BPM); LVESP and LVEDP - left ventricular end systolic and end diastolic pressures; SV-stroke volume, CO- cardiac output; EF- ejection fraction; SW- stroke work; PRSW-preload-recruitable stroke work; Ea- arterial elastance; Ees – left ventricular end-systolic elastance; Ea/Ees ratio - ventricular-arterial coupling; dP/dt_{max} and dP/dt_{min}- maximum and minimum of systolic pressure change over time; τ- tau time constant of isovolumic relaxation according to Weiss' method. All data are presented as mean±SEM. CON = untreated mice, TAD = Tadalafil, iNO = inhaled nitric oxide, iNO+TAD = combination therapy. * $P<0.05$ versus CON

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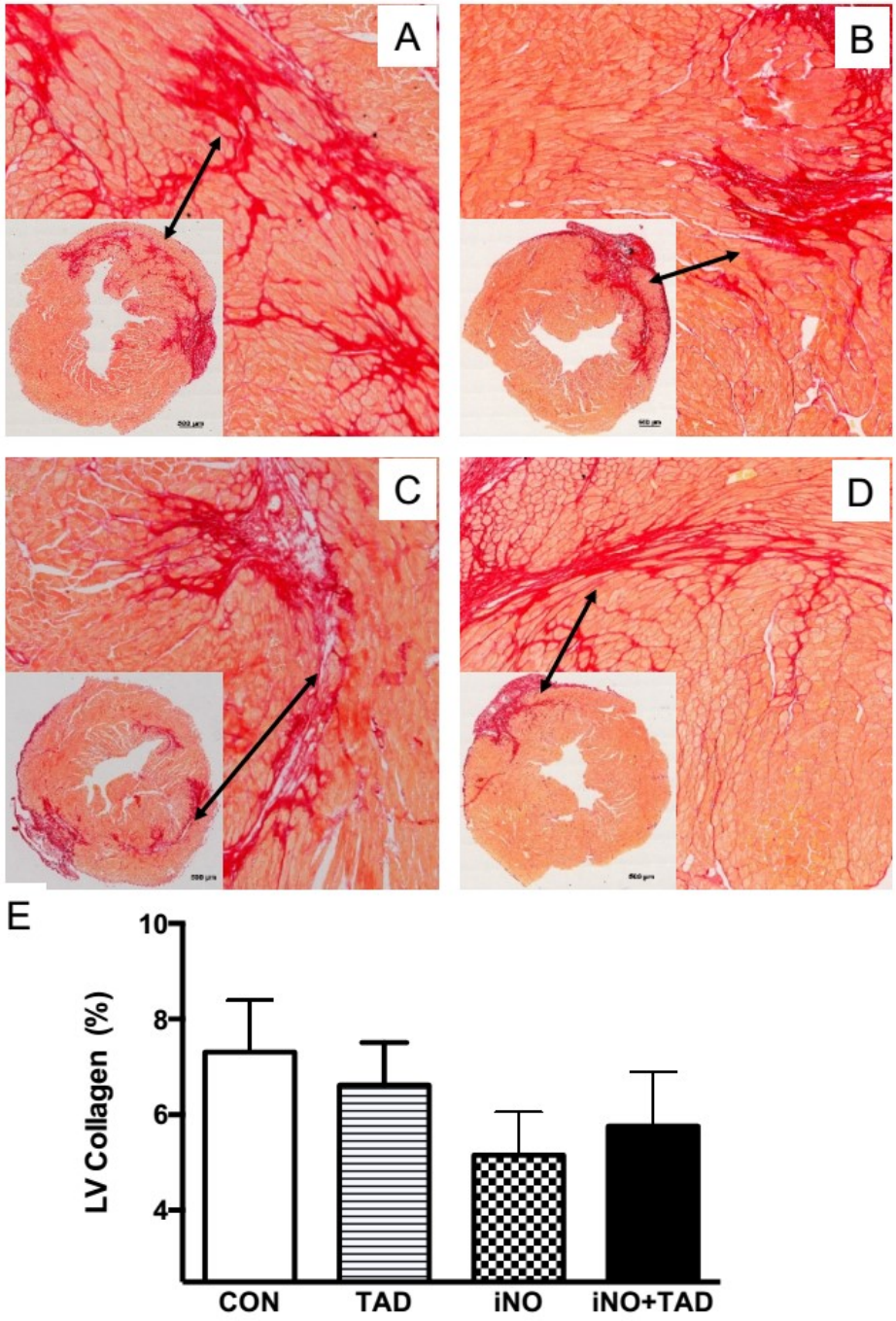

Figure 9. Histomorphometric analysis of LV collagen content. Collagen deposition was measured in a semi-quantitative manner on Sirius red-stained myocardial sections at three different planes and related to LV tissue area. Four weeks after ischemia-reperfusion (I/R) minimal reduction (non significant) of fibrosis was observed in all treatment groups (panel E). CON (panel A) = untreated mice; TAD (panel B) = Tadalafil; iNO (panel C) = inhaled Nitric Oxide; iNO+TAD (panel D) = combination therapy; black arrows point to the enlarged areas.

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4.1.2. iNO and tadalafil treatment modulate NO-cGMP signaling and cardiac nitrosative stress

After inhalation, NO readily forms nitrates, nitrites, nitrosothiols, and iron-nitrosyl adducts, collectively measured as NOx. Upon reaction with superoxide, NO also forms peroxynitrites, which modify tyrosine residues of various proteins. Plasma NOx levels measured in mice immediately after inhalation of iNO with or without TAD were 5- to 6-fold higher than in mice who did not inhale NO ($P<0.0001$ for both, Figure 10.A). Similarly, plasma nitrite concentrations in iNO and iNO+TAD were more than 3-fold higher than in CON and TAD ($P<0.0001$ for both, Figure 10.B). Of interest, nitrotyrosine content in the immediate reperfusion period trended to be lower in reperfused cardiac tissue after inhalation of NO with or without TAD ($P=0.10$ and 0.08, respectively, Figure 4D), while the effect was not detectable in plasma (Figure 10.C).

NO inhalation and PDE5-inhibition also increase cGMP bioavailability via stimulation of guanylate cyclase-mediated synthesis and prevention of phosphodiesterase-mediated cyclic nucleotide breakdown, respectively. To study whether cardioprotection was associated with increased cGMP bioavailability, we measured circulating and cardiac cGMP levels immediately after iNO administration was completed. Plasma cGMP levels showed an approximately 20% non-significant increase after iNO and TAD treatments (48±3 pmol/mL [n=8] and 51±7 pmol/mL [n=6], respectively) versus 38±6 pmol/mL [n=7] in CON, but nearly doubled after iNO-TAD (80±12 pmol/mL [n=7], $P<0.01$ vs CON and iNO, $P<0.05$ vs TAD). In parallel, cardiac tissue cGMP level increased significantly only after iNO-TAD treatment (iNO-TAD 0.15±0.02 pmol/mg [n=7] vs 0.05±0.01 pmol/mg in CON [n=7], $P<0.01$), while the increase with either iNO 0.07±0.01 pmol/mg [n=6], or TAD 0.10±0.02 pmol/mg [n=8] alone did not reach statistical significance (Figure 10.E-F).

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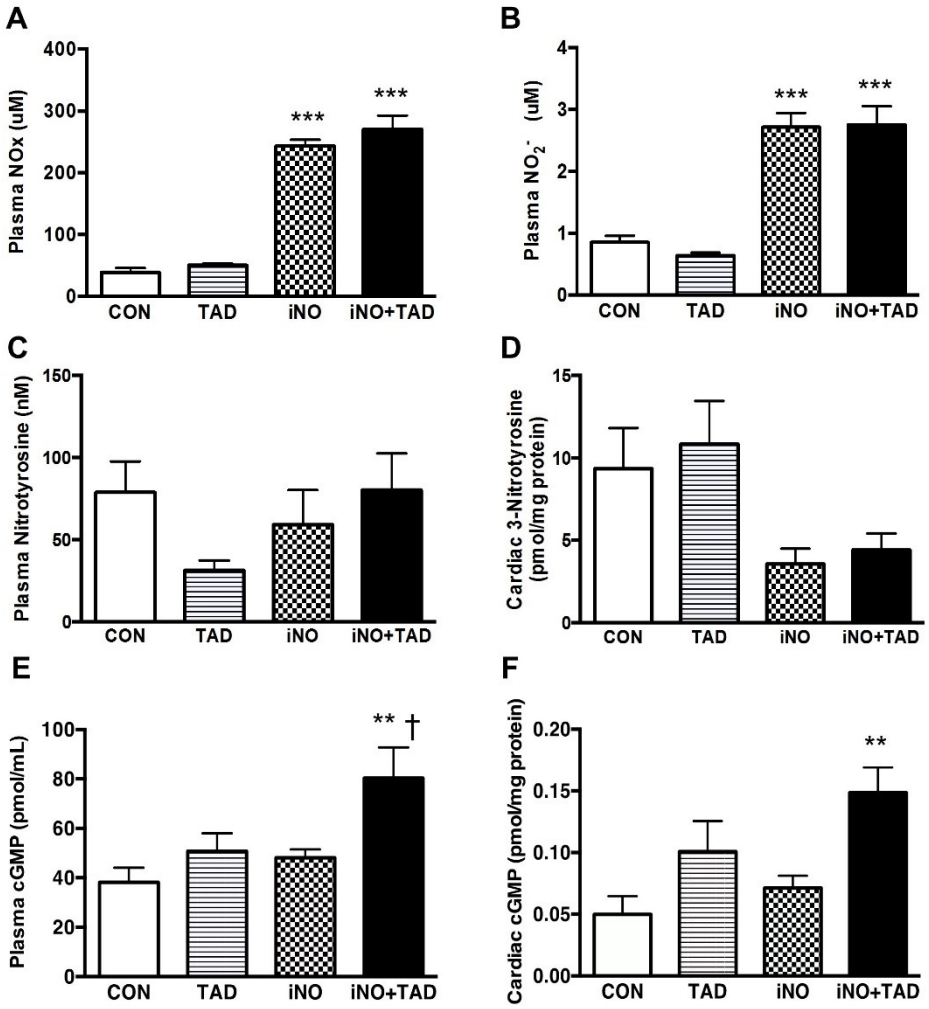

Figure 10. NO-derived oxidation products, 3-Nitrotyrosine and cGMP levels in plasma and in cardiac tissue at 20 min after reperfusion. Plasma levels of NOx, comprising nitrates, nitrites and S-nitroso compounds (Panel A) and nitrite (NO2-, Panel B) are measured using chemiluminiscence analysis in CON (n=8), TAD (n=7), iNO (n=7), and iNO+TAD (n=8) groups. Nitrosative stress is evaluated by measuring proteins in circulation or in cardiac tissue containing 3-nitrotyrosine residues (3-NT) using ELISA (Panel C-D). Circulating and cardiac cGMP contents were measured using enzyme immunoassay (Panels E-F). Plasma cGMP increased after iNO+TAD (n=7; P<0.01 vs CON and P<0,05 vs iNO) treatment, while TAD (n=6) and iNO (n=8) did not differ significantly from CON (n=7). Similarly iNO+TAD (n=7), but not TAD (n=8) or iNO (n=6), significantly increased tissue cGMP levels vs CON (P<0.01, panel F). CON = untreated mice, TAD = Tadalafil, iNO = inhaled nitric oxide, iNO+TAD = combination therapy. *** P<0.0001 vs CON and TAD, ** P<0.01 vs CON, † P<0.05 vs iNO

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4.1.3. Combined iNO and TAD therapy attenuates myocardial leukocyte infiltration, but not myocardial fibrosis.

To investigate additional mechanisms of cardioprotection, we analyzed myocardial infiltration of myeloperoxidase-positive cells using immunohistochemical stains, an established marker of the inflammatory reaction after I/R injury. After 3 days, the number of MPO-positive cells relative to mid-ventricular transversal tissue areas was significantly lower in iNO+TAD treated mice (P=0.02 vs CON, Figure 11.), but not in iNO- or TAD-treated mice.

Four weeks after I/R, we measured collagen fiber deposition in ~7% of the ischemic LV area, which was mostly restricted to the mid-ventricular wall and did not vary with treatment assignment. In the absence of transmural infarction, the interstitial fibrosis did not result in extensive adverse LV remodeling causing significant impairment in contractile function, as is usually observed after permanent LAD ligation. (Figure 9.)

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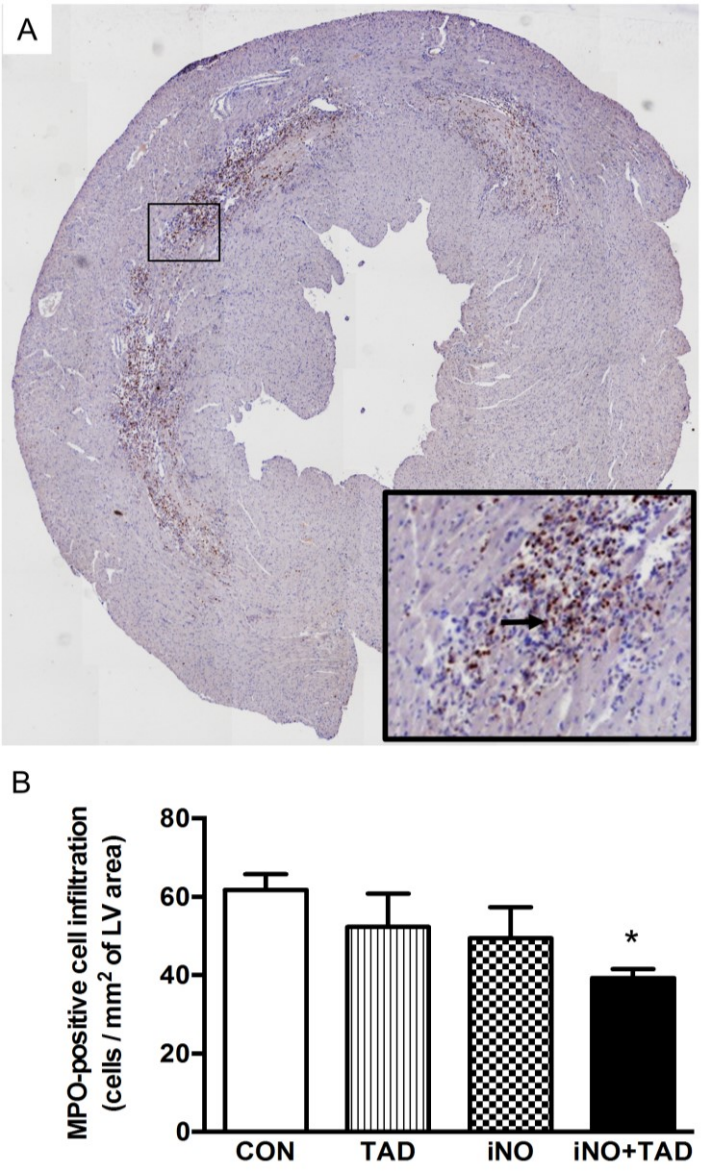

Figure 11. Myeloperoxidase-positive (MPO) cell infiltration three days after ischemia-reperfusion. MPO expressing cells (marked by arrow) infiltrate the ischemic area shown on a representative image (panel A). The composite image from the immunostained transversal myocardial section was assembled from mosaics (MozaiX, AxioVision) and contains an inlet with higher magnification of the black framed area. Treatment with iNO+TAD (n=6) significantly reduced MPO-positive cell infiltration compared to CON mice (n=5), while iNO (n=6) or TAD (n=7) had only a modest effect (Panel B). MPO-positive cells were counted on 3 different mid-ventricular transversal sections, averaged and expressed relative to the LV section area. CON = untreated mice, TAD = Tadalafil, iNO = inhaled nitric oxide, iNO+TAD = combination therapy. * $P<0.05$ vs CON

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4.2. Clinical study on the role of NOS3 in physiologic adaptation in elite athlethes

4.2.1. Characteristics of athletes and control individuals

Elite athletes, including men (n=94) and women (n=32) and Hungarian non-athletic controls (n=109 men and n=46 women) were screened. Age distribution and gender ratio (25.4% and 29.7% female in athletes and non-athletes, respectively) were not different between the study groups. Height, weight and body mass index were higher in men versus women and in athletes versus non-athletes. Body surface area, an important value in the interpretation of cardiac magnetic resonance imaging results, was also higher in athletes when compared with non-athletic controls (Table 7).

4.2.3. Cardiac morphology and function in athletes and controls

Left ventricular end diastolic and end systolic volumes and left ventricular myocardial mass, indexed to body surface area (LVEDVi, LVESVi, LVMi, respectively) were significantly higher in athletes than in the non-athletic group, suggestive of eccentric hypertrophy (Table 8). Resting LV ejection fraction (LVEF %) was not different between athletes and non-athletes (58.5±6.3% versus 59.4±4.3%, respectively, NS), but LV stroke volume index (LVSVi) was higher in athletes than in untrained controls (67.6±8.3 versus 54.8±7.8 ml/m2, $p<0.0001$). There were significant gender-related differences with higher end-diastolic and end-systolic LV volumes and larger LV mass in men versus women, irrespective of athletic activity (Table 8.). Similarly, RV end-diastolic and end-systolic volume indexes (RVEDVi, RVESVi) were higher in athletes compared to non-athletes and again, higher in men than in women (Table 8.). Resting RV stroke volume index (RVSVi) and RV mass index (RVMi) were both higher in athletes compared to non-athletes (for RVSVi 68.2±10.2 versus 56.7±7.0 ml/m2 , $p<0.05$ and for RVMi 29.9±6.1 versus 24.4±4.3 g/m2, $p<0.05$). Both, RVSVi and RVMi were higher in men than in women. At exhaustion both VO2 maximum, (60±7 versus 40±7 ml/kg/min, $p<0.0001$) and minute ventilation (VE, 150±15 versus 84±39 l/min, $p<0.0001$) were significantly higher in athletes than in non-athletic controls and within each group, also significantly higher in men than in women.

Table 7. Characteristics of athletes and non-athlete individuals

	Athletes			Non-athletes		
	All (n=126)	***Men (n=94)***	***Women (n=32)***	***All (n=155)***	***Men (n=109)***	***Women (n=46)***
Age (years)	25.9±5.5	26.9±5.7	24.9±5.3	27.4±5.2	27.1±5.1	28.0±5.7
Height (m)	1.87±0.11 *	1.95±0.04 †	1.77±0.07 ‡	1.76±0.11	1.83±0.07	1.67±0.09
Weight (kg)	85.2±15.6 *	97.5±8.3 †	72.3±10.2 ‡	70.5±15.3	81.4±13.6	59.9±7.2
Body mass index (kg/m^2)	24.8±2.6	25.8±2.2	23.4±2.5	22.8±4.3	24.1±4.6	21.6±3.9
Body surface area (m^2)	2.18±0.28 *	2.38±0.1 †	1.89±0.3 ‡	1.94±0.3	2.08±0.3	1.74±0.2

* $p<0.01$ versus all non-athletes, † $p<0.01$ versus men non-athletes, ‡ $p<0.01$ versus women non-athletes. Body mass index was different between athletes and non-athletes and between men and women ($p<0.05$). Height, weight, body surface area and body mass index were different between men and women individuals in the athlete and non-athlete groups ($p<0.05$). Age was not different between study groups.

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Table 8. Characteristics of athlete and non-athlete men and women

	Athletes			Non-athletes		
	All (n=126)	**Men (n=94)**	**Women**	**All (n=155)**	**Men (n=109**	**Women (n=46)**
LVEF (%)	58.5±6.3	57.8±4.3	60.4±9.7	59.4±4.3	59.5±4.4	58.4±4.2
LVEDVi (ml/m²)	116.2±17.4 *	121.4±14.9 ‡	102.4±16.2	92.9±12.9	97.3±11.1 ‡	84.4±13.2
LVESVi (ml/m²)	48.8±11.2 *	51.4±9.6 ‡	41.9±12.1	37.8±7.6	39.5±7.3 ‡	35.4±8.0
LVMi (g/m²)	81.1±19.6 *	88.1±15.9 ‡	62.4±16.0	61.3±13.9	68.1±10.4 ‡	47.0±10.1
LVSVi (ml/m²)	67.6±8.3 *	69.8±8.1 ‡	61.6±5.6	54.8±7.8	57.8±6.8 ‡	49.0±6.8
RVEF (%)	57.9±6.2	57.1±4.0	60.2±9.9	58.5±4.8	58.2±4.9	58.2±4.4
RVEDVi (ml/m²)	121.5±19.6 *	127.8±17.4 ‡	106.0±17.3	95.5±15.2	100.7±14.1 ‡	85.7±13.4
RVESVi (ml/m²)	53.2±11.3 *	55.8±10.9 ‡	46.6±9.9	39.8±9.6	42.6±9.9 ‡	35.3±7.3
RVSVi (ml/m²)	68.2±10.2 *	70.7±9.5 ‡	61.5±9.2	56.7±7.0	58.3±6.4 ‡	52.4±7.7
RVMi (g/m²)	29.9±6.1 *	32.5±4.6 ‡	23.5±5.4	24.4±4.3	25.3±3.5 ‡	20.1±3.0

n – number of individuals; LVEF – left ventricular ejection fraction; LVEDVi – left ventricular end-diastolic volume index; LVESVi – left ventricular end-systolic ventricular index; LVMi – left ventricular mass index; LVSVi – left ventricular stroke volume index; RVEF – right ventricular ejection fraction; RVEDVi – right ventricular end-diastolic volume index; RVESVi – right ventricular end-systolic volume index; RVSVi – right ventricular stroke volume index; RVMi – right ventricular mass index

* $p<0.01$ versus all non-athletes, ‡ $p<0.01$ versus women. Age was not different between study groups.

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4.2.4. Genotype distribution

Allelic distributions in athletes and controls were similar with a minor allelic frequency of 0.27 in athletes vs. 0.26 in controls. In 64 athletes the Glu homozygous genotype, in 56 the heterozygous while in 6 out of 126 athletes the Asp homozygous genotype was found. The genotype distribution was similar in the non-athletic control group (84/ G/G, 62/ G/A, and 9/ A/A, chi-square=0.62; p=0.73, Table 9.). Therefore the investigated SNP did not correlate with athletic status (p=0.59, with OR (CI 95) of 0.8 (0.5-1.4). The proportion of the G/G genotype varied between 49 and 57% in both genders in both groups. Overall, genotype distributions were in Hardy-Weinberg equilibrium for the SNP in the population.

Table 9. Nitric oxide synthase 3 gene 298 Glu/Asp genotype distribution in athletes and in non-athlete controls

	Athlete (VO_2 max >50ml/kg/min)		**Non-athlete (VO_2 max <50ml/kg/min)**	
	Men	**Women**	**Men**	**Women**
GG (Glu/Glu)	46 (49%)	18 (56%)	58 (53%)	26 (57%)
GT (Glu/Asp)	43 (46%)	13 (41%)	45 (41%)	17 (37%)
TT (Asp/Asp)	5 (5%)	1 (3%)	6 (6%)	3 (6%)

Number of individuals with different genotypes in the different groups. Percentage in brackets represent the allelic frequency within the subgroup.

4.2.5. Association of cardiac structure and function with the *NOS3* 298 genotype

Resting RV stroke volume index (RVSVi) and RV mass index (RVMi) were both higher in Aspartate carriers (athletes and non-athletes, n=133) compared to Glutamate homozygous participants (athletes and non-athletes, n=148), for RVSVi 60±9 versus 62±12 ml/m^2 (p=0.047, t-test) and for RVMi 26±6 versus 27±6 g/m^2 (p=0.019, t-test).

Athletes carrying the Asp allelic variant had higher right ventricular mass index, than those homozygous for the Glu allelic variant (31.7±5.5 versus 27.4±6.0 g/m^2,

p<0.01, Table 10. and 11.). RVMi represents right ventricular hypertrophy and was associated with higher right ventricular stroke volume index (RVSVi) in the Asp allele carrier versus the Glu homozygous athlete group (71.1±9.6 versus 64.3±9.8 ml/m^2, p<0.001, Table 10. and 11.). In non-athletic individuals no association was observed between genotype and right ventricular function and mass. Similarly, left ventricular function and anatomy was not associated with the genotype in any of the study groups (Table 11.). Oxygen consumption at exhaustion (VO_2 maximum) was not different between the Asp allelic variant carriers and the Glu homozygous individuals, irrespective of the athletic status (in athletes 61±8 versus 58±6 ml/kg/min and in non-athletes 40±7 versus 39±7 ml/kg/min, respectively, NS for both). There was, however, a trend towards higher maximal minute ventilation in Asp allelic variant carrier versus Glu homozygous athletes (152±13 versus 146±11 l/min, *p*=0.08). The genotype was not associated with ventilation in non-athletes.

Table 10. Characteristics of male and female athletes and athletes with, or without the Asp allele

		Resting right ventricular stroke volume index (RVSVi, ml/m^2)	Right ventricular mass index (RVMi g/m^2)
Gender	**Men**	70.7±9.5 ‡	32.5±4.6 ‡
	Women	61.5±9.2	23.5±5.4
NOS3	**Glu/Glu**	64.3±9.8	27.4±6.0
	Asp carrier	71.1±9.6 #	31.7±5.5 #

In this age matched population of elite athletes with a similar social and ethnic background linear regression analysis revealed that both male gender and the presence of the Asp allele are independent predictors for higher resting right ventricular stroke volume index (RVSVi) and right ventricular mass index (RVMi) values. (‡ p<0.001 vs women, #p<0.001 vs Aspartate carriers)

Table 11. Characteristics of different genotypes within athletes and non-athletes (irrespective of their gender)

	Athletes (VO2 max >50ml/kg/min)		Non-athletes (VO2 max >50ml/kg/min)		
	Glu/Glu	Glu/Asp + Asp/Asp	Glu/Glu	Glu/Asp + Asp/Asp	p
N	64	62	84	71	-
LVEF (%)	58.3±8.1	58.7±4.5	59.1±2.4	60.0±6.7	0.688
LVEDVi (ml/m²)	115.1±20.5 *	117.0±14.9 *	92.1±14.6	94.4±9.3	<0.001
LVESVi (ml/m²)	49.2±13.3 *	48.5±9.3 *	37.8±7.8	37.9±7.6	<0.001
LVMi (g/m²)	77.9±23.1 *	83.5±16.2 *	60.3±15.3	63.2±11.2	<0.001
LVSVi (ml/m²)	66.4±7.6 *	68.5±8.7 *	54.1±7.6	56.1±8.2	<0.001
RVEF (%)	57.7±8.1	58.1±4.4	58.3±4.1	58.4±5.8	0.427
RVEDVi (ml/m²)	117.8±19.9 *	124.4±19.1 *	95.6±17.6	95.4±9.8	<0.001
RVESVi (ml/m²)	53.0±10.5 *	53.4±12.1 *	40.4±11.0	38.7±6.5	<0.001
RVSVi (ml/m²)	64.3±9.8 #*	71.1±9.6 *	56.6±6.7	56.8±7.8	<0.001
RVMi (g/m²)	27.4±6.0 #*	31.7±5.5 *	25.3±4.7	23.3±3.4	<0.001

n – number of individuals; LVEF – left ventricular ejection fraction; LVEDVi – left ventricular end-diastolic volume index; LVESVi – left ventricular end-systolic ventricular index; LVMi – left ventricular mass index; LVSVi – left ventricular stroke volume index; RVEF – right ventricular ejection fraction; RVEDVi – right ventricular end-diastolic volume index; RVESVi – right ventricular end-systolic volume index; RVSVi – right ventricular stroke volume index; RVMi – right ventricular mass index

Analysis of variance showed significant differences among the inspected groups: athletes and non-athletes with and without the Aspartate allele. Post hoc tests revealed a significant influence of the genotype (# $p<0.001$ vs Asp carriers within the athlete group) on resting RVSVi and RVMi in athletes. Athletic status had significant influence on all parameters with the exception of LVEF and RVEF (* $p<0.001$ vs non-athletes irrespective of genotype).

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5. Discussion

5.1. Murine experiment on potentiation of cardioprotection from NO with PDE5 inhibition

In this study we report that combining inhalation of NO with oral administration of tadalafil (TAD) is safe and confers incremental myocardial protection during I/R injury in mice. For a similar risk area, combination therapy was associated with a greater reduction in troponin release during the acute phase, and less inflammatory cell infiltration when compared to either treatment alone. The early benefit at the time of reperfusion translated in markedly improved functional and structural remodeling after four weeks. LV end-systolic dimensions following combination treatment were reduced and associated with a better-preserved regional LV function on transthoracic echocardiography. In addition, invasive pressure volume analysis using conductance catheter technology confirmed improved contractile performance in mice treated with iNO and TAD with significantly higher stroke volumes. Finally, the superiority of the combination therapy was associated with significantly greater nitrite plasma concentration, a trend for lower cardiac nitrosative stress levels and significantly higher cGMP bioavailability in the heart and in the circulation, emphasizing the importance of this second messenger system in cardioprotection.

Several laboratories have previously reported that NO inhalation during ischemia and reperfusion effectively reduces myocardial infarct size and improves cardiac function in rodent and porcine models of I/R (Liu et al., 2007; Nagasaka et al., 2008; Neye et al., 2012). Moreover, when administered at 80 ppm for 24 hours, iNO prevented early increase in left ventricular dimensions and helped to preserve ejection fraction (Hataishi et al., 2006). At first glance it seems, that there is a discrepancy between our data and the work of Hataishi et al., as we failed to verify the same protection against LV dysfunction with iNO treatment alone. There are, however, important differences in study design, which could account for this difference. Hataishi and coworkers used the same ischemic time (60 min) and concentration of inhaled NO (80 ppm) but exposed their mice to NO in a sealed chamber for the entire duration of the experiment (e.g. 24h), while in our study NO delivery was limited for 50 minutes. Hataishi and colleagues reported reduced LV-EDV and better preserved EF at 24h after I/R, a timepoint that reflects early reperfusion

injury and cannot be compared to the functional and structural remodeling parameters measured at 4 weeks follow up. The lack of cGMP rise in the myocardium in Hataishi's study may be attributable to increased cardiac PDE5 activity which has been shown to be up-regulated in the early I/R phase by other groups (Hataishi et al., 2006; Kass, 2012). Others have shown that iNO conferred cardioprotection is at least partially cGMP mediated, as with the loss of the alpha form of soluble guanylate cyclase, the protective effect of NO was lost as well (Nagasaka et al., 2011). These observations are consistent with our findings of 100% increased cardiac cGMP content following pretreatment with TAD, while iNO alone was only able to increase cardiac cGMP by 50%. Changes in cardiac cGMP content during the early reperfusion phase strikingly mirror changes in plasma, further supporting the biological significance of these observations.

In the bloodstream, iNO is rapidly converted to nitrite, nitrate, or to various S- and N-nitrosated proteins (Bhatraju et al., 2015; Bloch et al., 2007). Red blood cells serve as a major reservoir of nitrite with a bidirectional reversible flux between red blood cell and plasma compartments. When exposed to lower hemoglobin oxygen saturations and acidic pH in ischemic myocardium, NO can be released from nitrites, hem-nitrosyls and S-nitrosothiols. Such a selective release of bound NO increases its bioavailability in areas of impaired perfusion and could confer cardioprotection (McMahon and Doctor, 2006; Neye et al., 2012; Schumacker, 2013; Terpolilli et al., 2012). In this study, we have observed 2.5-3 fold increased plasma nitrite levels and 6-7 fold increased total NOx levels, which may serve as source of NO. Conversely, NO may interact with superoxide radicals to generate peroxynitrites and partially offset cardioprotection or may scavenge free radicals and transiently reduce nitrosative stress, as suggested by reduced cardiac 3-nitrotyrosine levels in the early post-reperfusion phase in mice treated with inhaled NO (Figure 10D) (Ferdinandy et al., 2000; Gielis et al., 2011; Szabó et al., 2007).

At the same time, inhibition of phosphodiesterases (PDEs), the class of enzymes responsible for cGMP degradation, significantly reduced MI size and cardiac dilatation and preserved global LV function in mice (Das et al., 2015a; Kass et al., 2007a, 2007b). Under physiologic conditions, PDE5 expression levels in the cardiovascular system are very low and mainly confined to smooth muscle cells. Upregulation and activation of PDE5 was reported in ischemic and failing myocardium, in part via cGMP-dependent and PKG-I mediated phosphorylation (Das et al., 2015a; Kass et al., 2007a, 2007b), setting

the stage for successful administration of selective PDE5 inhibitors such as sildenafil in heart failure with reduced ejection fraction (Das et al., 2015a, p. 5; Guazzi et al., 2011; Lukowski et al., 2014; Redfield et al., 2013).

We hypothesized that during acute ischemic disease insufficient NO bioavailability to stimulate cGMP generation argues against the use of PDE5 inhibition as a viable strategy to sufficiently boost cGMP levels. Therefore, in this study we investigated whether combined iNO-induced stimulation of sGC with selective inhibition of PDE5-catabolized hydrolysis can safely confer cardioprotection. Ligation of the proximal LAD resulted in a territory at risk that encompasses more than 50% of the left ventricle. This degree of myocardial injury did not compromise hemodynamic stability or survival. Stable or very modest reductions in blood pressure were previously reported after application of iNO in acute MI (Bloch et al., 2007; Ichinose, 2013; Nagasaka et al., 2008). Conversely, single dose administration of the long-acting tadalafil was reported to have variable effects on blood pressure. In this study, monotherapy with iNO and TAD or combined application did not compromise blood pressure, one of the most critical parameters during I/R treatment protocols. However, rigorous dose escalation studies would be required to determine the most effective dose and the safety margins.

The applied dose of tadalafil, 4 mg/kg via gastric delivery was approximated from human studies and was reported to reach protective plasma concentrations in rodents (Ahmad et al., 2009; Koka et al., 2014; Salloum et al., 2014, 2009; Sesti et al., 2007). Some groups have used a higher dose of tadalafil (10mg/kg) 2h before coronary artery occlusion. The duration of occlusion was also shorter, 30 minutes compared to 60 minutes in our study (Sesti et al., 2007). Koka and coworkers reported on mice treated for 9 consectuvie days with oral gavage of tadalafil (4 mg/kg), in which they were able to measure a tadalafil plasma concentration of 534±89 ng/mL 1h after the last drug-administration (Koka et al., 2010). This dose was chosen based on the interspecies dose extrapolation scaling and would result in plasma concentrations equivalent to a human dose of 20 mg/day.

We measured a significant rise in plasma and cardiac cGMP levels only in animals that received combination therapy. Plasmatic cGMP does not necessarily reflects the cGMP bioavailability in organs, but increased cGMP signaling in the ischemic heart confers protection by attenuating β-adrenergic-stimulated contractility, preventing

progression of Ca^{2+}-triggered Ca^{2+} waves and limiting GAP-junction communication during cell-to-cell propagation of necrosis (Francis et al., 2010).

In addition to direct effects on cardiac myocytes, cGMP also affects multiple other pathways involved in I/R injury including platelet activation, vasorelaxation, expression of adhesion proteins and endothelial permeability, and neutrophil activation (Garcia-Dorado et al., 2009). The latter effect together with monocyte to macrophage differentiation is reflected by increased MPO-levels (Hataishi et al., 2006; Liu et al., 2007). Several mechanisms have been reported in the literature to account for the effect of NO on polymorphonuclear inflammatory cells: NO limits leukocyte chemotaxis, adherence and activation (Kubes et al., 1991). The mechanism may involve suppression of relevant circulating chemokines and expression of adhesion molecules. Inhaled NO or NO released after NOS3 gene transfer in a porcine model diminished MPO-activity and reduced the expression of intercellular endothelial adhesion molecule-1 (ICAM-1) expression in cardiac tissue (Liu et al., 2007; Szelid et al., 2009). Endogenous and exogenous NO dose-dependently reduces the release of interleukin 8 (IL-8), the main chemo-attractant factor of neutrophils (Cuthbertson et al., 1997). Moreover, in the coronaries of eNOS-deficient mice after I/R the expression of P-selectin was increased and exacerbated the adhesion of neutrophils (Jones et al., 1999). Leukocyte adhesion is at least partially mediated by the soluble guanylate cyclase (sGC), which was proven in vivo using the sGC activator BAY-41 2272 (Belhassen et al., 2001; Boerrigter and Burnett, 2007). More recently, TAD therapy has also been shown to ameliorate circulating inflammatory cytokines and chemokines (Varma et al., 2012). We observed reduction in MPO-positive cell infiltration exclusively after combined treatment and saw that following inhalation with or without concomitant TAD there is a trend for reduced cardiac 3-nitrotyrosine levels during the early reperfusion period. Unfortunately, whether reduced nitrosative stress is a cause or consequence of altered MPO-positive cell infiltration cannot be determined from our experiments.

At the molecular level, cGMP-dependent activation of protein kinase G-I (PKG-I) enhances ERK signaling and results in reduced opening probability of mitochondrial permeability transition pore (Francis et al., 2010). The latter represents a final common switch in the RISK pathway for cellular protection against ischemic damage and may partially be mediated through hydrogen sulfide (Das et al., 2015b; Salloum et al., 2009).

In our study combination treatment improved LV fractional shortening and prevented cardiac remodeling during the 4 weeks follow up period, while either treatment alone had only partial effects.

5.1.1. Limitations

First, the relatively short follow up period and limited transmurality of the infarction in our mice precludes extension of findings on survival and development of heart failure. Second, TTC-based planimetry of infarct size was not sensitive enough to discriminate between the three treatment groups. To delineate infarct size with greater precision, detailed morphometric analysis or high flux density magnetic resonance imaging would be required. Third, administration of TAD 60 min prior to I/R does not represent a therapeutic situation in mice, but the concept may be useful during clinical translation using the time window between MI detection and reperfusion.

5.1.2. Conclusion

Combined NO inhalation and selective PDE5 inhibition using tadalafil during myocardial ischemia-reperfusion confers superior protection against I/R injury in mice. The associated increase in cGMP-signaling after the combined treatment suggests the importance of this pathway for beneficial long-term structural and functional remodeling. Combined therapy may represent a promising strategy for translational research to improve the outcome of ischemia-reperfusion injury in patients.

5.1.3. Potential influence on human ischemia reperfusion therapy

Attenuating ischemia reperfusion injury is of major interest and in the last decades several attempts were made to breach this barrier (Ovize et al., 2013). Preconditioning has no clinical relevance, but remote-, post- and pharmacological post-conditioning are potential therapeutic means.

Our data suggest that inhaled NO therapy should be introduced at least 10-15 min before the reperfusion to effectively impact on mechanisms of reperfusion injury. Nitric oxide reaches the bloodstream very rapidly through the alveolar-capillary barrier and is transmitted to the ischemic tissues via NO-heme adducts, nitrites or S-nitrosylated proteins. Human pharmacokinetic studies demonstrated that tadalafil is a selective PDE-5 inhibitor, whose effects can last up to 36 hours (compared to sildenafil’s and

vardenafil's effects lasting for 4 to 8 hours). Tadalafil is also the only PDE-5 inhibitor whose activity is unaffected by food and has a relatively short time to onset of action (16 to 17 minutes). The pharmacokinetic profile of tadalafil shows maximal plasma concentration within 2.0 hours and an elimination half-life of 17.5 hours.

In STEMI pateints with normal systemic blood pressure, we would propose that the best time for tadalafil administration would be 16-30 min before reperfusion, which should provide enough time for NO to exert its cardoprotective effects as well.

Recently an international, multicenter, randomized, double blinded clinical trial was designed and executed by Janssens and coworkers at the Catholic University of Leuven to verify beneficial effects of inhaled nitric oxide therapy in patients with acute myocardial infarction. The *Nitric Oxide for Inhalation in ST-Elevation Myocardial Infarction* (NOMI) trial, however, failed to achieve reduction in infarct size (primary endpoint of the study). Major findings of the trial were improved functional recovery and an unforeseen significant interaction between iNO and nitroglycerin.

5.2. Clinical study on the role of NOS3 in physiologic adaptation in elite athletes

In this study, association between sport-related right ventricular adaptation and the Glu298Asp genetic variant of the endothelial nitric oxide synthase 3 gene was examined in elite athletes. The *NOS3* genotype and its relation with athletic performance and physiologic adaptation in selected elite athletes have already been described, however we are first to report that physical preconditioning evokes genotype-influenced right ventricular adaptation (Hand et al., 2006, p. 3; Rankinen et al., 2000, p. 3; Saunders et al., 2006).

Consistent with previous findings, in our study, no difference in genotype distribution was found between elite athletes and control individuals (Wolfarth et al., 2008). According to the dbSNP database Minor allele frequency was lower than in the HapMap-CEU population (0.27 vs. 0.34) and comparable with the largest population available (PA159018372, 0.27 vs. 0.29).

Although the adaptation of the left ventricle and its possible functional consequences on athletic performance represent a major focus of sport physiology research, there is increasing evidence that right ventricular adaptation may be a better

marker of athletic performance (Aaron et al., 2011; Scharhag et al., 2002; Steding et al., 2010). There is a near linear correlation between exercise intensity and pulmonary artery pressures, which results in a disproportionate increase in right, compared to the left ventricular afterload (La Gerche et al., 2014). Association between strenuous high intensity exercise and a disproportionate enlargement of the right ventricle was observed previously. (Maron, 1986; Perseghin et al., 2007). Beside chamber dilation athletic adaptation goes together with decreased resting RV ejection fraction and ventricular-arterial coupling alterations which, in turn, may be an early sign for contractile impairment (Claessen et al., 2014). In addition right ventricular mass and volume were also independent markers of self-reported physical activity in a recent community-based trial (Aaron et al., 2011). These changes in the RV structure represent physiologic athletic adaptations, however, extensive remodeling may also predict pathologic conditions. Distinguishing physiologic- from pathologic adaptation remains an important and challenging task (Ector et al., 2007; La Gerche et al., 2013). Therefore, a careful examination of the right ventricle in highly trained individuals is therefore critical, although it is not part of the current pre-participation screening protocols (La Gerche et al., 2010).

Because of its anti-hypertrophic myocardial effects nitric oxide (NO) could affect athletic performance via several mechanisms, including improved coupling of cardiac oxygen consumption to physical performance, enhanced LV relaxation and decreased LV end-diastolic pressure, increased NO-dependent myocardial Ca^{2+} influx and contractile force (Bito et al., 2010; Booz, 2005; Heusch et al., 2000; Matter et al., 1999). The primary source of NO is NOS3 in the cardiovascular system. Therefore, Glu to Asp amino acid substitution at codon 298 in the NOS3 enzyme and reduced NO production may result in altered biological effects, including impaired vasodilation and increased myocardial hypertrophy.

In our study, however, no connection was found between the *NOS3* genotype and left ventricular parameters neither in athletes, nor in untrained-individuals. Interestingly, without a significant effect on maximal oxygen uptake, there was a trend towards higher minute ventilation at peak exercise in Asp allelic carrier compared to Glu homozygous athletes. This phenomenon was not observed in non-athletes.

Bench-side and clinical data support the theory that the hypoxia-induced pulmonary vasoconstriction may be further enhanced by the increased cleavage of the Aspartate containing *NOS3* protein (La Gerche et al., 2014; Tesauro et al., 2000). Under excessive chronic load this could hasten the depletion of the marginally sufficient right ventricular contractile reserves, which was shown to lead to a disproportionate and exaggerated right ventricular remodeling in both animal- and clinical studies (Benito et al., 2011; La Gerche et al., 2014; Modesti et al., 2004). As sustained right ventricular fatigue and injury may result in pathologic fibrous remodeling, the clinical significance of our findings could lie with their potential influence on the development of RV cardiomyopathy (Gerche et al., 2012; Heidbüchel et al., 2003).

The influence of the *NOS3* Glu298Asp polymorphism on enzymatic cleavage and thus NO availability has been shown to reduce treatment efficacy in heart failure patients. In a genetic sub-study of the African-American Heart Failure Trial (A-Heft) Glu298Glu genotype predominance was associated with the positive impact of NO donors (isosorbide dinitrates and hydralazine) on heart failure survival (McNamara et al., 2009; Tesauro et al., 2000).

The *NOS3* Glu298Asp polymorphism has also been associated with cardiac adaptation to long-term athletic performance. This association included the cardiovascular adaptation of untrained individuals to long term endurance training with a blunted responsiveness of submaximal exercise, diastolic blood pressure and rate pressure product in previously sedentary Asp homozygous subjects. Higher stroke volume and lower heart rate during sub-maximal exercise was reported in postmenopausal women carrying the Asp allelic variant (Hand et al., 2006; Rankinen et al., 2000). The *NOS3* Glu298Asp variant was also associated with the actual contest performance in elite ultra-endurance athletes (Saunders et al., 2006).

In elite athletes we found larger right ventricular myocardial mass and increased RV stroke volume (both indexed to body surface area) in Asp allele carriers versus Glu homozygous individuals. The higher stroke volume observed in the Asp carrier athletes was associated with a non-significant trend of larger end diastolic volumes compared to the Glu homozygous athletes.

These associations could be the consequences of the increased afterload and adverse pulmonary remodeling brought forth by reduced NO bioavailability. Knockout murine

experiments highlighted the fact that albeit all three isoforms are present in the lung, NOS3-derived NO is the major regulator of pulmonary vascular tone. Loss of NOS3 function results in a markedly enhanced hypoxic pulmonary vasoconstriction, significantly elevates right ventricular pressure and induces airway hyper-responsiveness (Fagan et al., 1999; Feletou et al., 2001). In addition, even the endothelial response to ß-adrenergic stimuli could be compromised and thus protection from sympathetic hyperactivity could be hampered (Davel et al., 2015). During the last decade candidate gene approach and analysis of single genetic variants became overshadowed by systems biology, gene-environmental and gene-gene interaction studies. In the present study group however strict inclusion criteria make the analysis of correlations with a single genetic variant more plausible.

5.2.1. Limitations

Cross sectional study design limits insight on the interaction of the *NOS3* Glu298Asp polymorphism with athletic remodeling. As this study was focused on a highly selected group of athletes, individuals from several sports disciplines – although with very similar training regimes – had to be screened to reach an acceptable number of participants. Given our single gene candidate approach, other polymorphisms in linkage disequilibrium with the Glu298Asp polymorphism may be the true cause of this association.

5.2.1 Conclusion

In this study cardiac function and structure of elite athletes was determined and a previously unknown correlation was found between load-dependent right ventricular adaptation and a candidate genetic variant, the *NOS3* Glu298Asp polymorphism. Our study supplements evidence in the literature showing another aspect of NOS3 genotype influence on cardiac adaptation. Whether this has a positive or negative effect on the individuals' long term cardiovascular health and athletic performance has yet to be determined. Combined use of non-invasive cutting edge imaging modalities and genotyping tools may help to track the extent of influence both during training and late life deconditioning.

Given the high frequency of the nitric oxide synthase 3 gene 298 Glu/Asp polymorphism this could have substantial impact if there was more evidence related to its functional and long-term implications.

6. Future directions

After the NOMI trial's failure to achieve infarct size reduction and its puzzling findings of iNO and organic nitrate interaction, new questions emerge and novel treatment plans have to be established. One should, however, keep in mind, that drug interactions not only have the potential to nullify damage mitigation, but based on our data they may be able to release unexploited reserves of cGMP mediated cardioprotection. Beyond the search for an optimal combination of medications, therapy has to be individualy adjusted for concomitant diseases and new, most probably functional and not anatomical landmarks have to be indentified as markers of treatment efficacy. Potential research projects could assess the influence of concomitant diseases (e.g. diabetes mellitus) and the interaction among organic and inorganic nitrates or other drugs with influence on the cGMP mediated pathway (eg. sGC stimulators). To estimate the exact functional benefits of the iNO+TAD combination therapy long term follow up studies with exercise tests end less investigator dependent imaging endpoints (eg. cMRI) would be needed.

It has been proven that genetic heritage has the potential to influence the development of athlete's heart and although single candidate gene approach became somewhat outdated, in highly selected populations it may still help to assess genetic influence on performance and/or remodelling. To strengthen our data on the influence of the *NOS3* Glu298Asp polymorphism on right ventricular remodeling proscpective studies on reversibility and adverse cardiac events would be needed. Although costly, MRI guided and training plan adjusted follow-up studies seem to be feasible, the limited size of target population, however, makes it in my opinion almost impossible to conduct research with mortality or morbidity end-points.

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7. Summary

Nitric oxide signaling has remained on the frontlines of scientific interest in cardiovascular physiology and pathophysiology for almost 30 years. Animal models were constructed to study various diseases and physiologic adaptations. Numerous signaling pathways were identified and their role in cardiovascular regulation was explored. Transition to human physiology and pathophysiology resulted in FDA-approved drugs that became first line therapy for a variety of diseases.

Ischemia reperfusion injury, however, remains a field to be conquered. In animal models several compounds brought spectacular results in infarct size reduction and attenuation of adverse cardiac remodeling. Some of them passed phase I but none of them could prove their efficacy in phase II clinical trials. Research presented in this work aimed to evaluate the remaining potential of inhaled nitric oxide therapy and the NOS/sGc/cGMP signaling pathway. We found, that concomitant NO inhalation and PDE inhibition not only reduced infarct size, but conferred additional functional improvement when comapered with standalone treatments. Based on our data we hypothesize that cardioprotective signal induction and simultaneous signal stabilization could effectively enhance currently available therapeutic approaches.

Nitric oxide signaling is a key mediator in physiologic cardiac adaptation. It is known, that in selected populations individual differences in endogenous NO production and bioavailability may be associated with adverse cardiac remodeling and/or athletic performance. We were able to identify a previously not known correlation between exercise induced right ventricular remodeling and a common NOS3 polymorphism. Our findings support previous data on the influence of nitric oxide signaling and NOS polymorphisms on athletic adaptation and proved that under special circumstances the examined genetic variants may be capable to influence cardiac morphology and function.

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8. Összefoglalás

A nitrogén monoxid jelátvitel kardiovaszkuláris élettanra és kórélettanra kifejtett hatásainak vizsgálata több mint 30 éve áll a tudományos érdeklődés középpontjában. Számos állatmodell áll rendelkezésre a különböző betegségek és a fiziológiás adaptáció vizsgálatára. Különféle jelátviteli utakat azok szerepét azonosították a kardiovaszkláris rendszer szabályozásban. Mindezen eredmények humán élettanra és kórélettanra vonatkoztatása az FDA által is elismert, több betegség kezelésében élvonalbeli szerré előlépő hatóanyagok kifejlesztéséhez vezetett.

Az iszkémia-reperfúziós károsodás csökkentése azonban továbbra is megoldatlan megoldatlan maradt. Preklinikai modellekben számos hatóanyag igen látványos eredményt ért el az infarktus területének csökkentésében és hatékonyan előzte meg az rosszindulatú remodellációt. Néhány hatóanyag sikeresen túljutott a fázis I-es vizsgálatokon, azonban a fázis II vizsgálatok során egyik sem bizonyult hatásosnak. Az ebben a dolgozatban bemutatott kísérletes munka célja az inhalált nitrogén-monoxid kezelésben és a NOS/sGC/cGMP jelátvitelben rejlő terápiás és diagnosztikus lehetőségek vizsgálata volt. Az inhalált NO kombinálása PDE gátlókkal nem mérsékelte az egyes szerek infarktus-méretét csökkentő hatását, hanem eredményeink alapján további funkcionális javuláshoz is vezetett. Adataink alapján az egyidejűleg kiváltott kardioprotektív jelátvitel indukció és jel-stabilizáció tovább fokozhatja a már meglévő terápiás lehetőségek hatékonyságát.

A nitrogén-monoxid a szívizom fiziológiás sport-adaptációjában is kulcsfontosságú mediátor. Ismert, hogy bizonyos populáción belül az NO termelés és biohasznosulás egyéni eltérései nem csak a remodellációt, de a kardiovaszkuláris szabályozást és a sportolói teljesítményt is befolyásolhatják. Vizsgálatunkkal egy eddig nem ismert összefüggést tártunk fel a testmozgás indukálta jobb kamrai remodelláció és egy jól ismert NOS3 polimorfizmus között. Eredményeink tovább erősítik azokat a korábbi megfigyeléseket, amik szerint a nitrogén-monoxid jelátvitel és a NOS polimorfizmusok aktív szerepet tölthetnek be a sport-adaptációban és igazoltuk, hogy ilyen különleges körülmények között a NOS gén genetikai variánsai képesek a szív morfológiájának és működésének befolyásolására.

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10. List of publications

10.1. Publications related to the doctoral work

1. **Lux A**, Pokreisz P, Swinnen M, Caluwe E, Gillijns H, Szelid Z, Merkely B, Janssens SP. (2016) Concomitant Phosphodiesterase 5 Inhibition Enhances Myocardial Protection by Inhaled Nitric Oxide in Ischemia-Reperfusion Injury. Journal of Pharmacology and Experimental Therapeutics. 2016 Feb;356(2):284-92. doi: 10.112. IF: 3.972
2. Szelid Z, **Lux A**, Kolossváry M, Tóth A, Vágó H, Lendvai Z, Kiss L, Maurovich-Horvat P, Bagyura Z, Merkely B. (2015) Right Ventricular Adaptation Is Associated with the Glu298Asp Variant of the NOS3 Gene in Elite Athletes. PLoS One. 2015 Oct 30;10(10):e0141680. doi: 10.1371. IF: 3.234

10.2. Other publications

1. Oláh A, Németh BT, Mátyás Cs, Hidi L, Lux Á, Ruppert M, Kellermayer D, Sayour AA, Szabo L, Torok M, Meltzer A, Gellér L, Merkely B, Radovits T (2016) Physiological and pathological left ventricular hypertrophy of comparable degree is associated with characteristic differences of in vivo hemodynamics. AJP Heart and Circulatory Physiology. 2016 Mar 1;310(5):H587-97. doi: 10.1152. IF: 3,838
2. Szilveszter B., Elzomor H, Károlyi M, Kolossváry M, Raaijmakers R, Benke K, Celeng Cs, Bartykowszki A, Bagyura Zs, Lux A, Merkely B, Maurovich-Horvat P. (2016) International Journal of Cardiovascular Imaging. 2016 Jan;32(1):153-60. doi: 10.1007. IF: 1,810
3. Kovács A, Oláh A, Lux Á, Mátyás M, Németh BT, Kellermayer D, Ruppert M, Torok M, Szabo L, Meltzer A, Assabiny A, Birtalan E, Merkely B, Radovits T (2015) Strain and strain rate by speckle tracking echocardiography correlate with pressure-volume loop derived contractility indices in a rat model of athlete's heart. AJP Heart and Circulatory Physiology 01/2015; DOI: 10.1152/ajpheart.00828.2014 IF: 3,838

4. Bagyura Zs, Kiss L, Édes E, Lux Á, Polgár L, Soós P, Szenczi O, Szelid Zs, Vadas R, Józan P, Bagdy Gy, Merkely B (2014) Cardiovascular screening programme in the Central Hungarian region. The Budakalász Study. Orvosi Hetilap. 2014 Aug 24;155(34):1344-52. doi: 10.1556
5. Oláh A, Lux Á, Németh BT, Hidi L, Birtalan E, Kellermayer D, Mátyás Cs., Ruppert M., Merkely G., Szabó G., Merkely B., Radovits T. (2013) A sportszív részletes hemodinamikai jellemzése bal kamrai nyomás-térfogatanalízis segítségével. Cardiologica Hungarica 43:(5) pp. 224-232
6. Radovits T, Oláh A, Lux Á, Németh BT, Hidi L, Birtalan E, Kellermayer D, Mátyás Cs, Szabó G, Merkely B (2013) Rat model of exercise-induced cardiac hypertrophy - hemodynamic characterization using left ventricular pressure-volume analysis. AJP Heart and Circulatory Physiology 05/2013; IF: 4.012
7. Bagyura Zs., Szelid Zs., Soós P., Szenczi O., Maurovich-Horváth P., Édes E., Lux Á., Polgár L., Andrási Z., Tátrai A., Józan P., Merkely B. (2012) Magyarországi primer prevenciós felmérés: Budakalász Epidemiológiai Vizsgálat előzetes eredmények. Orvosképzés 87:(2) pp. 102-108
8. Szelid Zs, Kerecsen G, Maurovich-Horvát P, Lux A, Marosi E, Kovács A, Kiss RG, Préda I, Merkely B (2010): Determination of coronary in-stent restenosis using dual source computed tomography angiography. Interventional Medicine and Applied Science 2010; 2(1): 5-9.

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Supplement

Differences in both disease models and species make comparisons extremely difficult and all conclusions have to be treated with extreme caution. With all this in mind, I believe my work complements already available data on the importance of NO signaling under unusual circumstances. It may be safe to say that both ischemia reperfusion injury and extreme athletic performance put additional load on viable myocardium and accumulate free radicals in the myocardium. In both settings, better nitric oxide availability was associated with beneficial effects on cardiac remodeling. Lack of data on nitric oxide availability and cGMP levels in our human participants makes mechanistic comparisons impossible. Luckily extreme athletic performance associated free radical accumulation is known, thus we can make speculations. In addition to changes in the lesser circulation, it is not unthinkable that NO mediated scavenging of free radicals also conferred some cardioprotection for our athletes carrying the Asp allele. At this point there might be similarities between murine experiments and human observations, as in mice we were able to document beneficial changes in NOx and nitrotyrosine levels after NO inhalation. The role of cGMP signaling remains debatable, but if we accept the idea of increased NO levels in Asp carriers (it was not measured), than there might be differences in cGMP availability as well. In this case cGMP mediated alteration of cardiac remodeling, just as in murine I/R models, might be present as well.

There are however too many differences between my experiments, thus I would refrain myself from translating inhaled NO mediated I/R injury reduction into a potential complementary performance-enhancing treatment for athletes.