

Themed Section: Pharmacology of the Gasotransmitters

REVIEW

Cardiac NO signalling in the metabolic syndrome

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Received

11 February 2014

Revised

9 September 2014

Accepted

28 September 2014

It is well documented that metabolic syndrome (i.e. a group of risk factors, such as abdominal obesity, elevated blood pressure, elevated fasting plasma glucose, high serum triglycerides and low cholesterol level in high-density lipoprotein), which raises the risk for heart disease and diabetes, is associated with increased reactive oxygen and nitrogen species (ROS/RNS) generation. ROS/RNS can modulate cardiac NO signalling and trigger various adaptive changes in NOS and antioxidant enzyme expressions/activities. While initially these changes may represent protective mechanisms in metabolic syndrome, later with more prolonged oxidative, nitrosative and nitrative stress, these are often exhausted, eventually favouring myocardial RNS generation and decreased NO bioavailability. The increased oxidative and nitrative stress also impairs the NO-soluble guanylate cyclase (sGC) signalling pathway, limiting the ability of NO to exert its fundamental signalling roles in the heart. Enhanced ROS/RNS generation in the presence of risk factors also facilitates activation of redox-dependent transcriptional factors such as NF- κ B, promoting myocardial expression of various pro-inflammatory mediators, and eventually the development of cardiac dysfunction and remodelling. While the dysregulation of NO signalling may interfere with the therapeutic efficacy of conventional drugs used in the management of metabolic syndrome, the modulation of NO signalling may also be responsible for the therapeutic benefits of already proven or recently developed treatment approaches, such as ACE inhibitors, certain β -blockers, and sGC activators. Better understanding of the above-mentioned pathological processes may ultimately lead to more successful therapeutic approaches to overcome metabolic syndrome and its pathological consequences in cardiac NO signalling.

LINKED ARTICLES

This article is part of a themed section on Pharmacology of the Gasotransmitters. To view the other articles in this section visit <http://dx.doi.org/10.1111/bph.2015.172.issue-6>

Abbreviations

ADMA, asymmetric dimethylarginine; AF, atrial fibrillation; DPP-4, dipeptidyl peptidase-4; PARP-1, poly[ADP-ribose] polymerase-1; PETN, pentaerythryl tetranitrate; PKG, cGMP-dependent PK; ROCK, Rho-associated PK; ROS, reactive oxygen species; SNO, S-nitrosothiol; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; 15D-PGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂

Tables of Links

TARGETS	
Enzymes^a	GPCRs^b
AMPK	Angiotensin AT ₁ receptor
Caspase-3	β ₁ -adrenoceptor
DPP-4, dipeptidyl peptidase-4	β ₃ -adrenoceptor
eNOS	Ion channels^c
iNOS	ATP-sensitive K ⁺ channels, K _{ir} 6.x
nNOS	RyR, ryanodine receptors
PDE1	Transporters^d
PDE2	GLUT 4, glucose transporter
PDE5	(SLC2A4)
PKG	Nuclear hormone receptors^e
ROCK, Rho kinase	PPARα
SERCA	PPARβ/δ
sGC, soluble guanylate cyclase	PPARγ
Xanthine oxidase/dehydrogenase	

LIGANDS	
15D-PGJ ₂ , 15-deoxy-Δ ^{12,14} -prostaglandin J ₂	Leptin
ADMA, asymmetric dimethylarginine	L-NAME
Allopurinol	Lovastatin
Angiotensin II	Melatonin
Ataciguat	Metformin
Atenolol	Nebivolol
Atorvastatin	Niacin, nicotinic acid
Atrial natriuretic peptide	NO
BH4, tetrahydrobiopterin	Pioglitazone
Captopril	Pravastatin
cGMP	Rosiglitazone
Cinaciguat	Rosuvastatin
Enalapril	Sildenafil
Fasudil	Simvastatin
Fluvastatin	Tadalafil
GIP	Vardenafil
GLP-1	Vitamin C
GW7647	Zofenopril
Insulin	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^{a,b,c,d,e}Alexander *et al.*, 2013a,b,c,d,e).

Introduction

Although NO was discovered decades ago, scientific interest in this gasotransmitter is continuously increasing. Enzymic and non-enzymic formation of NO and cGMP-dependent and independent NO signalling has been reviewed in detail in the current Themed Issue (Csonka *et al.*, 2015) and elsewhere (Ferdinandy and Schulz, 2003; Stasch *et al.*, 2011; Tang *et al.*, 2013; Rassaf *et al.*, 2014). Intercellular and intracellular NO signalling is very complex, reflecting its many pathways and interactions with other free radicals to form additional signalling molecules. Reactive oxygen species (ROS), especially the superoxide anion radical, can react with NO non-enzymically with an extremely high-rate constant limited only by diffusion. These reactions produce peroxynitrite (ONOO⁻) and other highly reactive oxygen and nitrogen species (ROS/RNS), which in concert with NO act as signalling molecules and also account for oxidative, nitrative and nitrosative stress (Ferdinandy, 2006; Pacher *et al.*, 2007; Pechanova and Simko, 2009). Most techniques available for the measurement of NO and its reactive metabolites have numerous technical limitations (reviewed in this Themed Issue by Csonka *et al.*, 2015) which further complicates the interpretation of results on the role of NO signalling in physiology and pathology.

NO plays an important role in the regulation of cardiovascular functions in health and disease by, for example,

promoting vasodilation, inhibiting vascular smooth muscle cell growth, platelet aggregation, and leukocyte adhesion, apart from by regulating myocardial function and providing cardioprotection (see Pacher *et al.*, 2007; Ferdinandy and Schulz, 2003; and reviewed in this Themed Issue by Andreadou *et al.*, 2015). The metabolic syndrome, comprising hypertension, hyperlipidaemia and insulin resistance/diabetes, is the major cardiovascular risk factor and thus accounts for leading causes of morbidity and mortality in industrialized societies. Publications on the role of NO-related pathways in these pathologies are continuously growing. In this review, we attempt to summarize the knowledge related to the role of NO signalling in the heart in the presence of the major cardiovascular risk factors that are associated with the metabolic syndrome. Our review focuses on the effect of the metabolic syndrome on NO signalling in the non-ischaemic heart.

The role of NO in myocardial ischaemia/reperfusion injury and cardioprotection by ischaemic conditioning in the healthy heart and in different co-morbidities is reviewed in detail elsewhere (Ferdinandy and Schulz, 2003; Andreadou *et al.*, 2015). In brief, NO itself protects the heart against ischaemia/reperfusion injury. However, accumulation of excess NO during prolonged ischaemia contributes to reperfusion injury via an increased oxidative/nitrative stress. The role of endogenous NO in cardioprotection induced by ischaemic preconditioning is still controversial (Csonka *et al.*,

1999; Nakano *et al.*, 2000; Post *et al.*, 2000). Nevertheless, it seems that mild oxidative/nitrative stress induced by exogenous or endogenous NO is necessary to trigger both pre- and post-conditioning (Nakano *et al.*, 2000; Csonka *et al.*, 2001; Heusch, 2001; Kupai *et al.*, 2009).

NO signalling in the heart

In the heart tissue, coronary and endocardial endothelial cells and cardiac myocytes are major sources of NO. However, NO may also derive from intracardiac ganglia and some nerve fibres located close to cardiac blood vessels. Endothelial NOS (eNOS) is expressed typically in the coronary and cardiac endothelium, whereas neuronal NOS (nNOS) is mainly located in the cardiac myocytes (see Pacher *et al.*, 2007; Tirziu and Simons, 2008). In coronary vascular endothelial cells, the eNOS-caveolin-1 interaction in the caveolae is important for normal eNOS activity (Feron and Balligand, 2006). The physiological triggers for NO release from endothelial cells are the flow-induced shear stress and mechanical deformations of the endothelium during the cardiac cycle (Michel, 2010). In cardiac myocytes, eNOS is co-localized with caveolin-3 in the T tubules of plasmalemmal caveolae, nNOS is localized in the sarcoplasmic reticulum (Shah and MacCarthy, 2000), and the putative mitochondrial NOS (mtNOS) in cardiac mitochondria (Dedkova and Blatter, 2009). The normal intracellular function of eNOS and nNOS in cardiomyocytes depends on discrete coupling mechanisms in the local cytosolic environments. These mechanisms can be affected by altered metabolism due to the metabolic syndrome (see Huang, 2009; Pechanova and Simko, 2010).

In fact, NO generated by inducible NOS (iNOS) may have its origin in the myocytes or neutrophils that migrate in the proximity of myocytes during inflammation and also in activated fibroblasts. iNOS, when expressed in cardiac myocytes, can regulate the response to β -adrenoceptor stimulation. However, as the neutrophils migrate to sites close to the myocytes, iNOS becomes essential for the ability of neutrophils to damage myocytes (Poon *et al.*, 2003). Indeed, an increase in iNOS expression in the heart with substrate limitation leads to uncoupled iNOS producing superoxide anions and contributing to contractile dysfunction (Heusch *et al.*, 2010). These findings demonstrate that cellular source and local cytosolic environment strongly modulate the effects of different NOS isoforms, as reviewed elsewhere (Tirziu and Simons, 2008; Huang, 2009; Pechanova and Simko, 2010).

NO may affect myocytes in a number of different ways. NO signalling via cGMP-dependent or independent pathways modulates the function of downstream proteins via specific post-translational modifications, such as phosphorylation by cGMP-dependent PK (PKG) or S-nitrosylation. Interestingly, an increase in intracellular cGMP induced by natriuretic peptides or cGMP analogues was recently shown to modulate both sarcolemmal and mitochondrial ATP-sensitive K^+ channel opening in ventricular cardiomyocytes suggesting further diverse actions of NO (Burley *et al.*, 2014).

NO also affects mitochondrial function and dynamics, thus regulating cardiac energy metabolism. Under

pathological conditions, it may also contribute to the development of myocardial dysfunction and heart failure (Davidson and Duchon, 2006; Azevedo *et al.*, 2013; Dai *et al.*, 2013; Miller *et al.*, 2013). Localization of NO production within mitochondria seems to provide a distinct reciprocal regulation between mtNOS and intramitochondrial Ca^{2+} , pH, L-arginine and oxygen. NO produced by the putative mtNOS may represent a mechanism of fine regulation of the respiratory complexes, enzymes of the citric acid cycle and energy metabolism as well (see Zaobornyj and Ghafourifar, 2012; Csonka *et al.*, 2015; Andreadou *et al.*, 2015). However, the existence of mitochondrial mtNOS is still a controversial issue (Pacher *et al.*, 2007), and NO, which rapidly diffuses into mitochondria from other cellular compartments or cells, is sufficient to efficiently regulate energy metabolism. Despite these facts, very few studies investigated the role of NO signalling in mitochondrial function in hearts with the metabolic syndrome.

The main physiological role of NO derived from eNOS and nNOS includes reduction of contractile frequency of cardiomyocytes, attenuation of cardiac contractility, acceleration of relaxation and increasing distensibility of cardiomyocytes, and improvement of the efficiency of myocardial oxygen consumption. In conditions of enhanced cardiac reserve and cardiac hypertrophy, NO derived from eNOS modulates receptor-mediated signalling which ultimately leads to a moderate inhibition of cardiac contractility (Shah and MacCarthy, 2000; Yue and Yu, 2011). NO derived from the complex of nNOS-ryanodine receptor (RyR) stabilizes RyR calcium release and increases the efficiency of Ca^{2+} cycling in sarcoplasmic reticulum by the inhibitory effects (Yue and Yu, 2011). In swine, intracoronary infusion of an NO synthesis inhibitor, N- ω -nitro-L-arginine, markedly decreased left ventricle (LV) function, while peak LV pressure and mean coronary arterial pressure were increased (Post *et al.*, 2001). Similarly, in healthy humans, inhibition of endogenous NO release also reduced, whereas replenishment with exogenous NO increased left ventricular function, further emphasizing that NO contributes to normal left ventricular function (Rassaf *et al.*, 2006). Thus, dysfunction of NOS induced by altered expression, location, coupling and activity may contribute to the contractile dysfunction, adverse remodelling and myocardial hypertrophy – changes associated with various cardiac disease conditions, such as heart failure and infarction (Tang *et al.*, 2013).

Interestingly, eNOS expression was not affected by cardiovascular risk factors like hypertension, obesity and insulin resistance (Fulton *et al.*, 2004; Bouvet *et al.*, 2007), and paradoxically was found to be increased in various pathological states associated with oxidative stress (Li *et al.*, 2002; Ding *et al.*, 2007; Zhen *et al.*, 2008). This effect may be partly mediated by limiting the availability of NO, thereby exerting a negative feedback on NOS expression through activation of NF- κ B (Zhen *et al.*, 2008; Pechanova and Simko, 2009; 2010; Vrankova *et al.*, 2009) (Figure 1).

In conclusion, signalling functions of NO produced by specific NOS isoforms seem to be compartmentalized in distinct cellular microdomains and thus modulate cardiac function differently. Moreover, they may be further affected by risk factors of the metabolic syndrome.

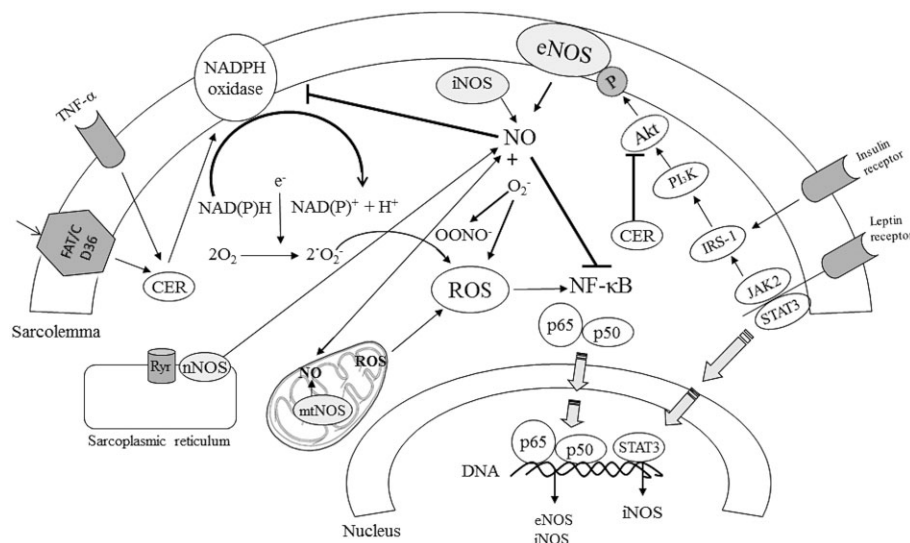


Figure 1

NO signalling and metabolic syndrome-related pathways. ROS generated by NADPH oxidases and other sources (e.g. mitochondria, XO, uncoupled NOS, among others) leads to increased NF- κ B activity followed by eNOS and iNOS up-regulation. eNOS produces NO which prevents activation of both NADPH oxidase and NF- κ B. The leptin/STAT3 pathway may also up-regulate the gene for iNOS whereas the leptin/JAK2/IRS-1 pathway increases eNOS activity via Akt stimulation, as does insulin. Increased circulating free fatty acids lead to ceramide elevation with increasing effects on NADPH oxidase activity and diminishing effects on Akt activation. NO produced by neuronal NOS (nNOS) and putative mtNOS may affect heart function in metabolic syndrome by different specific routes.

NO signalling in the hypertensive heart

Left ventricular remodelling and heart failure represent major pathological consequences of chronic arterial hypertension. During the development of hypertension, differential signals and metabolic abnormalities lead to the structural remodelling of the cardiovascular system, as characterized by myocardial hypertrophy and/or fibrosis, and coronary artery wall hyperplasia which finally result in heart injury known as cardiomyopathy (Kristek and Gerová, 1996; Babal *et al.*, 1997; Pechanova *et al.*, 1997; Tribulova *et al.*, 2000; Cebova and Kristek, 2011). Pathological remodelling of the hypertensive heart is due to an imbalance of stimulatory and inhibitory signals of tissue proliferation. Angiotensin II (Ang II), aldosterone or endothelin, with their vasoconstrictor and pro-proliferative effects, stand on one side of the balance and NO, prostacyclin, bradykinin or atrial natriuretic peptide, exerting vasodilating and antiproliferative activities, provide the counteracting factors (Swynghedauw, 1999; Cuspidi *et al.*, 2006; Pechanova and Simko, 2010). NO antagonizes the effects of Ang II on vascular tone, cell growth and renal sodium excretion, while it down-regulates the synthesis of ACE and angiotensin AT₁ receptor. On the other hand, Ang II decreases NO bioavailability by promoting oxidative stress (Zhou *et al.*, 2004). Mice infused with Ang II displayed an increase in blood pressure, cardiac hypertrophy and fibrosis associated with enhanced collagen I content, TGF- β 1 activity and endoplasmic reticulum stress markers, which were, however, blunted after endoplasmic reticulum stress inhibition (Kassan *et al.*, 2012). Recently, however, Jin *et al.* (2012)

demonstrated that myocardial nNOS is up-regulated by Ang II which functions as an early adaptive mechanism to attenuate NADPH oxidase activity and facilitate myocardial relaxation by promoting the cGMP/PKG pathway. It was also documented that activation of this pathway by novel soluble guanylate cyclase (sGC) stimulators, including riociguat (BAY 63-2521), attenuates systemic hypertension and systolic dysfunction, as well as fibrotic tissue remodelling in the myocardium in a rodent model of pressure and volume overload (Geschka *et al.*, 2011). This is in line with earlier data showing impaired NO-sGC signalling pathways in hypertension and heart failure, and beneficial effects of sGC stimulators/activators in preclinical models of hypertension in attenuating myocardial hypertrophy and remodelling (Evgenov *et al.*, 2006; Stasch *et al.*, 2011). Validating this concept, recent clinical trials with riociguat in pulmonary hypertension and chronic thromboembolic pulmonary hypertension showed encouraging results, which lead to the FDA approval of the drug for these indications (Ghofrani *et al.*, 2013a,b).

It is generally believed that increased production of ROS plays an important role in the pathology of hypertension, but so far the limited number of clinical studies using non-specific antioxidants yielded mixed results. Complicating the picture, it should also be noted that temporarily increased ROS generation in hypertension is not necessarily harmful, as it may stimulate the activity of the antioxidant defence system and improve the NO signalling pathway, resulting in the establishment of a new equilibrium between increased oxidative load and the stimulated NO pathways, thus maintaining sufficient NO availability (Dröge, 2002). However, in hypertension associated with obesity or diabetes, ROS may favour activation of pro-inflammatory NF- κ B-dependent

pathways (Figure 1). In these conditions, activation of NF- κ B increases levels of cytokines such as IL-6 and TNF- α that may affect the phosphorylation of tyrosine kinases and decrease NOS activity with a final decrease in NO generation (see Belin de Chantemele and Stepp, 2012).

In conclusion, increased ROS formation during hypertension may activate NF- κ B and promote pro-inflammatory and pro-oxidant changes (increased expression of TNF- α , COX2, iNOS, NADPH oxidase, etc.) or compensatory adaptive mechanisms (increased expression of eNOS and antioxidant enzymes). Prolonged ROS/RNS formation may also lead to uncoupling of eNOS/iNOS and impaired NO-sGC signalling in hypertensive cardiovascular system.

NO signalling in the obese/hyperlipidaemic heart

In obesity, cardiac output increases to serve the larger body mass of the obese individual (Kardassis *et al.*, 2012). The increase in cardiac output is due to a larger blood volume resulting in elevated venous return and an increased activation of the sympathetic nervous system, both prevalent in the obese population. An increase in cardiac output elevates cardiac oxygen consumption. Consequently, the need for perfusion is increased (Alvarez *et al.*, 2002; Frohlich and Susic, 2008). In mice fed a high-fat diet, obesity suppressed left ventricular ejection fraction, increased left ventricular remodelling, and led to diminished circulating endothelial progenitor cells level and impaired recovery of damaged endothelium (Tsai *et al.*, 2012).

The importance of two adipocyte-derived hormones – leptin and angiotensinogen – in the pathological consequence of obesity has been highlighted (Coatmellec-Taglioni and Ribière, 2003). Leptin regulates energy balance and metabolism by a variety of peripheral and central mechanisms through specific cell surface receptors (Koh *et al.*, 2008). Leptin infusion was shown to reduce blood pressure and heart rate, which may be reversed by an increased NO synthesis (Frühbeck, 1999). *In vitro* studies demonstrated that leptin elicited endothelium-dependent NO-mediated vasorelaxation in rats (Lembo *et al.*, 2000). In the mouse heart, disruption of leptin signalling may contribute to obesity-related cardiac disease, as leptin-deficient (*ob/ob*) mice display cardiac hypertrophy, increased cardiac apoptosis and reduced survival. These changes were linked to decreased cardiac expression of nNOS and NO production, with a concomitant increase in xanthine oxidase (XO) activity and oxidative stress, resulting in nitroso-redox imbalance (Saraiva *et al.*, 2007). Furthermore, cardiac β_3 -adrenoreceptor expression and function were shown to be dependent on leptin as they were severely diminished in the same model (*ob/ob* mice). It was proposed that diminished β_3 -adrenoreceptor signalling may be the critical element to explain the direct effects of leptin on the myocardium and suggest an important role of leptin in obesity-related cardiac hypertrophy and heart failure (Larson *et al.*, 2012). Leptin may up-regulate iNOS to generate large amounts of NO that induce nitrosative and nitrative stress and impair endothelial and myocyte functions (Koh *et al.*, 2008). In ventricular myocytes isolated from male

Sprague-Dawley rats, leptin-induced NO generation inhibited myocyte contraction which was prevented by the NOS inhibitor L-NAME (Nickola *et al.*, 2000). In addition, hyperleptinaemia may result in the overdrive of hypothalamus-pituitary-adrenal axis (HPA axis) and the sympathetic nervous system, as well as in impaired insulin secretion and insulin resistance. HPA axis overdrive would account for metabolic abnormalities such as central adiposity, hyperglycaemia, dyslipidemia, hypertension and other cardiovascular diseases which are well-known clinical aspects of the metabolic syndrome (Peters *et al.*, 2002).

Cardiac lipotoxicity caused by the accumulation of lipids has been well described in rodent models of obesity, hyperlipidaemia and diabetes (Zhou *et al.*, 2000; Chiu *et al.*, 2001; 2005; Young *et al.*, 2002). Feeding mice a palmitate-rich diet led to the accumulation of medium- and long-chain ceramides and sphingomyelins, which were incorporated into cellular membrane, thus changing the micro-domain structure of the plasma membrane of cardiomyocytes. The palmitate-rich diet also resulted in a decreased expression of caveolins, structural components of plasmalemmal rafts, the caveole (Knowles *et al.*, 2011; 2013). In addition, ceramides may activate NADPH oxidase leading to an increased oxidative stress (Zhang *et al.*, 2003) (Figure 1). In cardiomyocytes, eNOS localizes to caveolae, which contains β -adrenoceptors and L-type calcium channels as well (Garcia-Cardena *et al.*, 1996; Feron and Balligand, 2006). The co-localization of caveolin-3 and eNOS may facilitate both eNOS activation by cell surface receptors as well as NO release at the cell surface for intercellular signalling (Feron and Balligand, 2006). Immunohistochemistry findings in human cardiac tissue samples from obese humans showed a drastic reduction of caveolin-3 expression in cardiomyocytes (Knowles *et al.*, 2013), further signifying the role of caveolin proteins in obesity.

In conclusion, it seems that the dual effect of leptin in the obese heart depends on eNOS or iNOS activation by different mechanisms. Elevation of ceramide levels in obesity may inhibit eNOS activity by decreasing caveolin proteins and promoting oxidative stress.

NO signalling in the hypercholesterolaemic heart

It is well documented that hypercholesterolaemia profoundly affects cardiac NO metabolism. It has been previously reported that in cholesterol-fed rats, cardiac NO level decreases (Ferdinandy *et al.*, 1997; Giricz *et al.*, 2003; Onody *et al.*, 2003) and that hypercholesterolaemia blunts activity of downstream signalling elements of NO as indicated by a lower PKG activity (Giricz *et al.*, 2009). Reports on the effect of hypercholesterolaemia on the phosphorylation of myocardial eNOS, which reflects its activity, however, are controversial. In cholesterol-fed rats, Zhang showed a decreased p-eNOS level in parallel with an elevated apoptosis (Zhang *et al.*, 2012); meanwhile, in hearts of hypercholesterolaemic LDLr(-/-) mice, eNOS phosphorylation was unchanged (Ou *et al.*, 2011). Similarly, eNOS protein concentrations were found to be unchanged in cholesterol-fed rabbits

(Rajamannan *et al.*, 2005) and rats (Giricz *et al.*, 2003). These discrepancies might be attributed to the vast differences between the animal models. It has been also uncovered that the decrease in NO content in hypercholesterolaemic animals is supposedly not due to a decreased activity of NOS isoenzymes, but instead a result of an increased clearance of NO, as assessed by elevated markers of oxidative stress, such as dityrosine, nitrotyrosine (Giricz *et al.*, 2003) and superoxide anion formation due to at least, in part, XO activity (Onody *et al.*, 2003), and elevated expression and activity of NADPH oxidase (Onody *et al.*, 2003; Varga *et al.*, 2013). These reports were confirmed by Stokes *et al.* (2009) who found cardiac S-nitrosothiol (SNO) levels elevated and cardiac nitrite levels decreased in hypercholesterolaemic mice. In genetic models of hypercholesterolaemia, similar findings were reported. In human apoB100 transgenic mice, cholesterol-enriched diet increased cardiac superoxide anion generation and NADPH oxidase expression in parallel with an elevated cardiac nitrotyrosine level (Csont *et al.*, 2007). LDLr(-/-) mice also have a higher net production of ROS and susceptibility to develop membrane permeability transition, and increased ROS production in mitochondria can be observed (Oliveira *et al.*, 2005). These findings strongly emphasize that cardiac NO production is diminished, while its elimination is accelerated in diet-induced and genetic models of hypercholesterolaemia as well. Meanwhile, there is an apparent dearth of reports on the successful pharmacological restoration of hindered NO-related mechanisms: fasudil, a selective Rho-associated PK (ROCK) inhibitor elevated activity of antioxidant enzymes and the expression of eNOS as well as cardiac NO, and elsewhere atorvastatin increased eNOS protein concentrations and serum nitrite concentrations in cholesterol-fed rabbits (Rajamannan *et al.*, 2005). This scarcity of direct evidence is quite interesting, especially in view of the high number of antioxidant and anti-hyperlipidaemic treatments that have been under development recently. Therefore, it is likely that novel pharmacological targets will have to be explored aiming to restore cardiac NO homeostasis in hypercholesterolaemia.

One can speculate that disturbed NO metabolism might affect cardiac function. Indeed, it has been demonstrated in guinea pigs fed with a cholesterol-enriched diet that increased plasma XO activities were associated with a profound myocardial and coronary endothelial dysfunction (Schwemmer *et al.*, 2000). Similarly, cholesterol feeding resulted in the deterioration of cardiac function in rats (Onody *et al.*, 2003). This notion is further supported by other studies where positive chronotropic effect of atropine was selectively lost in genetically hypercholesterolaemic apoE^{-/-} mice, which was restored after a rosuvastatin treatment (Pelat *et al.*, 2003). This latter paper also reported that cardiac expression of caveolin-1 was elevated in apoE^{-/-} mice, further evidencing a disturbed NO metabolism in hypercholesterolaemia. Similarly, LDLr(-/-) mice demonstrated a decrease in left atrial contractility and eNOS expression relative to wild-type mice. Interestingly, LDLr(-/-) mice fed with an atherogenic diet for 15 days showed increased left ventricular mass and enhanced expression of NOS isoforms, which was reversed by the administration of S-nitroso-N-acetylcysteine (Garcia *et al.*, 2008). These results highlight that, although it is well studied, the contribution of disturbed

NO signalling to the deteriorated cardiac function in hypercholesterolaemia is not completely understood.

Isolated hypercholesterolaemia in humans is rarely seen; however, it is a major contributor to numerous pathological conditions, such as atherosclerosis and diabetes. NO metabolism in the human heart has been studied in even rarer cases. In hypercholesterolaemic patients, tetrahydrobiopterin (BH4) attenuated acetylcholine (ACh)-induced decrease in coronary diameter and restored ACh-induced increase in coronary blood flow, which was not shown in normocholesterolaemic patients (Fukuda *et al.*, 2002). Asymmetric dimethylarginine (ADMA) is an endogenous NOS inhibitor and an established cardiovascular risk factor in adults (Wu, 2009; Wu *et al.*, 2009). Serum concentration of ADMA is elevated in hypercholesterolaemic adults, which contributes to NO-dependent endothelial dysfunction (Böger *et al.*, 1998; for review, see Horowitz and Heresztyn, 2007), but not in children with hypercholesterolaemia type II, possibly due to an increase in dimethylarginine dimethylaminohydrolase activity (Chobanyan-Jürgens *et al.*, 2012). However, whether ADMA influences NO bioavailability in the heart, it has yet to be assessed.

In addition to decreased NO bioavailability, the NO-sGC signalling is also pathologically impaired in atherosclerosis, which can be successfully restored by novel sGC stimulators/activators in preclinical rodent models of atherosclerosis and restenosis, where these drugs attenuate inflammation and other pathological changes (Evgenov *et al.*, 2006; Stasch *et al.*, 2011).

In conclusion, in animal models and humans, hypercholesterolaemia hinders cardiac NO metabolism and, in these conditions, increased oxidative stress plays a major role. Furthermore, diminished NO availability and, most likely, impaired NO-sGC signalling in the heart tissue manifests in deteriorated cardiac function and would contribute to the development of other cardiovascular pathologies.

NO signalling in the diabetic heart

In diabetic patients, independent of vascular complications, a specific form of cardiomyopathy develops known as diabetic cardiomyopathy. Many factors may contribute to the evolution of this pathology, including metabolic disturbances (glucotoxicity, lipotoxicity), inflammatory processes, mitochondrial uncoupling, enhanced oxidative stress and deteriorated NO signalling (Pacher *et al.*, 2005). Several publications highlight the role of altered NO metabolism in diabetic cardiomyopathy, but surprisingly there is limited information on the direct measurement of cardiac NO levels obtained by strictly NO-specific methods (see Csonka *et al.*, 2015). As assessed by electron paramagnetic resonance spectrometry, a gold standard NO-specific method, NO level was increased in the hearts of streptozotocin-induced diabetic rats (Amour *et al.*, 2007). In line with this finding, an increase in cardiac NO metabolites (nitrite, nitrate) has been reported in the Goto-Kakizaki rat model of type 2 diabetes (Desrois *et al.*, 2010). Although these reports indicate that cardiac NO metabolism is influenced by diabetes, to date no data have been published on cardiac levels or bioavailability of NO from diet-induced animal models, let alone diabetic patients.

Diverse mechanisms have been proposed in diabetes-induced dysfunction of NO signalling. The pivotal role of altered eNOS function as the rate-limiting step in NO bioavailability is emphasized in the pathomechanism (Münzel *et al.*, 2005; Zhang *et al.*, 2011). The mechanisms responsible for eNOS dysfunction, however, remain elusive. Availability of cofactors for the eNOS complex, especially of BH₄, determines the ratio of NO or superoxide anion produced by the enzyme (Gielis *et al.*, 2011). Furthermore, a decrease in the dimer to monomer eNOS ratio within the myocardium of diabetic animals has been reported (Zou *et al.*, 2002; Jo *et al.*, 2011). Monomerization and subsequent uncoupling of NOS results in increased oxidative stress and decreased NO bioavailability that has been implicated in the pathophysiology of many cardiovascular diseases.

Of the three major NOS isoforms, two (iNOS and eNOS) are known to be increased in the diabetic heart (Stockklauser-Färber *et al.*, 2000; Farhangkhoei *et al.*, 2003; Jesmin *et al.*, 2006; Rajesh *et al.*, 2012). The increase in NOS expression in the diabetic heart is associated with an increase in lipid peroxidation and nitrotyrosine formation, which might be related to the uncoupled and monomer state of the enzyme. Indeed, inhibition of NOS activity in diabetes (by L-NAME or L-NMMA) improves myocardial function, suggesting that the increased production of superoxide anion and peroxynitrite rather than NO is a major contributor of suppressed contractile function (Smith *et al.*, 1997; Esberg and Ren, 2003). Moreover, it seems that peroxynitrite-induced nitrosative stress contributes to inactivation of succinyl-CoA:3-oxoacid CoA transferase causing deterioration of energy metabolism of the diabetic heart (Turko *et al.*, 2001). In addition, restoration of iNOS coupling by BH₄ administration improves ischaemic tolerance, reduces iNOS-derived superoxide anion generation, and increases NO bioavailability in the diabetic heart. The authors also imply that iNOS-derived NO-mediated cardioprotection occurs through protein S-nitrosylation but not cGMP-dependent signalling in the diabetic heart (Okazaki *et al.*, 2011). The central role of oxidative stress in impaired NO bioavailability and signalling in diabetic hearts is further substantiated by Rajesh *et al.* (2009), demonstrating that the XO inhibitor allopurinol not only attenuated the myocardial oxidative stress, but also attenuated the pathologically increased nitrosative/nitrosative stress, cell death, remodelling and cardiac dysfunction in diabetic mice hearts (see Ansley and Wang, 2013).

Much less is known about the NO-related downstream pathways (cGMP-PKG and NO-dependent post-translational modifications) in the diabetic heart. Recently, in patients with heart failure with preserved ejection fraction (obese and diabetic subjects), myocardial cGMP content as well as PKG activity is decreased, which might be related to the increase in oxidative/nitrosative stress (van Heerebeek *et al.*, 2012). However, it seems that natriuretic peptide-induced cGMP-PKG signalling is not affected by diabetes, as shown by Rosenkranz *et al.* (2003). They reported that B-type natriuretic peptide is a suitable anti-hypertrophic strategy in the diabetic myocardium, where NO-dependent (bradykinin – ACE inhibitor) mechanisms fail to positively affect the development of hypertrophy (Rosenkranz *et al.*, 2003). cGMP-independent effects of NO are mainly mediated by S-nitrosylation, the covalent modification of a protein

cysteine thiol by an NO group to generate SNO. Puthanveetil *et al.* reported recently that in the diabetic myocardium, iNOS-dependent S-nitrosylation of GAPDH and caspase-3 contributes to increased poly[ADP-ribose] polymerase-1 (PARP-1) activity, and thereby initiates cell death activation in hyperglycaemia (Puthanveetil *et al.*, 2012). This is also in line with data confirming the central role of PARP in diabetic cardiac complications (Pacher *et al.*, 2002; Pacher and Szabó, 2005).

In conclusion, diabetes markedly decreases NO availability in the heart that is related to increased superoxide (from various sources including uncoupled NOS) and peroxynitrite formation. As a consequence of increased oxidative/nitrosative stress, downstream signalling of NO (cGMP-PKG and protein S-nitrosylation) is also profoundly affected.

Cardiac NO signalling as a pharmacological target

NO donors

NO donors are pharmacologically active substances that spontaneously release NO, or are metabolized to NO or its redox congeners and provide a wide scope for pharmacotherapy in cardiovascular medicine (Ignarro *et al.*, 2002). Several NO donors have been used in clinical settings for decades, such as nitroglycerin and sodium nitroprusside. Nitrate tolerance, however, has become a limiting factor for their clinical use (Kojda *et al.*, 1995; 1998; Csont and Ferdinandy, 2005). The underlying mechanisms responsible for nitrate tolerance may include neurohormonal counter-regulatory factors, intravascular volume or intrinsic abnormalities such as desensitization of the target enzyme guanylate cyclase or a decrease in biotransformation of NO donors (Munzel *et al.*, 1995; Dikalov *et al.*, 1997; 1998; 1999). Molsidomine and pentaerythrityl tetranitrate (PETN) represent more effective tolerance-devoid NO donors with a pharmacodynamically beneficial effect. Molsidomine is one of the sydnonimines and it is metabolized to the active linsidomine. PETN is the nitrate ester of pentaerythritol, structurally very similar to nitroglycerin. It was found to be the most active drug in cGMP production (Hinz *et al.*, 1998; Mollnau *et al.*, 2005). Despite these facts, neither molsidomine nor PETN was able to improve pathological changes of the cardiovascular system in adult spontaneously hypertensive rats (Kristek *et al.*, 2003).

The compound LA-419 is an analogue of isosorbide mononitrate containing a protected thiol group in its molecular structure. Preclinical studies have shown that this compound has anti-atherogenic and antioxidant properties that make it applicable for the treatment of chronic cardiovascular disorders (Megson and Leslie, 2009). Ruiz-Hurtado and Delgado (2010) demonstrated that LA-419 prevents left ventricular remodelling in rats with aortic stenosis at doses not affecting arterial blood pressure. In their experiment, LA-419 even restored cardiac eNOS expression and enhanced the interaction between eNOS and its positive regulator, heat shock protein 90, and re-established the normal cardiac levels of cGMP. The thiol group of LA-419 improved also NO stability by converting NO into nitrosothiols and protecting the

formed NO from reaction with ROS (Ruiz-Hurtado *et al.*, 2007; Ruiz-Hurtado and Delgado, 2010).

In conclusion, there are very few data about the effects of NO donors on heart and/or cardiomyocyte functions in the metabolic syndrome. Nevertheless, the beneficial effect of compound LA-419 seems to be a promising therapeutic approach against cardiac remodelling due to the metabolic syndrome and the associated risk factors as well. However, the impaired NO-sGC signalling in the metabolic syndrome by oxidative stress is likely to represent a major obstacle for the success of this approach.

ROS scavengers

ROS are involved in several physiological cellular signalling mechanisms. However, pathological increase in oxidative stress contributes to different pathologies including the metabolic syndrome and cardiovascular disorders. Accordingly, one of the most powerful antioxidants, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol), prevents cardiovascular damage in different experimental hypertension and diabetes models (Ebenezer *et al.*, 2009; Hasdan *et al.*, 2002; Nagase *et al.*, 2007), decreases hypertrophic responses to atrial natriuretic peptide in neonatal rat cardiac myocytes (Laskowski *et al.*, 2006), and reduces apoptosis in cardiac cells exposed to hyperglycaemia or in diabetic rats (Fiordaliso *et al.*, 2007). Tempol decreased apoptosis in response to increased aldosterone signalling via a non-genomic pathway in cardiomyocytes (Hayashi *et al.*, 2008) and inhibited the Ca²⁺ transient within cardiac myocytes stimulated by pressure-flow stress (Belmonte and Morad, 2008). Tempol improved insulin sensitivity and dyslipidemia, reduced weight gain and diastolic dysfunction and heart failure in diet-induced preclinical models of the metabolic syndrome (see Wilcox, 2010). Moreover, infusion of tempol into hyperglycaemic dogs normalized their coronary endothelial dysfunction and coronary wall shear stress in type 1 and 2 diabetes models (Gross *et al.*, 2003). Chronic treatment with another antioxidant, N-acetylcysteine (NAC), partially attenuated the increase in blood pressure in young, but not in adult spontaneously hypertensive rats (SHR). The antioxidant action of NAC on lipid peroxidation, inhibition of NF-κB expression and eNOS activation was greater in young than in adult SHR, indicating preventive rather than therapeutic effect of NAC (Pechánová *et al.*, 2006). Melatonin, an indoleamine with antioxidant properties, has been shown to decrease blood pressure even in the established form of the spontaneous hypertension. An *in vitro* study revealed that melatonin lowered the tone of phenylephrine-precontracted femoral artery via both NO-dependent and NO-independent components since vasorelaxation was preserved even after the blockade of sGC by oxadiazolo[4,3-a]quinoxalin-1-one (Pechánová *et al.*, 2007). Melatonin treatment also prevented the development or induced a reversal of left ventricular fibrosis in the model of L-NAME-induced hypertension or in spontaneously hypertensive rats (see Simko and Pechanova, 2010). It has been documented that melatonin reduces blood pressure in patients with hypertension or non-dipping blood pressure (Reiter *et al.*, 2009). Interestingly, melatonin, leptin and insulin have been found to activate the same intracellular signalling pathways, particularly PI3K and STAT-3 (Carvalho *et al.*, 2001). As a consequence, melatonin may

attenuate or reverse insulin resistance in obesity by mimicking the actions of insulin and leptin signalling via crosstalk between these pathways (see Nduhirabandi *et al.*, 2012).

Several studies described positive effects of different polyphenolic compounds on the heart by restoring the balance between ROS and NO production, in hypertension as well as in other components of the metabolic syndrome (Pechánová *et al.*, 2004; Galleano *et al.*, 2010). Sutra *et al.* (2008) showed the preventive effects of different polyphenolic molecules, like catechin, resveratrol, delphinidin and gallic acid, on cardiac fibrosis associated with the metabolic syndrome. Similarly, protection of ROS/NO balance was suggested to be involved in the beneficial effect of resveratrol. The results of Penumathsa *et al.* (2008) suggested that the effect of resveratrol is non-insulin-dependent but triggers some of the intracellular insulin signalling components such as eNOS and Akt through the AMPK pathway in the myocardium. Furthermore, resveratrol was shown to regulate the caveolin-1 and caveolin-3 status that might play an essential role in GLUT-4 translocation and glucose uptake in streptozotocin-induced type 1 diabetic myocardium (Penumathsa *et al.*, 2008). Similarly, olive leaf extract containing polyphenols, such as oleuropein and hydroxytyrosol, was shown to reverse chronic inflammation and oxidative stress in rat model of diet-induced obesity and diabetes (Poudyal *et al.*, 2010). Resveratrol also protected against diabetic cardiac dysfunction by inhibiting oxidative/nitrative stress and improving NO availability (Zhang *et al.*, 2010).

Despite the fact that antioxidants represent great promise in the treatment of hypertension and other components of the metabolic syndrome, data from clinical studies and trials with non-specific antioxidants are not conclusive. For example, in the HOPE (Heart Outcomes Prevention Evaluation) study, involving patients with atherosclerotic complications or diabetes mellitus, vitamin E in the dose of 400 IU daily, was not able to reduce blood pressure and morbidity and mortality from cardiovascular reasons (Yusuf *et al.*, 2000; Ward and Croft, 2006). In contrast, a more recent study confirmed that subjects with type 2 diabetes after a 3 month long supplementation of vitamins C and E or their combination demonstrated significantly lower level of hypertension, decreased levels of blood glucose, and increased superoxide dismutase (SOD) and GSH enzyme activity that could probably reduce insulin resistance by attenuating oxidative stress (Rafiqi *et al.*, 2013). Vitamin C was also shown to increase BH4 levels by preventing its oxidation, which reduced eNOS uncoupling (Landmesser *et al.*, 2003). Thus, preservation of BH4 may also explain the effects of long-term ascorbate treatment on blood pressure in patients with hypertension (Duffy *et al.*, 1999).

Several studies suggest that imbalance between ROS production and mitochondrial antioxidants also contributes to the pathogenesis of hypertension and associated vascular pathologies. Ito *et al.* (1995) found that hypertension and cardiac hypertrophy were associated with decreased expression of SOD1 and SOD2 in spontaneously hypertensive rats compared with Wistar-Kyoto rats. Indeed, overexpression of mitochondrial SOD2 and thioredoxin 2 reduced the production of both mitochondrial and cytoplasmic ROS (Widder *et al.*, 2009). SOD2 overexpression also attenuated H₂O₂-induced apoptosis, decreased lipid peroxidation, reduced

age-related decline in mitochondrial ATP levels and decreased blood pressure (see Dikalov and Ungvari, 2013).

Recently, extracellular antioxidant enzymes like EC-SOD or covalent henzymes like SOD-CHS-CAT conjugate (superoxide dismutase-chondroitin sulphate-catalase) started to be of particular interest, as they demonstrated protective actions against development of hypertension, heart failure and diabetes mellitus *in vivo* (see Maksimenko and Vavaev, 2012).

Taken together, based on numerous promising preclinical studies with mitochondrial antioxidants, XO and/or NADPH oxidase inhibitors in models of hypertension, diabetes and atherosclerosis, it appears that, instead of using non-specific antioxidants, selectively targeting the sources of ROS with more specific drugs may represent a better approach to overcome metabolic syndrome and its complications.

PDE inhibitors

The cGMP-dependent NO signalling is largely influenced by the family of PDEs that control cGMP levels and therefore affect the downstream effects of NO including PKG stimulation. Several PDEs, including PDE1, PDE2 and PDE5, play a role in the regulation of cGMP in both vascular smooth muscle cells and cardiac myocytes. PDEs are compartmentalized providing selective interactions of a certain source of cGMP and PDE hydrolysis. PDE1 and/or PDE5 are up-regulated in chronic disease conditions such as atherosclerosis, cardiac pressure-load stress, and heart failure, as well as in response to long-term exposure to nitrates. In pathophysiological states with reduced NO availability, such as, for example, diabetes and hyperlipidaemia (see above), using selective PDE inhibitors may be particularly helpful (see Kass *et al.*, 2007). Because PDE-5 is widely distributed in the body, selective PDE-5 inhibitors have been extensively developed. The first PDE-5 inhibitor sildenafil on the market is used for the indication of erectile dysfunction. However, recent studies revealed several beneficial pleiotropic cardiovascular effects of PDE-5 inhibitors in patients with erectile dysfunction and multiple co-morbidities, including coronary artery disease, heart failure, hypertension and diabetes mellitus (see Chrysant and Chrysant, 2012). For example, tadalafil attenuates oxidative stress and inflammation and induces cardioprotection in type 2 diabetic mice models (Varma *et al.*, 2012; Koka *et al.*, 2013). Moreover, vardenafil attenuated diabetes-induced cardiac dysfunction in type 1 diabetic rats (Radovits *et al.*, 2009).

In conclusion, PDE inhibition is a promising tool to restore the downstream signalling pathway of NO in the metabolic syndrome.

sGC stimulators and activators

Activation of sGC has traditionally been achieved with nitrovasodilator drugs extensively used in ischaemic heart disease. However, these drugs are associated with the rapid development of tolerance and potentially deleterious cGMP-independent actions (see Csont and Ferdinandy, 2005). Furthermore, the NO-sGC signalling pathway is impaired in hypertension, heart failure and atherosclerosis by ROS/RNS, limiting the ability of NO to activate its own signalling machinery (Evgenov *et al.*, 2006; Stasch *et al.*, 2011). There-

fore, NO- and haem-independent sGC activators have been developed, such as, for example, cinaciguat and ataciguat. These compounds selectively activate the oxidized/haem-free enzyme via binding to the haem pocket of the enzyme, thereby causing strong vasodilatation. Accordingly, activators of sGC may be beneficial in the treatment of a variety of pathologies including systemic and pulmonary hypertension, heart failure, atherosclerosis and peripheral arterial disease (Evgenov *et al.*, 2006; Stasch *et al.*, 2011). Indeed, NO-insensitive sGC activators attenuated left ventricular hypertrophy, preserved cardiac function, and increased survival in spontaneously hypertensive stroke-prone rats with high-salt high-fat diet (Costell *et al.*, 2012), in salt-sensitive Dahl rats (Geschka *et al.*, 2011), as well as in chronic L-NAME-treated rats (Zanfolin *et al.*, 2006). sGC activators have demonstrated beneficial effects not only in hypertension and heart failure models but also in models of atherosclerosis and restenosis (see Evgenov *et al.*, 2006; Stasch *et al.*, 2011). Following successful recent clinical trials, riociguat received FDA approval for the treatment of pulmonary hypertension and chronic thromboembolic pulmonary hypertension in humans, and clinical trials with other similar drugs are ongoing in heart failure.

In conclusion, the pharmacological activation of sGC may be the most promising tool to restore the downstream signalling pathway of NO in the metabolic syndrome, which should be validated in future clinical trials.

Interaction of pharmacological treatment of metabolic syndrome with cardiac NO signalling

Interaction of antihypertensives with cardiac NO signalling

Three approaches have been developed to correct the imbalance between increased oxidative stress and simultaneously decreased NO synthesis in the cardiovascular system: (1) reducing ROS bioavailability by administration of antioxidant compounds; (2) increasing NO levels via administration of NO donors such as nitroglycerin or mono/dinitrates; and (3) reducing ROS production and stimulating NO production, for example, by treatment with statins, ACE inhibitors, angiotensin AT₁ receptor antagonists, or β -adrenoceptor antagonists (β -blockers) with NO-dependent properties such as nebivolol (see Münzel *et al.*, 2010).

Among antihypertensives, the third-generation β -blockers with stimulating effect on NOS and/or β_3 -adrenoceptors have the best described effect on cardiac NO signalling. Nebivolol achieved a marked improvement on cardiac mass, coronary flow, mRNA expression levels of sarcoplasmic reticulum Ca²⁺ ATPase (SERCA2a), and atrial natriuretic peptide and phospholamban (PLN)/SERCA2a and phospho-PLN/PLN ratio in rats treated with isoprenaline (Ozakca *et al.*, 2013). In Zucker diabetic fatty rats, nebivolol and atenolol showed a comparable reduction in blood pressure; however, nebivolol appeared to achieve a better lipid profile, left ventricular function and less left ventricular hypertrophy, compared with atenolol. Moreover, a reduction

in platelet aggregation and an increased endothelium-dependent and endothelium-independent relaxation were observed in the nebivolol group versus the atenolol group. Together with an attenuation of oxidative stress parameters, nebivolol also better preserved antioxidant defence markers (Toblli *et al.*, 2010). Concerning NO signalling, nebivolol has been shown to stimulate endogenous production of NO by inducing phosphorylation of eNOS (Maffei *et al.*, 2006) which determines its favourable effects on cardiac function in patients with heart failure when compared with classical β -blockers. The action of nebivolol on iNOS was also confirmed by real-time PCR experiments, showing cardiac overexpression of iNOS, but not nNOS or eNOS, in male C57BL/6N mice (Maffei *et al.*, 2007).

Among other promising antihypertensives with NO increasing and ROS reducing effect are the ACE inhibitors with a thiol group such as captopril and the newer zofenopril. In our earlier studies, both captopril and enalapril increased NOS activity in the heart of spontaneously hypertensive animals but did not increase the expression of eNOS. Both ACE inhibitors increased the level of cGMP. However, cGMP levels were significantly higher in the captopril group. Captopril, besides inhibition of ACE, prevented hypertension by increasing NOS activity and by simultaneous decrease of oxidative stress which resulted in increase of cGMP concentration (Pechánová, 2007). Most of the clinical studies revealed that captopril, besides decreasing blood pressure, has also vasodilator effects and attenuates left ventricular hypertrophy (Konstam *et al.*, 2000). The SMILE (Survival of Myocardial Infarction Long-term Evaluation) program indicates that zofenopril may favourably affect the prognosis of patients with a recent myocardial infarction (Lombardi *et al.*, 2012) and even of patients with the metabolic syndrome (Borghi *et al.*, 2008). Accordingly, a 12 week zofenopril treatment significantly decreased lipid peroxidation, reduced cardiac hypertrophy and improved NO pathway in patients with essential hypertension (Napoli *et al.*, 2004).

In conclusion, antihypertensive drugs, such as β -blockers with NO-dependent effects and ACE inhibitors with a thiol group, may successfully restore NO signalling in the heart in the metabolic syndrome.

Interaction of antidiabetic drugs with cardiac NO signalling

For the treatment of diabetes, several classes of drugs are available with markedly different mechanisms of action. Besides various synthetic insulin analogues, several other non-insulin-related drugs were developed and marketed in the last years. The mechanism of action involves the stimulation of endogenous insulin secretion, the sensitization of peripheral tissues to insulin or the increase in incretin levels. Although these mechanisms are directly not related to NO signalling, all of these drugs have some degree of interaction with NO-related pathways.

Insulin itself is a strong regulator of cardiac NO level by affecting eNOS phosphorylation. Administration of insulin *in vivo* to healthy rats activates Akt through a PI3K-dependent mechanism. Phosphorylation of the eNOS and the concurrent increase in NO production is a result of Akt activation (Gao *et al.*, 2002). However, this NO-related effect of insulin is attenuated in the diabetic myocardium (Zakula *et al.*, 2011).

Sulfonylurea drugs are potent stimulators of endogenous insulin secretion by acting on ATP-sensitive K^+ channels. Although these drugs do not interact directly with myocardial NO production, experimental and clinical data suggest considerable interaction with NO signalling. Cardioprotection mediated by NO is mainly related to the opening of mitochondrial ATP-sensitive K^+ channels (Han *et al.*, 2002; Ljubkovic *et al.*, 2007). The non-selective nature of K^+ channel inhibition results in the attenuation of NO-mediated cardioprotection by sulfonylureas, limiting their clinical applicability in diabetic patients with ischaemic heart diseases (Garratt *et al.*, 1999).

Insulin-sensitizing drugs include biguanides (metformin is the most often used) and the thiazolidinedione class of antidiabetic drugs (rosiglitazone and pioglitazone). These drugs mainly act at peripheral tissues by sensitizing them to the action of insulin. Metformin facilitates the activation of AMP-activated PK (AMPK) in the heart that has been shown to be cardioprotective during heart failure. Metformin-induced positive effects were associated with increased AMPK and eNOS phosphorylation, and reductions in insulin, TGF- β 1, basic fibroblast growth factor, and TNF- α levels in the circulation and/or in the myocardium (Gundewar *et al.*, 2009; Wang *et al.*, 2011). Withdrawal of rosiglitazone from the market due to adverse cardiovascular effects (increased mortality, accentuation of ischaemic heart diseases) highlighted the controversial cardiovascular effects of thiazolidinediones. In experimental studies, both rosiglitazone (Gonon *et al.*, 2007) and pioglitazone (Ye *et al.*, 2008a) reduced infarct size possibly via increased eNOS phosphorylation. However, the mechanisms that resulted in adverse effects in humans are still not known.

Dipeptidyl peptidase-4 (DPP-4) inhibitors are a relatively new class of antidiabetics. By the inhibition of DPP-4, they increase the level of incretins (GIP and GLP-1), inhibiting glucagon release, which in turn increases insulin secretion, decreases gastric emptying and decreases blood glucose level (Figure 2). DPP-4 inhibitors were proven to be atheroprotective (Matheeußen *et al.*, 2013) and to affect positively diastolic function in the insulin-resistant Zucker diabetic fatty rat model by increasing phosphorylation of eNOS (Ser¹¹⁷⁷) and the expression of total eNOS (Aroor *et al.*, 2013).

In conclusion, antidiabetics (except for sulfonylureas) may positively affect tissue NO availability and NO signalling, thereby providing a promising tool to treat cardiac complications of the metabolic syndrome.

Interactions of anti-hyperlipidaemic treatments and the cardiac NO signalling

Statins are the most frequently prescribed anti-hyperlipidaemic medications. Apart from their HMG-CoA reductase inhibitory function, statins reduce cardiovascular risks associated with hypercholesterolemia via a wide range of well-documented pleiotropic effects. For instance, in the heart, atorvastatin increases phosphorylation of a host of mediators associated with NO signalling, such as ERK, PDK-1, Akt and eNOS itself, plausibly via an adenosine receptor-dependent mechanism (Merla *et al.*, 2007; Ye *et al.*, 2008b). Direct modulation of NO signalling by statins downstream of NOS was also suggested by another study, where rosuvastatin administration reverted the elevation in mean arterial blood

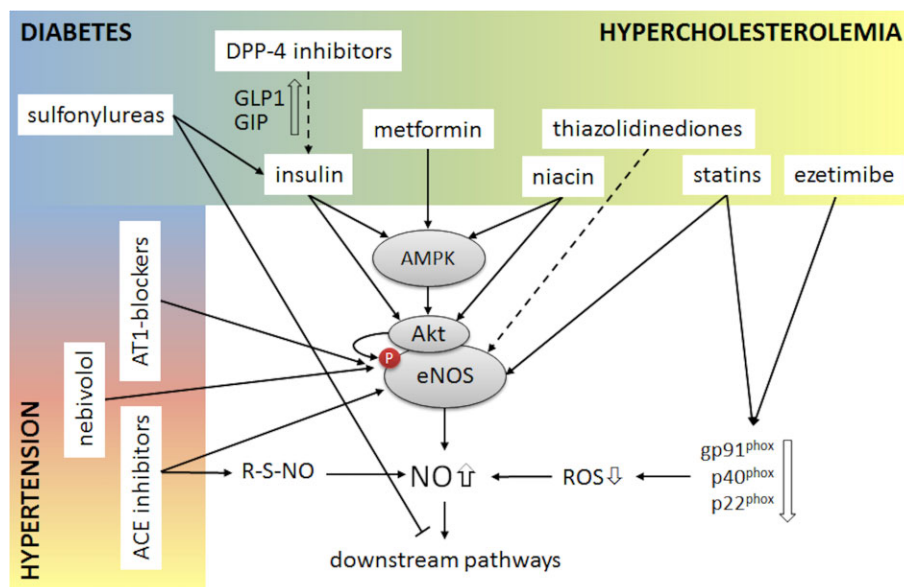


Figure 2

Effect of drugs used in the metabolic syndrome on cardiac NO signalling. Antihypertensives activate primarily eNOS or potentiate the release of NO from SNOs. Antidiabetics activate predominantly the kinases AMPK and Akt upstream of eNOS to induce its phosphorylation. Sulfonylureas may, however, interfere with NO-related downstream effectors (i.e. mitochondrial K_{ATP} channels). Anti-hyperlipidaemic drugs have pleiotropic effects on NO signalling, serving as antioxidants and inducers of eNOS phosphorylation.

pressure and cardiac remodelling caused by a treatment with a NOS inhibitor, L-NAME (Baraka *et al.*, 2009). Statins modulate cardiac NO metabolism under hyperlipidaemic conditions as well. In OLETF rats, both atorvastatin and pravastatin up-regulated cardiac eNOS expression compared with their genetic controls (Yu *et al.*, 2004; Chen *et al.*, 2007). Interestingly, not all statins are equally effective in the modulation of cardiac NO metabolism. For example, pravastatin induced eNOS more effectively than atorvastatin (Chen *et al.*, 2007), and we have previously shown that the first-generation statin, lovastatin, does not affect NO production or NOS activity in cholesterol-fed animals (Giricz *et al.*, 2003). Similarly, in spontaneously hypertensive rats, pravastatin treatment failed to modulate the expression of nNOS, eNOS, sGC or the NADPH oxidase subunits p40Phox and Gp91 in myocardial tissue (Herring *et al.*, 2011), which highlights that NO modulation is not a general characteristic of the whole class of statins and the effects are strongly model dependent. Newer statins have been also shown to alter cardiac NO bioavailability in other pathologies unrelated to hyperlipidaemia. For instance, in a hypertension model of rats over-expressing renin, rosuvastatin decreased the accentuated myocardial gp91(phox), p40(phox), p22(phox) expression and reduced the myocardial lipid peroxidation, nitrotyrosine formation and malondialdehyde content, suggesting that it increased NO bioavailability by reducing ROS formation (Habibi *et al.*, 2007). Elsewhere, simvastatin reduced iNOS expression in cytokine-treated H9C2 cardiac myoblasts, which appeared to be related to the cholesterol biosynthesis-modulating effect of statins, since mevalonate, and geranylgeranyl pyrophosphate could reverse these effects (Madonna *et al.*, 2005). However, other statins exerted

seemingly opposing effects on the cardiac NO production modulated by pro-inflammatory signals: lipophilic statins fluvastatin and lovastatin increased IL-1 β -induced nitrite production by cardiac myocytes, whereas hydrophilic pravastatin did not. Fluvastatin also increased iNOS expression (Ikeda *et al.*, 2001). These data demonstrate clearly that, before initiating a statin treatment, compounds must be evaluated individually in the view of the other coexisting pathologies. Statins might have positive effects on age-related disturbance of cardiac NO metabolism as well. In 20-month-old rats, atorvastatin administration for 4 months reversed the age-related increase in cardiac malondialdehyde and decrease of SOD, catalase and NOS activity (Han *et al.*, 2012). Direct effects of statins on cardiac NO signalling have been studied in humans in a few publications. Perioperative simvastatin therapy of patients undergoing non-coronary cardiac surgery increased nitrite and nitrate levels, expression and phosphorylation of eNOS at Ser¹¹⁷⁷, phosphorylation of Akt, HSP90, and its association with eNOS in right atrial appendage (Almansob *et al.*, 2012). Furthermore, atorvastatin induced a mevalonate-reversible inhibition of NOX2-NADPH oxidase activity in right atrial samples from patients who developed post-operative atrial fibrillation (AF); however, it did not affect ROS, or NOS uncoupling in patients with permanent AF (Reilly *et al.*, 2011). Although the general notion is that statins improve cardiac NO metabolism, these data also suggest that differences in the biochemical background of diverse pathologies might profoundly influence the beneficial pleiotropic effects of several of the statins.

The PPAR family of nuclear receptors has been a target for numerous antidiabetic and anti-hyperlipidaemic agents, many of which are shown to modulate NO metabolism.

GW7647, a potent PPAR α inducer, enhanced cardiac eNOS activation in isolated papillary muscles of rat hearts (Xiao *et al.*, 2010). WY-14643, another PPAR α agonist, has also been shown to increase the expression of eNOS and iNOS, as well as nitrite/nitrate levels in the ischaemic myocardium of Goto-Kakizaki and Wistar rats (Bulhak *et al.*, 2009). This publication also demonstrated that PPAR α activation leads to the induction of the downstream mediators of NO, as shown by an elevated cardiac phosphorylation of Akt at Ser⁴⁷³ and Thr³⁰⁸. However, elsewhere, fenofibrate, also a PPAR α inducer, did not alter cardiac NO or its metabolites in LPS-treated Wistar rats (Jozefowicz *et al.*, 2007). Similarly, PPAR β/δ agonist GW0742 reduced the ischaemia/reperfusion-induced increase in the expression of iNOS and normalized the phosphorylation of Akt and glycogen synthase kinase-3 β in a rat model of regional myocardial I/R *in vivo* (Kapoor *et al.*, 2010), demonstrating the involvement of these PPAR isoforms in NO metabolism. More information is available on the effects of PPAR γ induction on cardiac NO balance. The endogenous PPAR γ ligand, 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (15D-PGJ₂), attenuated the cardiac ischaemia/reperfusion-induced increase in iNOS mRNA expression in rats (Wayman *et al.*, 2002). The inhibition of iNOS expression by 15D-PGJ₂, but not by rosiglitazone, a synthetic PPAR γ agonist, was confirmed in neonatal cardiomyocytes pretreated with LPS (Hovsepian *et al.*, 2010) or IL-1 β (Mendez and LaPointe, 2003). Negative correlation between the activity of PPAR γ and NOS enzymes was confirmed in another study, where pioglitazone down-regulated iNOS expression in a murine cardiac allotransplantation model (Hasegawa *et al.*, 2011). However, pioglitazone seems to have opposing effects on eNOS. In diabetic OLETF rats, cardiac expression of eNOS and phosphorylation of Akt was reduced compared with non-diabetic controls, which was reversed by the induction of PPAR γ by pioglitazone (Makino *et al.*, 2009). The notion that disturbed eNOS signalling is restored by PPAR γ activation seems to be strengthened by other experiments. Phosphorylated eNOS was increased in mice receiving rosiglitazone before ischaemia/reperfusion (Gonon *et al.*, 2007), and in diabetic *db/db* mice, the reduced dilations of coronary arterioles in response to ACh and the NO donor NONOate were augmented by rosiglitazone (Bagi *et al.*, 2004).

There is only a limited amount of data on the effect of other less frequently prescribed anti-hyperlipidaemic agents on cardiac NO signalling. Although dietary supplementation of niacin is often recommended for obese patients, its effect on the NO-cGMP-PKG system has been revealed indirectly in a single publication. Niacin-bound chromium induced myocardial phosphorylation of Akt, AMPK and eNOS in streptozotocin-induced diabetic rats after ischaemia-reperfusion injury, suggesting that beneficial effects of niacin and chromium are mediated not only through the modulation of metabolic pathways, but via the activation of the NO pathway as well (Penumathsa *et al.*, 2009). Inhibition of cholesterol absorption by ezetimibe, an inhibitor of the intestinal Niemann-Pick C1-like 1 protein, has been shown to decrease cardiac NADPH oxidase-mediated oxidative stress in hyperlipidaemic *db/db* mice (Fukuda *et al.*, 2010), therefore, plausibly increase NO bioavailability, known to be depressed in hyperlipidaemia (Ferdinandy *et al.*, 1997; Gircz *et al.*, 2003; Onody *et al.*, 2003).

In conclusion, experimental evidence shows that administration of various cholesterol-reducing medications leads to a restoration of the balance in NO metabolism disturbed by hyperlipidaemia.

Conclusions and perspectives

Published data show that NO availability and its signalling in the heart is impaired in the presence of risk factors associated with the metabolic syndrome. The decreased tissue availability of NO is a consequence of increased oxidative and nitrosative/nitrate stress rather than a decreased cardiac NO synthesis. The impaired NO signalling in the heart due to the metabolic syndrome leads to different pathophysiological processes including myocardial hypertrophy, fibrosis and eventually heart failure. Therefore, in addition to treating the individual risk factors related to the metabolic syndrome, restoration of NO signalling in the heart by pharmacological tools may be a promising therapeutic avenue to alleviate cardiac pathologies related to the metabolic syndrome.

Acknowledgements

This study was elaborated within the projects APVV-0742-10, VEGA 2/0183/12 and 2/0144/14, the New Horizons Grant of the European Foundation for the Study of Diabetes, Hungarian Scientific Research Fund (OTKA K109737) and COST Action BM1005, and the Intramural Program of NIH/NIAA. All authors contributed equally on literature search and metabolic syndrome models preparation.

Conflicts of interest

The authors declare no conflict of interest.

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