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Review

Reactive oxygen species as cardiovascular mediators: Lessons from endothelial-specific protein overexpression mouse models

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ABSTRACT

The term reactive oxygen species (ROS) summarizes several small chemical compounds such as superoxide, peroxynitrite, hydrogen peroxide and nitric oxide. The stoichiometry of the chemical reactions underlying generation and metabolism is subject of tight enzymatic regulation resulting in well balanced steady-state concentrations throughout the healthy body. ROS are short-lived and usually active at the site of production only, e.g. in vascular endothelial cells. Although an increase of vascular ROS-production is considered an important pathogenic factor in cardiovascular diseases, there is evidence for physiological or even beneficial effects as well. We have generated several transgenic mice using the Tie-2 promotor which expresses an enzyme of interest specifically in vascular endothelial cells. Here, we review some results obtained with mice carrying a Tie-2-driven overexpression of catalase or endothelial nitric oxide synthase (eNOS). Tie-2-catalase mice have a strongly reduced steady-state concentration of vascular hydrogen peroxide and show profound hypotension that is not dependent on the bioavailability of endothelial nitric oxide but is completely reversible by treatment with the catalase inhibitor aminotriazole. A similar hypotension was observed in transgenic mice with an endothelial-specific overexpression of eNOS but this hypotension is entirely dependent on vascular eNOS activity. These observations suggest a tonic effect of hydrogen peroxide on vascular smooth muscle. Further studies suggested that hydrogen peroxide promotes the exercise-induced increase of vascular eNOS expression and inhibits the release of endothelial progenitor cells induced by exercise training. In summary, our data support the concept of a dual role of ROS in the vascular system.

1. Introduction

Reactive oxygen species generated in the vasculature are important signaling molecules but act in a deleterious way as well [1]. Among the different cell types in vessels both endothelial cells and smooth muscles cells are the source and the effectors of ROS at the same time. Important molecules belonging to the group of vascular ROS are nitric oxide (NO), superoxide, hydrogen peroxide, peroxynitrite and hydroxyl radicals (Fig. 1). Many different enzymes regulate the homeostasis of vascular ROS. As for superoxide, NADPH-oxidases existing in various subtypes in smooth muscle and endothelial cells appear to be the most important generators [2], while three forms of superoxide dismutases which convert superoxide to hydrogen peroxide and oxygen can be viewed as hydrogen peroxide generators [3]. Catalase and glutathione peroxydases regulate steady-state levels of hydrogen peroxide by conversion to water [4]. Although there is a general consent that NO produced by endothelial NO-synthase (eNOS) is a rather beneficial, i.e. vasoprotective, type of ROS [5], some studies reported an impairment of endothelial function and acceleration of atherosclerosis in transgenic mice with massive overexpression of eNOS specifically targeted to vascular endothelial cells [6,7]. The major reason for this appears to be a reaction between NO and superoxide yielding peroxynitrite, an “ugly” metabolite of NO [8]. Hydroxyl radicals are formed either by myeloperoxidase-catalyzed or by Fe^{2+}-catalyzed conversion of hydrogen peroxide and are believed to accelerate atherosclerosis as well [1,9]. The aim of this short review is to summarize data obtained from transgenic animals which overexpress either eNOS or catalase specifically in vascular endothelial cells. Studies with these mice have revealed some significant signaling events induced by vascular NO and hydrogen peroxide in vivo.

2. Hydrogen peroxide

Hydrogen peroxide is non-radical uncharged oxidant which is chemically more stable then other reactive oxygen species (ROS) and can permeate through the vascular wall. Hydrogen peroxide produced intracellularly can mediate local signaling by operating as a second

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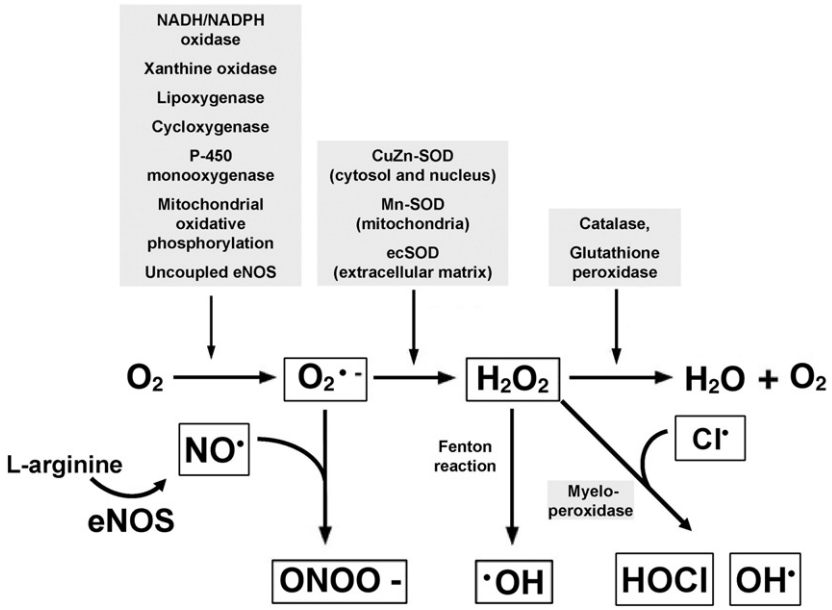

Fig. 1. Generation and metabolization of reactive oxygen species in the vasculature. Multiple enzymes may induce generation of superoxide (O_2^{-}.) including NADH/NADPH oxidase, xanthine oxidase, lipoxygenase, cyclooxygenase, P-450 monooxygenase, and the enzymes of mitochondrial oxidative phosphorylation. Hydrogen peroxide (H_2O_2) is mainly formed by 3 forms of superoxide dismutases (SOD) and rapidly degraded by catalase and glutathione peroxidase. Hydroxyl radicals ($OH^{\cdot}$) are formed either by myeloperoxidase-catalyzed or by Fe^{2+}-catalyzed conversion of hydrogen peroxide. Nitric oxide (NO) produced by endothelial NO-synthase (eNOS) can react with superoxide yielding peroxynitrite ($ONOO^{-}$).

messenger [4]. It can also accumulate extracellularly in the tissue and survive long enough to induce numerous paracrine functions, even in more distant cells. While hydrogen peroxide is mainly formed by superoxide dismutases, it is rapidly degraded by catalase and glutathione peroxidase. In vivo, steady-state levels of hydrogen peroxide in tissues and plasma are still unknown but are estimated to be in the micromolar range [10,11].

A large number of reports have suggested that hydrogen peroxide is an important mediator in the vasculature, e.g. a regulator of smooth muscle hypertrophy and proliferation, of eNOS expression and of arterial vasomotor tone [12–15]. However, in vitro studies have yielded conflicting results on the effect of hydrogen peroxide on vasomotor tone [16,17] suggesting that the vasomotor response to hydrogen peroxide depends on experimental conditions such as the type of vessel studied, the species studied and the concentration range used. Furthermore, it is not clear whether exogenous application of hydrogen peroxide is physiologically relevant, and what concentration, dose or treatment duration reflects the in vivo situation. To investigate the effect of endogenous hydrogen peroxide on the vasomotor tone of resistance vessels in vivo we generated transgenic mice with endothelial-specific overexpression of human catalase.

2.1. Transgenic mice with endothelium-specific overexpression of catalase

We used a transgenic construct, in which human catalase was inserted between murine Tie-2 promotor (2.1 kb) and a 10 kb Tie-2 intron fragment, designated as Tie-2-enhancer and this construct was used to target catalase gene expression to the vascular endothelium (Fig. 2). In the original publication describing the discovery of the Tie-2 promotor Schläger et al. suggested an endothelium-specific expression induced by this promoter [18]. Our transgenic cat^{++} mice are the first published mice where a mammalian gene (catalase) was overexpressed using the Tie-2 promotor. The endothelium-specific overexpression of catalase was evident in both conductance and myocardial resistance vessels and associated with an increase of catalase mRNA, protein and activity as well as a marked reduction of the endothelial steady-state concentration of reactive oxygen species including H_2O_2 [19,20]. Furthermore, endothelial specificity was investigated by measuring dihydroethidine fluorescence in leucocytes by FACS analysis and no difference between cat^{n} and cat^{++} was found suggesting absence of catalase overexpression in non-vascular cells [19]. Since Tie-2 might be erexpressed in monocytes and neutrophils [21,22] we have measured catalase protein expression in the bone

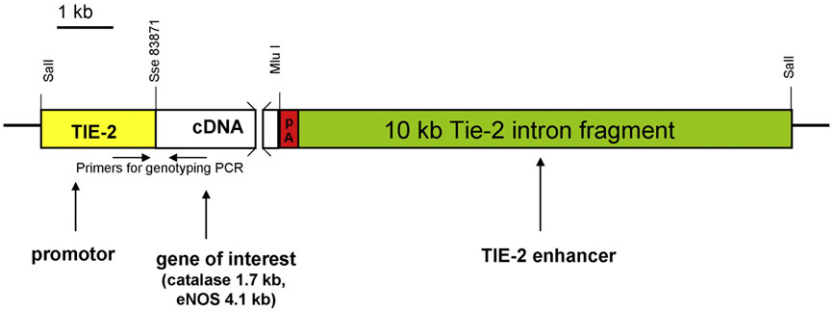

Fig. 2. Scheme of the construct inserted into fertilized eggs of C57BL/6 mice to generate mice with endothelial-specific overexpression of catalase (human catalase cDNA, 1.7 kb) and eNOS (bovine eNOS cDNA, 4.1 kb) driven by Tie-2 promotor (2.1 kb). Sal I restriction sites used for linearization of the plasmid and Sse83871/MluI sites added to cDNA for ligation are indicated. PCR primers used for genotyping PCR are indicated by horizontal arrows.

marrow mononuclear cells of cat^{n} and cat^{++} mice and have found no difference between the strains (our unpublished data). Taken together, these data strongly suggest endothelial-specific overexpression of catalase in our transgenic cat^{++} mouse model.

2.1.1. Vascular effects of endothelial-specific overexpression of catalase

Our main finding is that a reduction of steady-state concentrations of vascular hydrogen peroxide induced by an endothelial-specific overexpression of human catalase resulted in a marked reduction of systolic blood pressure in mice [19]. This hypotension was completely reversible by the catalase inhibitor aminotriazole (Fig. 3A). In contrast, Yang et al. reported that catalase overexpressing mice generated by means of an 80-kb P1 clone containing the entire human catalase gene show no change of hydrogen peroxide release from the aorta and have normal blood pressure [23]. Thus, specific targeting of catalase overexpression to the vasculature appears to be important to unmask a vasotonic effect of endogenous hydrogen peroxide.

It has been shown that vascular hydrogen peroxide has many associations to the vascular NO/cGMP pathway [12,14–17]. For example, hydrogen peroxide may either enhance endogenous NO generation by increasing the activity and expression of eNOS [24–26] or impair endothelial production of NO in response to mediators such as the calcium ionophore A23187, bradykinin and adenosine diphosphate [27]. Hydrogen peroxide might also play a role in flow-mediated vasodilation and catalase likely consumes NO which partially inhibits the enzyme's reaction with hydrogen peroxide [28,29]. However, profound hypotension in our cat^{++} mice was not dependent on endogenous nitric oxide bioavailability (Fig. 3A). There were no difference in vascular eNOS protein content, eNOS activity and the efficiency of the NO/cGMP pathway between cat^{n} and cat^{++} mice as evidenced by western blot analysis, chronic treatment with the NOS-inhibitor L-NAME and organ bath experiments, respectively. These data do not support the hypothesis that endogenous hydrogen peroxide impairs endogenous NO production and bioavailability in blood pressure-regulating skeletal resistance vessels.

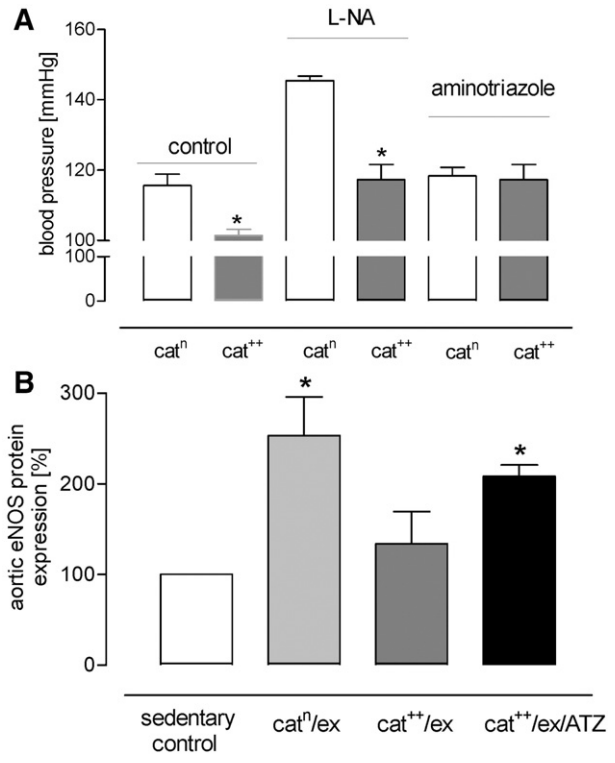

Fig. 3. (A) Effects of NO-synthase inhibitor L-nitro-arginine (L-NA, 100 mg/kg BW/day) and catalase inhibitor aminotriazole (670 mg/kg/day) on systolic blood pressure of cat^{++} and cat^{n}. Measurements were obtained in resting awake animals using a tail-cuff method (*P<0.0001, One way ANOVA). Endothelial-specific overexpression of catalase resulted in significant reduction of systolic blood pressure in cat^{++} as compared to cat^{n} and this difference remained after chronic inhibition of eNOS. In contrast, aminotriazole treatment restored systolic blood pressure in cat^{++}, but had no influence on blood pressure in cat^{n}. (B) Effect of 3 weeks of exercise training (ex) on the aortic eNOS protein expression in cat^{n} and cat^{++} as measured by western blot. In striking contrast to control mice (cat^{n}), 3 weeks of exercise had no effect on vascular eNOS expression in cat^{++} mice. Treatment of cat^{++} mice with the catalase inhibitor aminotriazole (ATZ) restored exercise-induced aortic upregulation of eNOS.

Organ bath experiments performed in aortric rings of cat^{n} and cat^{++} mice revealed no difference in concentration-dependent vasoconstriction to phenylephrine. However, inhibition of catalase activity by aminotriazole unmasked a potentiation of adrenergic vasoconstriction by phenylephrine in aortic rings of cat^{n} which was attenuated in cat^{++}, as expected. To investigate vasoconstrictor effects of exogenous hydrogen peroxide, denuded aortic rings of cat^{n} and cat^{++} were subjected to increasing concentrations of hydrogen peroxide. Small vasoconstrictor effects were observed up to 50 μmol/L hydrogen peroxide in cat^{n} only, while higher concentrations induced strong and irreversible vasodilator effects in cat^{n} and cat^{++}. These data suggest that concentrations of hydrogen peroxide which likely resemble in vivo conditions induce vasoconstrictor effects in aortic rings submaximally precontracted with phenylephrine. Thus, endogenous hydrogen peroxide contributes to adrenergic vasoconstriction in conductance vessels. Interactions of hydrogen peroxide with some proteins of $\alpha_{1A/C}$-receptors signal transduction pathway such as phospholipase C, protein kinase C and phosphoinositide 3-kinase [30] might explain the molecular mechanisms underlying hydrogen peroxide-induced increases of vascular tone.

The results of these organ bath experiments are consistent with the observed change of systolic blood pressure, with the effects of aminotrizole in vivo and with a previous report showing that increases of blood pressure in response to vasoconstrictor agents such as norepinephrine and angiotensin II were less pronounced in mice with an unspecific overexpression of catalase [23]. Thus, the involvement of endogenous hydrogen peroxide in adrenergic constriction of resistance vessels in vivo most likely contributes to hypotension in cat^{++}.

Hydrogen peroxide may contribute to pathologic events resulting in hypertension. Experimental as well as clinical hypertension is associated with oxidative stress [1,31]. Furthermore, previous studies indicate that in hypertension the plasma concentration of hydrogen peroxide is increased and is positively correlated to plasma renin activity and systolic blood pressure, while negatively correlated to cardiac contractility and renal function [10]. Yet, it is not known whether vascular oxidative stress is a result of the disease or maybe one underlying cause. Treatment options that have been shown to reduce vascular oxidative stress also reduce blood pressure [1,31]. Thus, the direct demonstration of hypotension in mice carrying an endothelial-specific overexpression of catalase strongly argues for both, direct vasoconstrictor effects of endogenous hydrogen peroxide on resistance vessels and a possible contribution of these effects to the development of hypertension.

2.1.2. Role of endogenous hydrogen peroxide in exercise-induced upregulation of eNOS

Previous studies have shown that hydrogen peroxide increases the expression and activity of eNOS in endothelial cells [24,32,33]. The increase of eNOS activity in response to hydrogen peroxide comes along with a change of eNOS phosphorylation and is thought to be an acute cellular adaptation to an increase in oxidant stress [25,26,34]. This is relevant to exercise, which is associated with an increase in oxidative stress within the skeletal muscle and an increase in circulating levels of H_2O_2 and markers of lipid oxidation. In vivo studies in animals and humans have demonstrated that exercise results in an increased vascular expression of eNOS suggesting an important role of endogenous NO production for beneficial effects of

exercise on vascular biology [35]. We have also demonstrated that exercise training increases expression of vascular extracellular superoxide dismutase (ecSOD) which, in turn, facilitates the generation of hydrogen peroxide from superoxide [36]. Using our transgenic mouse model with endothelial-specific overexpression of catalase we investigated whether endogenously produced hydrogen peroxide contributes to the upregulation of eNOS expression induced by exercise training.

Although vascular-specific overexpression of catalase had no effect on basal expression of eNOS, it almost completely inhibited the increase of eNOS expression induced by exercise in the aorta and in left ventricular arterioles [20]. Furthermore, inhibition of the artificially overexpressed catalase by oral treatment with catalase inhibitor aminotriazole can rescue the physiologic upregulation of endothelial eNOS expression induced by exercise (Fig. 3B). These results strongly indicate that the activity of the overexpressed catalase protein which results in a strong reduction of the vascular steady-state concentration of hydrogen peroxide is indeed responsible for the dysfunctional eNOS-expression in response to exercise.

Regulation of vascular eNOS expression is a highly complex. Shear stress is considered as an important stimulus for eNOS expression [37]. Previous studies in endothelial cells have shown that hydrogen peroxide increases eNOS expression as well [24]. The mechanism underlying this upregulation includes a calcium-dependent increase of calmodulin kinase II phosphorylation leading to activation of janus kinase II [32]. The latter tyrosine kinase phosphorylates other protein kinases such as Ras which directly activates transcription factors (for review see [38]).

Previous studies have shown that c-Src plays a central role in modulation of eNOS expression in response to shear stress via divergent pathways involving a short-term increase in eNOS transcription (c-Src activation of Ras/Raf and ERK1/2) and a longer-term stabilization of eNOS mRNA [33]. Further studies showed that exercise training increased eNOS protein >2-fold in the aorta and 1.7-fold in the heart in C57Blk/6 mice, but had no effect on eNOS protein levels in c-Src$^{+/-}$ mice [34]. Taken together with our current data, it seems that both vascular H_2O_2 and c-Src are critical in allowing endothelial cells to increase eNOS expression during exercise training (Fig. 4). The manner in which these signals interact in vivo are to be defined.

A variety of studies have indicated that exercise is associated with increased ATP synthesis in skeletal and myocardial muscle [39] and we have found a more than 3-fold increase of citrate synthase activity, which is known to correlate with training efficacy [40]. During ATP synthesis, the coenzyme Q radical can transfer its unpaired electron to molecular oxygen which increases superoxide and subsequently the generation of hydrogen peroxide [41]. Thus, mitochondrial hydrogen peroxide may contribute to increased vascular oxidative stress during exercise, particularly in myocardial and skeletal muscle arterioles where the diffusion distance is comparatively small. Furthermore, exercise increases heart rate and this will almost certainly enhance the mechanical forces of blood flow such as shear stress, pressure and cyclic strain on the vascular wall [35] and shear stress has been shown to increase endothelial superoxide generation [42]. Further studies have identified the endothelial NADPH oxidase as a major source of vascular superoxide induced by laminar and oscillatory shear [43]. In addition, shear stress is a potent stimulus for the expression of endothelial cell Cu/Zn SOD [44]. Thus, superoxide generated during exercise training may be more readily dismutated to hydrogen peroxide.

Our findings may have implications for the use of antioxidants and drugs that suppress the production of reactive oxygen species in vivo. While reactive oxygen species produced in large quantities clearly mediate cellular damage, lower levels of reactive oxygen species may have important signaling properties [45]. Therefore, suppression of cellular production of reactive oxygen species might lead to a loss of important vascular signaling events as is the case illustrated in this study: an inability to increase eNOS in response to exercise training which, over the long run, presumably increase the antioxidative capacity of the vascular wall and protect the vasculature against permanent oxidative stress known to be present in cardiovascular disease.

2.1.3. Role of endogenous hydrogen peroxide in cardiovascular ischemia–reperfusion injury

It is well known that myocardial ischemia–reperfusion depresses myocardial function and causes deleterious morphological changes that lead to heart failure and cardiac cell death [46,47]. Several reports suggest that increased oxidative stress and burst of free radical production including hydrogen peroxide are important mediators of myocardial ischemic damage [48,49]. However, most of the studies on the role of catalase and hydrogen peroxide in myocardial ischemia–reperfusion are performed with in vivo supplementation of catalase by injection or in vitro addition of hydrogen peroxide or catalase to perfusion buffers. Data interpretation from these studies is limited, since the physiologically relevance of such in vitro experiments are for the most part unclear. Using our transgenic animals we have investigated whether endogenous hydrogen peroxide produced in the vasculature is involved in ischemia–reperfusion injury. We hypothesized that endothelial-specific overexpression of catalase might improve coronary or/and myocardial reperfusion function via protection during ischemia and reperfusion. These processes are

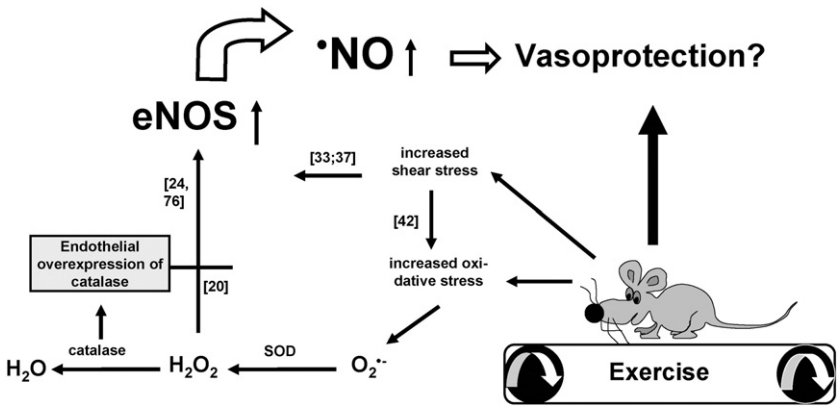

Fig. 4. Summary of the effects of exercise on endothelial-specific expression of endothelial NO-synthase. One of the most important changes within the cardiovascular systems during exercise is the increase of heart rate and of blood pressure. This induces an increase of physical forces such as shear stress and an increase of oxidative stress. Shear stress has been shown to increase oxidative stress [42] and to induce eNOS expression and phosphorylation [37,76]. Oxidative stress results in increased formation of hydrogen peroxide which has been shown in cell culture experiments to induce eNOS expression as well. Overexpression of catalase blunts the effect of exercise on eNOS expression (numbers in brackets indicate citation numbers).

associated with increased oxidative stress [50]. In contrast, endothelial-specific overexpression of catalase had only little effects on myocardial contractility, coronary function or electrical stability in hearts subjected to 20 min of global ischemia followed by 30 min of reperfusion [51]. However, hearts from cat^{++} mice subjected to ischemia–reperfusion showed better inotropic and lusitropic responses to exogenous norepinephrine, suggesting that hydrogen peroxide is involved in oxidant-mediated ischemia–perfusion injury. Thus, overexpression of catalase in the endothelium is only weakly protective against myocardial ischemia–reperfusion injury, but appears to preserve the responsiveness of the heart to adrenergic stimulation.

2.1.4. Role of endogenous hydrogen peroxide in exercise-induced increase of circulating stem cells with endothelial progenitor capacity

Vascular oxidative stress has been shown to promote many pathophysiologic changes resulting in cardiovascular remodeling underlying cardiovascular diseases [1]. Recent evidence suggest that regeneration of damaged vascular endothelium involves the participation of stem cells mobilized from the bone marrow. Asahara et al. first published that circulating CD34^{+}-angioblasts within human peripheral blood are able to differentiate in vitro to endothelial phenotype [52]. These cells were named “endothelial progenitor cells (EPCs)”. EPCs participate in endothelial homeostasis by contribution to re-endothelialization and improve organ blood flow by homing into ischemic regions and forming entirely new vessels or by releasing angiogenic factors in a paracrine manner [53–55]. These beneficial effects are impaired when the number and/or functional activities of EPCs are reduced. Clinical trials revealed that the number of circulating EPCs is decreased in subjects with cardiovascular risk factors, such as such as hypercholesterolemia, diabetes and smoking [56] and a similar situation likely holds true for EPC homing [57]. Since these pathologies are all associated with increased vascular oxidative stress, depletion of circulating EPCs is considered a marker of the progression of cardiovascular disease [58]. Whether oxidant damage directly affects EPC mobilization is an important, but unanswered question.

It is well known that the effect of reactive oxygen species on vascular function is critically concentration-dependent. Under physiological conditions, i.e. at low concentrations, reactive oxygen species can act as intracellular second messenger, modulating proangiogenic pathways such as VEGF-A signaling and postnatal vasculogenesis [59], while higher concentrations of reactive oxygen species, e.g in cardiovascular disease, likely impairs vessel growth. Therefore, reactive oxygen species may directly influence stem cell-induced vascular repair mechanisms by reducing the number of circulating EPCs.

We investigated the effect of hydrogen peroxide on the number of circulating EPCs in sedentary C57Bl/6, cat^{n} and cat^{++} mice. We have trained cat^{n} and cat $^{++}$ using different training protocols and methods and measured peripheral EPCs levels. The major findings are that exercise apparently doesn't change circulating EPCs in young healthy C57Bl/6 mice [60]. In striking contrast, exercise strongly increased EPCs in cat++-mice and this was completely inhibited by oral treatment with the catalase inhibitor aminotriazole. Likewise, inhibition of catalase by oral aminotriazole strongly decreased circulating EPCs in both catn and cat++-mice independent of exercise. These data suggest a new pathophysiologic role of hydrogen peroxide as one player of vascular oxidative stress, i.e. a reduction of the number of circulating EPCs.

3. Nitric oxide

3.1. Transgenic mice with endothelial-specific overexpression of eNOS

Nitric oxide derived from eNOS, a major isoform of NOS in the cardiovascular system has a highly diverse biological profile including vasodilation, antiaggregation, antiapoptosis, antiantiadhesion, antiproliferation and antioxidation [5]. In vivo studies of eNOS using murine knockout models have provided the wealth of information regarding the role of eNOS in cardiovascular physiology and disease. However, genetic approach not only provided new insight into the actual role of eNOS, but also uncovered possible secondary alterations which compensate for the induced change. In the case of eNOS knockout mice, upregulation of other NOS isoforms, induction of signal molecules such as prostaglandins or endothelium-derived hyperpolarizing factor may compensate for the lack of eNOS.

Along with existing complementary knockout models, transgenic mouse models overexpressing eNOS provided important findings regarding the crucial role of eNOS in preventing cardiovascular septic shock, regulating blood pressure and modulating the baroreceptor response [61] and highlighted the complexity of eNOS regulation. Here, we describe the existing mouse models for eNOS overexpression and compare them to our recently generated mouse transgenic line with endothelial-specific overexpression of bovine wild type eNOS driven by murine Tie-2 promotor.

3.1.1. Existing mouse models for eNOS overexpression

The first transgenic mouse model overexpressing bovine eNOS in the vascular wall was generated by Ohashi et al. using murine preproendothelin-1 promotor [7]. Targeting the transgene to the endothelium resulted in exogenous bovine eNOS mRNA and protein expression in endothelium of the thoracic aorta, large pulmonary arteries and medium to small sized coronary arteries. These eNOS^{++} mice have hypotension which was completely reversed by chronic L-NAME treatment suggesting NO-mediated reduction of blood pressure. Furthermore, eNOS^{++} mice showed significantly decreased aortic endothelial-dependent relaxation and depressed vascular responses to exogenous NO. Decreased soluble guanylyl cyclase activity and cGMP-dependent protein kinase levels have been suggested as a mechanism for impaired endothelial-dependent relaxation [62]. Further studies on importance of eNOS cofactor tetrohydrobiopterin (BH_4) in regulating eNOS activity revealed that eNOS-derived superoxide production is enhanced in eNOS^{++} mice in cardiac tissue lysates and intact aorta indicating increased eNOS ucoupling in these animals [63]. In accordance with having increased uncoupled eNOS and depleted BH_4 levels, specific eNOS enzymatic activity was markedly attenuated in eNOS^{++} (elevated only 2-fold compared with wild type) relative to eNOS protein levels (elevated 8-fold compared with wild type). The authors concluded that eNOS uncoupling in eNOS^{++} mice is an independent and direct consequence of a stoichiometric discordance between enzyme and its cofactor BH_4.

Another group has generated a mouse model overexpressing human eNOS gene [64] by a random insertion of the human eNOS DNA fragment and the native eNOS promotor that contains all the regulatory sequences of eNOS. Mice overexpressing eNOS were hypotensive and had a 20% lower systemic vascular resistance which was sensitive to administration of NOS-inhibitor L-NAME. Immunohistochemistry revealed eNOS localisation in the endothelial cells of smaller and larger blood vessels of several organs (aorta, heart, kidney). eNOS protein level was tremendously increased as evident by western blot analysis and was accompanied by a 10-fold increase in NOS activity measured by the L-arginine to L-citrulline conversion assay in aortic tissue homogenates. Furthermore, studies of integrated systemic resistance using isofurane-anesthetized open-chest mouse model showed a decreased responsiveness of soluble guanylyl cyclase (sGC) to exogenous NO and lower sGC activity, but no evidence of eNOS uncoupling or adaptation in other vasoregulatory pathways [65].

It is well known that impairment or deficiency of eNOS gives rise to accelerated atherosclerosis indicating that physiological levels of eNOS are anti-atherogenic [66]. Two strains of eNOS overexpressing mice have been bred with apolipoprotein E-deficient mice to study the

potential role of NO in atherosclerosis. Interestingly, conflicting results have been reported. When eNOS^{++} mice generated by van Haperen et al. were crossbred with Apo E knockouts and fed with "western-type" diet, atherosclerotic lesion size was significantly reduced by eNOS overexpression. As for the underlying mechanisms, reduction in blood pressure and 15% lower plasma cholesterol levels in eNOS^{++} mice were suggested. The authors conclude that elevation of eNOS activity could be beneficial for patients at risk of developing atherosclerotic disease [64].

In striking contrast, Ozaki et al. reported that atherosclerotic lesions areas were significantly larger in Apo E/eNOS^{++} mice then in Apo E knockouts both fed with "high-cholesterol" diet [6]. They have found the presence of eNOS dysfunction, demonstrated by lower NO production relative to eNOS protein levels. This was associated with increased superoxide production and decreased vascular BH_4 levels suggesting that chronic overexpression of eNOS does not inhibit, but accelerates atherosclerosis under hypercholesterolemia. Several potential mechanisms might explain the difference between the outcomes of these studies. First, the difference in promoter by which eNOS was targeted resulted in different expression levels of eNOS. Second, this discrepancy may be due to the difference between "high-cholesterol" and "western style" diets used to promote atherosclerosis and therefore due to the difference in balance between NO and superoxide production from the endothelium. Finally, it appears that eNOS^{++} mice generated by Ohashi et al. overexpressed uncoupled eNOS as a consequence of a stoichiometric discordance between enzyme and its cofactor BH_4 [67].

3.1.2. Mice with endothelial-specific overexpression of eNOS driven by Tie-2 promotor

Recently we have generated a mouse model in which bovine eNOS was inserted between murine Tie-2 promotor and a 10 kb Tie-2 intron fragment, designated as Tie-2-enhancer (Fig. 2). In agreement with the original publication of Tie-2 promotor [18] and our previous studies [19,20], fluorescent immunohistochemistry revealed an endothelial-specific eNOS overexpression in coronary arterioles and in carotid artery of eNOS^{++} mice (Fig. 5A). Western blot and densitometric analysis detecting eNOS protein in mouse aorta showed 3.2-fold overexpression in eNOS^{++}. The overexpressed eNOS protein was functionally active as indicated by a significant reduction of blood pressure (~15 mm Hg) in eNOS^{++} as compared to transgen-negative littermates. Furthermore, the reduction of blood pressure was completely blunted by oral treatment with the NOS-inhibitor L-NA (Fig. 5B). Electron spin resonance measurements with $Fe^{2+}(DETC)_2$ (iron/diethyldithiocarbamic acid, 0.2 mmol/L) spintrap revealed an increased concentration of nitric oxide in the aorta of eNOS^{++} (Fig. 6).

Using the CPH (1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine) spintrap, neither an increase of oxidative stress nor an increase of peroxynitrite concentration was detected in eNOS^{++} heart tissue. Similar results were obtained in aortic, muscle and lung tissues (our unpublished data). An important advantage of our transgenic model is relatively moderate level of eNOS overexpression which is sufficiently low to avoid increased $OONO^-$ generation. The higher the expression strength is, the more peroxynitrite appears to be generated as predictable from the law of mass action. The reaction constant for the reaction of NO with superoxide is about 3 times higher (1.9×10^{10} M^{-1} s^{-1}) than the reaction constant for the reaction of superoxide with SOD (2.4×10^{9} M^{-1} s^{-1}) [5]. It is assumed that generation of $OONO^-$ is outcompeted by the 100–1000-fold higher concentration of SOD as compared to NO. The higher the steady-state concentration of NO the higher is the possibility of $OONO^-$ generation. In our mind, this advantage of our model enables us to investigate in vivo NO-physiology rather than in vivo NO-toxicology.

3.1.2.1. Influence of eNOS overexpression on vascular soluble guanylyl cyclase (sGC) in vivo. In contrast to the results of Yamashita et al.

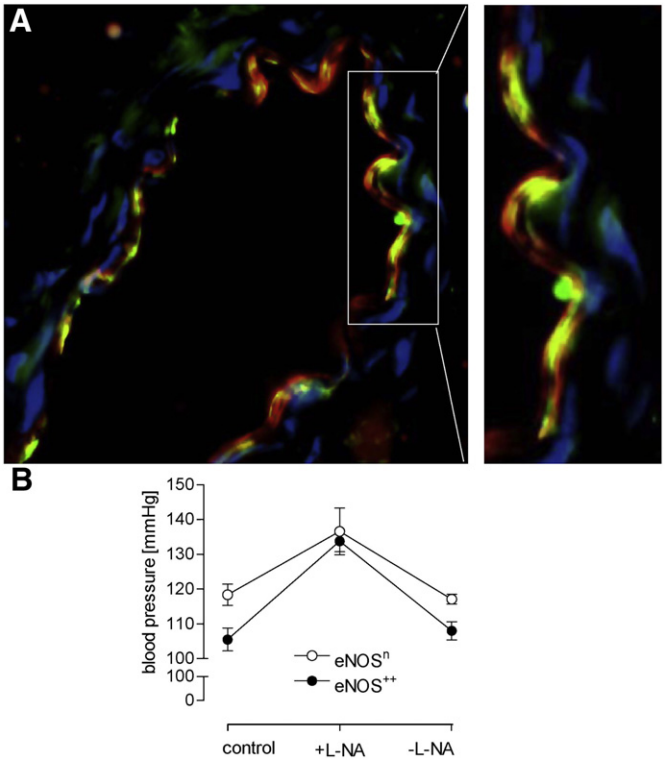

Fig. 5. (A) Representative fluorescent immunohistochemical image performed in carotid artery of eNOS^{++} mice double stained for eNOS (green) and for the endothelial marker CD31 (red). Nuclei are stained blue. Original magnification: 200×. (B) Reduced blood pressure in eNOS $^{++}$ mice was completely dependent on eNOS activity as evidenced by oral treatment with L-nitro-arginine which increased the blood pressure to a similar degree in eNOSn and eNOS++ mice. Termination of L- nitro-arginine treatment induced a complete restoration of systolic blood pressure to pretreatment values in both strains.

endothelium-dependent vasodilation induced by acetylcholine in aortic rings of our eNOS^{++} mice was not altered. Furthermore, overexpression of eNOS did not lead to a significant rightward shift of the relaxation response to the NO-donor SNAP suggesting no changes in vascular sensitivity to exogenous NO. Moreover, endothelial-specific overexpression of eNOS did not change the protein expression of sGC-α_1 and sGC-β_1 subunits In contrast, we have found a strong decrease in maximal sGC activity in eNOS overexpressing mice and phosphorylation of VASP at serine 239, which is characteristic for cGMP-dependent phosphorylation was significantly decreased in eNOS^{++} mice [68].

These data suggest that NO does not regulate sGC expression in vivo and that even marked reductions of sGC activity do not translate into a functional impairment of the vascular NO/cGMP pathway. No changes of sCG expression by endogenous chronically elevated nitric oxide production are in agreement with our recently published study showing no effect of therapeutic treatment with long-acting NO-donor PETN and ISMN on the expression and function of vascular sGC in vivo [69]. Furthermore, other report failed to show the existence of a NO-dependent feed-back loop controlling sGC expression in vivo [62]. Mice overexpressing eNOS in the vasculature driven by preproendothelin-1 promotor showed a resistance to endothelium-dependent and NO-induced vasodilatation but not a decrease of sGC expression [62]. Instead, the authors describe a 50% reduction of basal unstimulated sGC activity and a 20% reduction of PKG expression. Reduced activity of sGC in the peripheral resistance vessels, but not in aorta, was also reported in another mouse model overexpressing eNOS, although signal transduction pathway downstream was unaffected by eNOS overexpression in these mice [65]. Other groups reported that changes of PKG activity do not occur in eNOS knockout

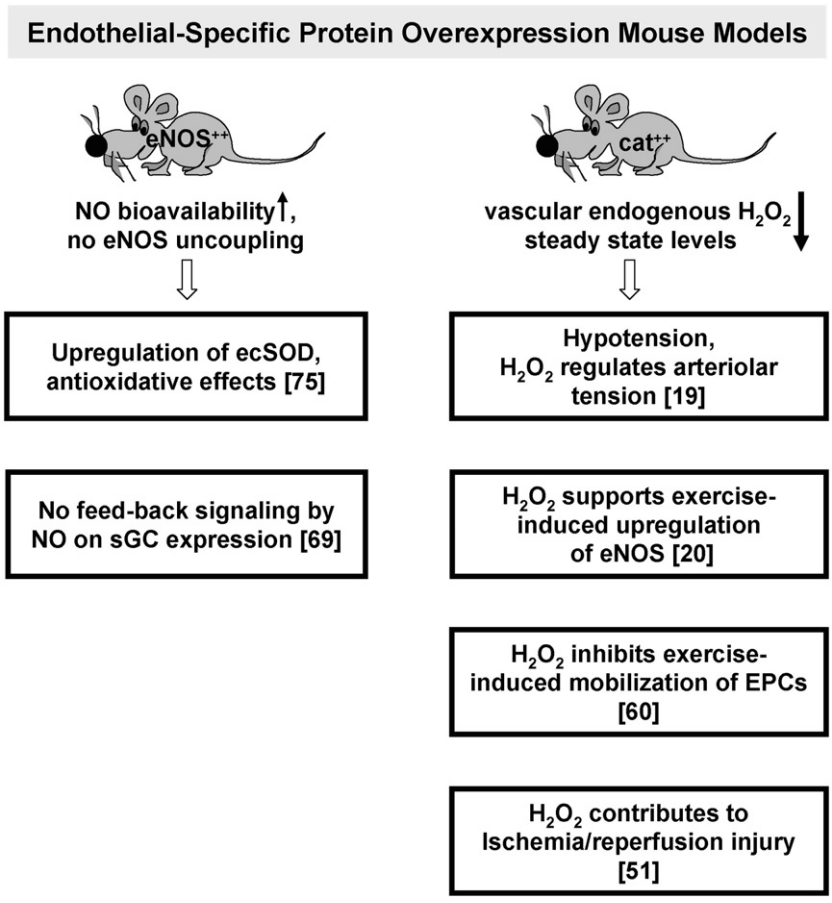

Fig. 6. Summary of the effects of endothelial-specific overexpression of catalase and endothelial NO-synthase driven by the Tie-2 promotor.

mice and that there is no change of sGC protein expression in this animal model [70,71].

The results of our experiments indicate that the NO-cGMP pathway has a great capacity which ensures full functional activity even in the setting of strong desensitization of the central enzyme sGC. These results are in line with a recent report demonstrating that the majority of NO-sensitive sGC is not required for cGMP-forming activity [72]. In this investigation, a complete NO-dependent vasodilation was achieved in aortic rings of sGC-α_1 deficient mice. This vasodilation occurred despite a low increase in vascular cGMP and was mediated by the sGCα_2/sGC-β_1 heterodimer which accounts for only 6% of total vascular sGC.

In summary, our results refute the hypothesis that therapeutic [69] and endogenous NO play a significant role in regulation of vascular sGC protein expression. Feed-back signaling by NO of vascular sGC expression did not occur in vivo, not even at continuous NO-concentrations inducing a strong reduction of blood pressure.

3.1.2.2. Antioxidative effects of eNOS overexpression. It is generally accepted that endothelial dysfunction is a consequence of increased vascular oxidative stress, a condition characterized by a misbalance of endogenous production of vascular ROS and the vascular antioxidative capacity. Although a variety of mediators have been described to contribute to vascular oxidative stress, both the generation and detoxification of superoxide most likely play a major role in this process. Superoxide is generated by physiologic reactions as a metabolic by-product, for example in mitochondria, by CYP-oxygenases, by xanthine oxidase, by nitric oxide synthases and particularly by NAD(P)H-oxidases [13] (Fig. 1). There are highly specific mechanisms to rapidly detoxify superoxide and thereby prevent unwanted oxidatative processes in cells and tissues. Of these, the superoxide dismutases I (CuZnSOD), II (MnSOD) and III (extracellular (ec)SOD) are of utmost importance. While CuZnSOD and MnSOD provide intracellular and intramitochondrial protection against superoxide, respectively, ecSOD does so in the interstitium. It is expressed in vascular smooth muscle cells and consecutively secreted to the interstitium [73]. It rapidly binds to polyanionic sites such as heparan sulfates at the outer membrane of endothelial cells and protects endothelial NO while it traverses to the smooth muscle cell layer of the vascular wall [74]. In fact, the highest ecSOD concentrations can be found between the endothelial cell layer and the smooth muscle and 99% of ecSOD is tissue-bound.

Our previous investigations have shown that endogenous NO is an essential stimulus of ecSOD expression in large conductive vessels such as the aorta and that an increase of eNOS expression by exercise results in a consecutive overexpression of ecSOD [36]. Hence, driving the expression of ecSOD appears to be an important mechanism underlying the antioxidative effects of NO. Therefore we investigated whether endothelial-specific overexpression of eNOS and increase of endogenous NO levels influence ecSOD expression and activity in the microvasculature.

We found a significant upregulation of ecSOD protein expression and activity in lung cytosols of eNOS^{++} mice suggesting that NO is the active mediator [75]. To further challenge this hypothesis, we treated both eNOS^{n} and eNOS^{++} mice with the NOS-inhibitor L-NA and observed a strong downregulation of ecSOD protein expression. This result provides additional evidence for a regulatory role of vascular NO for ecSOD protein expression. Likewise, this result demonstrates that the increase of ecSOD in eNOS^{++} is not an unspecific consequence of genetic manipulation. Similar increase in the expression and activity of microvascular and macrovascular

ecSOD in vivo was observed in mice treated with the organic nitrate PETN, further suggesting that an increased bioavailability of vascular NO is the underlying cause [75].

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References

[1] G. Kojda, D.G. Harrison, Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure, Cardiovasc. Res. 43 (1999) 562.
[2] C.A. Papaharalambus, K.K. Griendling, Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury, Trends Cardiovasc. Med. 17 (2007) 48.
[3] S.I. Liochev, I. Fridovich, The effects of superoxide dismutase on H2O2 formation, Free Radic. Biol. Med. 42 (15-5-2007) 1465.
[4] H. Cai, Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences, Cardiovasc. Res. 68 (1-10-2005) 26.
[5] M.T. Gewaltig, G. Kojda, Vasoprotection by nitric oxide: mechanisms and therapeutic potential, Cardiovasc. Res. 55 (2002) 250.
[6] M. Ozaki, S. Kawashima, T. Yamashita, T. Hirase, M. Namiki, N. Inoue, K. Hirata, H. Yasui, H. Sakurai, Y. Yoshida, M. Masada, M. Yokoyama, Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice, J. Clin. Invest. 110 (2002) 331.
[7] Y. Ohashi, S. Kawashima, Ki Hirata, T. Yamashita, T. Ishida, N. Inoue, T. Sakoda, H. Kurihara, Y. Yazaki, M. Yokoyama, Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase [see comments], J. Clin. Invest. 102 (15-12-1998) 2061.
[8] J.S. Beckman, W.H. Koppenol, Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly, Am. J. Physiol. 271 (1996) C1424–C1437.
[9] S.J. Nicholls, S.L. Hazen, Myeloperoxidase and cardiovascular disease, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 1102.
[10] F. Lacy, M.T. Kailasam, D.T. O'Connor, G.W. Schmid-Schonbein, R.J. Parmer, Plasma hydrogen peroxide production in human essential hypertension: role of heredity, gender, and ethnicity, Hypertension 36 (2000) 878.
[11] B. Halliwell, M.V. Clement, L.H. Long, Hydrogen peroxide in the human body, FEBS Lett. 486 (1-12-2000) 10.
[12] A. Ellis, C.R. Triggle, Endothelium-derived reactive oxygen species: their relationship to endothelium-dependent hyperpolarization and vascular tone, Can. J. Physiol. Pharmacol. 81 (2003) 1013.
[13] K.K. Griendling, D. Sorescu, M. Ushio-Fukai, NAD(P)H oxidase: role in cardiovascular biology and disease, Circ. Res. 86 (17-3-2000) 494.
[14] M.S. Wolin, Interactions of oxidants with vascular signaling systems, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1430.
[15] T. Matoba, H. Shimokawa, M. Nakashima, Y. Hirakawa, Y. Mukai, K. Hirano, H. Kanaide, A. Takeshita, Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice, J. Clin. Invest. 106 (2000) 1521.
[16] R.D. Jones, A.H. Morice, Hydrogen peroxide—an intracellular signal in the pulmonary circulation: involvement in hypoxic pulmonary vasoconstriction, Pharmacol. Ther. 88 (2000) 153.
[17] T.M. Griffith, Endothelium-dependent smooth muscle hyperpolarization: do gap junctions provide a unifying hypothesis? Br. J. Pharmacol. 141 (2004) 881.
[18] T.M. Schlaeger, S. Bartunkova, J.A. Lawitts, G. Teichmann, W. Risau, U. Deutsch, T.N. Sato, Uniform vascular–endothelial-cell-specific gene expression in both embryonic and adult transgenic mice, Proc. Natl. Acad. Sci. U. S. A. 94 (1-4-1997) 3058.
[19] T. Suvorava, N. Lauer, S. Kumpf, R. Jacob, W. Meyer, G. Kojda, Endogenous vascular hydrogen peroxide regulates arteriolar tension in vivo, Circulation 112 (18-10-2005) 2487.
[20] N. Lauer, T. Suvorava, U. Rüther, R. Jacob, A. Meyer, D.G. Harrison, G. Kojda, Critical involvement of hydrogen peroxide in exercise-induced upregulation of endothelial NO-synthase, Cardiovasc. Res. 65 (1) (2005) 254.
[21] C. Lemieux, R. Maliba, J. Favier, J.F. Theoret, Y. Merhi, M.G. Sirois, Angiopoietins can directly activate endothelial cells and neutrophils to promote proinflammatory responses, Blood 105 (15-2-2005) 1523.
[22] M. De Palma, C. Murdoch, M.A. Venneri, L. Naldini, C.E. Lewis, Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications, Trends Immunol. 28 (2007) 519.
[23] H. Yang, M. Shi, H. VanRemmen, X. Chen, J. Vijg, A. Richardson, Z. Guo, Reduction of pressor response to vasoconstrictor agents by overexpression of catalase in mice, Am. J. Hypertens. 16 (2003) 1.
[24] G.R. Drummond, H. Cai, M.E. Davis, S. Ramasamy, D.G. Harrison, Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide, Circ. Res. 86 (18-2-2000) 347.
[25] H. Cai, Z. Li, M.E. Davis, W. Kanner, D.G. Harrison, S.C. Dudley Jr., Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide, Mol. Pharmacol. 63 (2003) 325.
[26] S.R. Thomas, K. Chen, J.F. Keaney Jr., Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway, J. Biol.Chem. 277 (22-2-2002) 6017.
[27] E.A. Jaimes, C. Sweeney, L. Raij, Effects of the reactive oxygen species hydrogen peroxide and hypochlorite on endothelial nitric oxide production, Hypertension 38 (2001) 877.
[28] Y. Liu, H. Zhao, H. Li, B. Kalyanaraman, A.C. Nicolosi, D.D. Gutterman, Mitochondrial sources of H2O2 generation play a key role in flow-mediated dilation in human coronary resistance arteries, Circ. Res. 93 (2003) 573.
[29] L. Brunelli, V. Yermilov, J. Beckman, Modulation of catalase peroxidatic and catalatic activity by nitric oxide, Free Radic. Biol. Med. 30 (1-4-2001) 709.
[30] K.K. Griendling, D. Sorescu, B. Lassegue, M. Ushio-Fukai, Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 2175.
[31] U. Landmesser, D.G. Harrison, Oxidative stress and vascular damage in hypertension, Coron. Artery Dis. 12 (2001) 455.
[32] H. Cai, M.E. Davis, G.R. Drummond, D.G. Harrison, Induction of endothelial NO synthase by hydrogen peroxide via a Ca(2+)/calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 1571.
[33] M.E. Davis, H. Cai, G.R. Drummond, D.G. Harrison, Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways, Circ. Res. 89 (23-11-2001) 1073.
[34] M.E. Davis, H. Cai, L. McCann, T. Fukai, D.G. Harrison, Role of c-Src in regulation of endothelial nitric oxide synthase expression during exercise training, Am. J. Physiol. Heart Circ. Physiol. 284 (2003) H1449–H1453.
[35] G. Kojda, R. Hambrecht, Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? Cardiovasc. Res. 67 (31-5-2005) 187.
[36] T. Fukai, M.R. Siegfried, M. Ushio-Fukai, Y. Cheng, G. Kojda, D.G. Harrison, Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training, J. Clin. Invest. 105 (2000) 1631.
[37] M. Uematsu, Y. Ohara, J.P. Navas, K. Nishida, T.J. Murphy, R.W. Alexander, R.M. Nerem, D.G. Harrison, Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress, Am. J. Physiol., Cell Physiol. 269 (1995) C1371–C1378.
[38] S.R. Thomas, P.K. Witting, G.R. Drummond, Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities, Antioxid. Redox Signal. 10 (2008) 1713.
[39] L.L. Ji, Antioxidants and oxidative stress in exercise, Proc. Soc. Exp. Biol. Med. 222 (1999) 283.
[40] T. Suvorava, N. Lauer, G. Kojda, Physical inactivity causes endothelial dysfunction in healthy young mice, J. Am.Coll. Cardiol. 44 (15-9-2004) 1320.
[41] T. Finkel, N.J. Holbrook, Oxidants, oxidative stress and the biology of ageing, Nature 408 (9-11-2000) 239.
[42] F.R.M. Laurindo, M. De Almeida Pedro, H.V. Barbeiro, F. Pileggi, M.H.C. Carvalho, O. Augusto, P.L. Da Luz, Vascular free radical release: ex vivo and in vivo evidence for a flow-dependent endothelial mechanism, Circ. Res. 74 (1994) 700.
[43] G.W. De Keulenaer, D.C. Chappell, N. Ishizaka, R.M. Nerem, R.W. Alexander, K.K. Griendling, Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase, Circ. Res. 82 (1-6-1998) 1094.
[44] N. Inoue, S. Ramasamy, T. Fukai, R.M. Nerem, D.G. Harrison, Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells, Circ. Res. 79 (1996) 32.
[45] T. Suvorava, G. Kojda, Prevention of transient endothelial dysfunction in acute exercise: a friendly fire? Thromb. Haemost. 97 (2007) 331.
[46] L.H. Opie, Reperfusion injury and its pharmacologic modification, Circulation 80 (1989) 1049.
[47] L.C. Becker, G. Ambrosio, Myocardial consequences of reperfusion, Prog. Cardiovasc. Dis. 30 (1987) 23.
[48] R. Southworth, M.J. Shattock, D.J. Hearse, F.J. Kelly, Developmental differences in superoxide production in isolated guinea-pig hearts during reperfusion, J. Mol. Cell. Cardiol. 30 (1998) 1391.
[49] A.M. Lefer, P.S. Tsao, D.J. Lefer, X.-L. Ma, Role of endothelial dysfunction in the pathogenesis of reperfusion injury after myocardial ischemia, FASEB J. 5 (1991) 2029.
[50] K.A. Kaminski, T.A. Bonda, J. Korecki, W.J. Musial, Oxidative stress and neutrophil activation—the two keystones of ischemia/reperfusion injury, Int. J. Cardiol. 86 (2002) 41.
[51] G. Wolkart, G. Kaber, G. Kojda, F. Brunner, Role of endogenous hydrogen peroxide in cardiovascular ischaemia/reperfusion function: studies in mouse hearts with catalase-overexpression in the vascular endothelium, Pharmacol. Res. 54 (2006) 50.
[52] T. Asahara, T. Takahashi, H. Masuda, C. Kalka, D. Chen, H. Iwaguro, Y. Inai, M. Silver, J.M. Isner, VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells, EMBO J. 18 (15-7-1999) 3964.
[53] S. Dimmeler, A.M. Zeiher, M.D. Schneider, Unchain my heart: the scientific foundations of cardiac repair, J. Clin. Invest. 115 (2005) 572.
[54] H. Kamihata, H. Matsubara, T. Nishiue, S. Fujiyama, Y. Tsutsumi, R. Ozono, H. Masaki, Y. Mori, O. Iba, E. Tateishi, A. Kosaki, S. Shintani, T. Murohara, T. Imaizumi, T.

Iwasaka, Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines, Circulation 104 (28-8-2001) 1046.
[55] L. Zentilin, S. Tafuro, S. Zacchigna, N. Arsic, L. Pattarini, M. Sinigaglia, M. Giacca, Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels, Blood 107 (1-5-2006) 3546.
[56] A. Zampetaki, J.P. Kirton, Q. Xu, Vascular repair by endothelial progenitor cells, Cardiovasc. Res. 78 (1-6-2008) 413.
[57] C. Urbich, E. Dernbach, L. Rossig, A.M. Zeiher, S. Dimmeler, High glucose reduces cathepsin L activity and impairs invasion of circulating progenitor cells, J. Mol. Cell Cardiol. 45 (2008) 429.
[58] N. Werner, S. Kosiol, T. Schiegl, P. Ahlers, K. Walenta, A. Link, M. Bohm, G. Nickenig, Circulating endothelial progenitor cells and cardiovascular outcomes, N. Engl. J. Med. 353 (8-9-2005) 999.
[59] S. Roy, S. Khanna, C.K. Sen, Redox regulation of the VEGF signaling path and tissue vascularization: hydrogen peroxide, the common link between physical exercise and cutaneous wound healing, Free Radic. Biol. Med. 44 (15-1-2008) 180.
[60] T. Suvorava, S. Kumpf, V. Adams, G. Kojda, Vascular oxidative stress inhibits mobilization of circulating stem cells with endothelial progenitor capacity in mice, Eur. J. Cardiovasc. Prev. Rehabil. 15 (Suppl.) (2008) S41.
[61] I.N. Mungrue, D.S. Bredt, D.J. Stewart, M. Husain, From molecules to mammals: what's NOS got to do with it? Acta Physiol. Scand. 179 (2003) 123.
[62] T. Yamashita, S. Kawashima, Y. Ohashi, M. Ozaki, Y. Rikitake, N. Inoue, K. Hirata, H. Akita, M. Yokoyama, Mechanisms of reduced nitric oxide/cGMP-mediated vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase, Hypertension 36 (2000) 97.
[63] J.K. Bendall, N.J. Alp, N. Warrick, S. Cai, D. Adlam, K. Rockett, M. Yokoyama, S. Kawashima, K.M. Channon, Stoichiometric relationships between endothelial tetrahydrobiopterin, endothelial NO synthase (eNOS) activity, and eNOS coupling in vivo: insights from transgenic mice with endothelial-targeted GTP cyclohydrolase 1 and eNOS overexpression, Circ. Res. 97 (28-10-2005) 864.
[64] R. Van Haperen, M. de Waard, E. van Deel, B. Mees, M. Kutryk, T. van Aken, J. Hamming, F. Grosveld, D.J. Duncker, R. de Crom, Reduction of blood pressure, plasma cholesterol, and atherosclerosis by elevated endothelial nitric oxide, J. Biol. Chem. 277 (13-12-2002) 48803.
[65] E.D. van Deel, D. Merkus, R. Van Haperen, M.C. de Waard, R. de Crom, D.J. Duncker, Vasomotor control in mice overexpressing human endothelial nitric oxide synthase, Am. J. Physiol. Heart Circ. Physiol. 293 (2007) H1144–H1153.
[66] C. Napoli, F. De Nigris, S. Williams-Ignarro, O. Pignalosa, V. Sica, L.J. Ignarro, Nitric oxide and atherosclerosis: an update, Nitric Oxide 15 (2006) 265.
[67] T. Takaya, K. Hirata, T. Yamashita, M. Shinohara, N. Sasaki, N. Inoue, T. Yada, M. Goto, A. Fukatsu, T. Hayashi, N.J. Alp, K.M. Channon, M. Yokoyama, S. Kawashima, A specific role for eNOS-derived reactive oxygen species in atherosclerosis progression, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 1632.
[68] M. Opperman, J. Stoodt, T. Suvorava, G. Kojda, Nitric oxide does not regulate guanylyl cyclase expression in-vivo, Naunyn-Schmiedeberg's Arch. Pharmacol. 371 (Suppl. 1) (2005) R38 (Abstract).
[69] M. Oppermann, V.T. Dao, T. Suvorava, M. Bas, G. Kojda, Effect of oral organic nitrates on expression and activity of vascular soluble guanylyl cyclase, Br. J. Pharmacol. 155 (2008) 335.
[70] R.P. Brandes, D.Y. Kim, F.H. Schmitz-Winnenthal, M. Amidi, A. Gödecke, A. Mülsch, R. Busse, Increased nitrovasodilator sensitivity in endothelial nitric oxide synthase knockout mice — role of soluble guanylyl cyclase, Hypertension 35 (2000) 231.
[71] M.B. Hussain, A.J. Hobbs, R.J. MacAllister, Autoregulation of nitric oxide-soluble guanylate cyclase-cyclic GMP signalling in mouse thoracic aorta, Br. J. Pharmacol. 128 (1999) 1082.
[72] E. Mergia, A. Friebe, O. Dangel, M. Russwurm, D. Koesling, Spare, guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system, J. Clin. Invest. 116 (2006) 1731.
[73] P. Stralin, K. Karlsson, B.O. Johansson, S.L. Marklund, The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase, Arterioscler. Thromb. Vasc. Biol. 15 (1995) 2032.
[74] O. Jung, S.L. Marklund, H. Geiger, T. Pedrazzini, R. Busse, R.P. Brandes, Extracellular superoxide dismutase is a major determinant of nitric oxide bioavailability: in vivo and ex vivo evidence from ecSOD-deficient mice, Circ. Res. 93 (3-10-2003) 622.
[75] M. Oppermann, V. Balz, V. Adams, M. Bas, T.V. Dao, T. Suvorava, G. Kojda, Pharmacologic induction of vascular extracellular superoxide dismutase expression in-vivo, J. Cell Mol. Med. (in press), doi:10.1111/j.1582-4934.2008.00627.x.
[76] R. Hambrecht, V. Adams, S. Erbs, A. Linke, N. Krankel, Y. Shu, Y. Baither, S. Gielen, H. Thiele, J.F. Gummert, F.W. Mohr, G. Schuler, Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase, Circulation 107 (1-7-2003) 3152.