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REVIEW

NOX1, 2, 4, 5: counting out oxidative stress

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For decades, oxidative stress has been discussed as a key mechanism of endothelial dysfunction and cardiovascular disease. However, attempts to validate and exploit this hypothesis clinically by supplementing antioxidants have failed. Nevertheless, this does not disprove the oxidative stress hypothesis. As a certain degree of reactive oxygen species (ROS) formation appears to be physiological and beneficial. To reduce oxidative stress therapeutically, two alternative approaches are being developed. One is the repair of key signalling components that are compromised by oxidative stress. These include uncoupled endothelial nitric oxide (NO) synthase and oxidized/heme-free NO receptor soluble guanylate cyclase. A second approach is to identify and effectively inhibit the relevant source(s) of ROS in a given disease condition. A highly likely target in this context is the family of NADPH oxidases. Animal models, including NOX knockout mice and new pharmacological inhibitors of NADPH oxidases have opened up a new era of oxidative stress research and have paved the way for new cardiovascular therapies.

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Abbreviations
Ang II, angiotensin II; apo-sGC, heme-free sGC; CAD, coronary artery disease; CVD, cardiovascular disease; eNOS, endothelial NO synthase; GPCR, g-protein coupled receptor; GPx, glutathione peroxidase; HASMC, human aortic smooth muscle cells; HMG-CoA, 3-hydroxy-3-methyl-glutaryl; HUVEC, human umbilical vein endothelial cells; LDL, low density lipoproteins; MPT, mouse proximal tubule cells; NO, nitric oxide; NOX, NADPH oxidase; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMA, phorbol-12 myristate-13-acetate; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; SHR, spontaneously hypertension rats; STZ, streptozocin; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule

Introduction

Cardiovascular diseases (CVD) are the leading cause of disability and death worldwide, causing a huge burden for affected individuals and the society as a whole. Many cardiovascular disorders are associated with endothelial dysfunction, an impairment of vasodilatation in response to stimuli (e.g. acetylcholine) acting through enhancement of nitric oxide (NO) formation by endothelial NO synthase (eNOS). In endothelial dysfunction, the bioavailability of NO is most likely affected by its reaction with elevated levels of superoxide (Gryglewski *et al*., 1986; Vanhoutte, 2009). In the vasculature, superoxide and other reactive oxygen species (ROS) can be derived from several sources. These include xanthine oxidases, lipoxygenases, cyclooxygenases, mitochondria, uncoupled NOS, and peroxidases (Williams and Griendling, 2007). However, NADPH oxidases as a source of ROS stand out, as they are the only known enzymes where

ROS generation is the main and only known function. An increasing amount of data has demonstrated clearly that NADPH oxidases expression and activity correlate with the development and progression of those CVD that are associated with endothelial dysfunction.

In this review, we will focus on the importance of a redox equilibrium and continue with a discussion on the current options to measure oxidative stress. Based on this evidence, we will explain why all clinical approaches to prevent and treat CVD with supplemented antioxidants have failed despite solid animal experimental data on the role of oxidative stress in these disorders. We here review the two main alternative approaches to tackle oxidative stress-related diseases with the focus on inhibiting the disease relevant sources of ROS. In addition, we also briefly touch on how to repair damage caused by oxidative stress.

With respect to sources of ROS, we focus on NADPH oxidases without dismissing the possibility that other enzymatic and non-enzymatic sources of ROS may also be of relevance in certain disease states. While NADPH oxidases have been suggested to be involved in many CVD, we here concentrate on their role in hypertension and ischaemia-reperfusion damage, exemplified by ischaemic stroke. We then introduce and review the latest advancements in the pharmacological inhibition of NADPH oxidases.

Redox balance: there is oxidative but also reductive stress

The cellular redox balance is essential for many physiological processes and probably also for cellular homeostasis and survival (Figure 1). While most research has been directed towards oxidative stress, its counterpart, reductive stress, has only very recently been getting more attention since it may explain many of the disappointing findings with the clinical application of antioxidants. Oxidative stress refers to a state with an excess of ROS resulting from an overproduction and/or compromised degradation of ROS (Stocker and Keaney, 2004; Williams and Griendling, 2007). However, oxidative stress is poorly defined in quantitative terms (Dotan *et al.*, 2004). Oxidative stress has been suggested to be a major cause of a variety of pathologies, including endothelial dysfunction and associated cardiovascular pathologies such as hypertension, ischaemic injury, cardiac hypertrophy and congestive heart failure (Cai *et al.*, 2003; Bedard and Krause, 2007; Williams and Griendling, 2007).

Importantly, ROS are not only detrimental, as they are important signalling molecules mediating vital physiologic functions such as the innate immune response, extracellular matrix dynamics, cell proliferation, cell migration, cell differentiation, inflammation as well as vascular contraction and relaxation (Bedard and Krause, 2007; Williams and Griendling, 2007). Reductive stress is characterized by an abnormal increase of reducing equivalents, such as an elevated ratio of reduced (GSH)/oxidized glutathione (GSSG) and NADPH/NADP (Rajasekaran *et al.*, 2007; Zhang *et al.*, 2010). After the discovery that reductive stress can have deleterious effects in lower eukaryotes (Simons *et al.*, 1995; Trotter and Grant, 2002) it was reported that reductive stress can also cause damage in mammals. For example, mice expressing the human mutant αB-crystalline protein suffer from protein aggregation and increased levels of heat shock protein (Hsp)25 that were associated with cardiomyopathy (Rajasekaran *et al.*, 2007). More recently, it was shown that high levels of another Hsp, Hsp27, in hearts resulted in reductive stress, development of cardiac dysfunction and reduced life span in mice, which was attenuated by partial glutathione peroxidase inhibition (Zhang *et al.*, 2010). Therefore, a balanced redox equilibrium is key to maintain cellular homeostasis and health. Disturbance of this balance by supplementation of antioxidants may be one explanation why clinical attempts to prevent and treat CVD (and other chronic diseases) have failed (see 'The ROS scavenging approach' below).

How do we know that there is oxidative stress?

With respect to the measurement of oxidative stress, we focus on *ex vivo* ROS assays and biomarkers that could also be utilized *in vivo* and for diagnostic purposes. We will not discuss the measurement of reductive stress in this review.

ROS assays

To determine if ROS are formed in a given system, for example cells, a tissue or an organ, a variety of ROS assays can be applied. The most commonly used ones are based on spectrophotometry (cytochrome c reduction, aconitase, nitro blue tetrazolium), chemiluminesence (e.g. lucigenin, luminol, L-012), electron-spin resonance and fluorescence [e.g. dihydroethidium (DHE), and its mitochondrially targeted derivative, MitoSOX, DCF-DA and Amplex Red]. For details of these and further assays, we refer to previous publications on this topic (Munzel *et al.*, 2002; Daiber *et al.*, 2004; Dikalov *et al.*, 2007; Rhee *et al.*, 2010).

In general, measuring ROS is confounded by several factors: (i) ROS levels are often very low. (ii) ROS, as the name suggests, are highly reactive, and short-lived. As such, (iii) their formation is influenced by, for example transition metals, which are often contaminants of buffers used in assays, resulting in analytical flaws. (iv) ROS can be highly localized and are not uniformly distributed within an organ or cell. This may not be adequately assessed by an ROS assay of the entire cell or an organ segment. It may thus remain impossible to accurately localize and quantify ROS in all relevant subcellular compartments. (v) For the same reason, scavengers of ROS, if not properly targeted, may not block the effect, as they do not reach the relevant subcellular compartment(s). (vi) Scavenging of ROS by antioxidants may result in the formation of other ROS with biological effects, for example scavenging of superoxide by SOD and some antioxidant vitamins results in increased levels of H_2O_2, another ROS with many biological effects (Dikalov *et al.*, 2007) (Munzel *et al.*, 2002).

Another consideration is that identification of the enzymatic source of ROS is often accomplished by substrate addition. For instance, with respect to NADPH oxidases, addition of NADPH to cell/tissue lysates or subcellular fractions may indeed be a tool to investigate NADPH oxidase activities. However, it is not known how NADPH can enter the cells

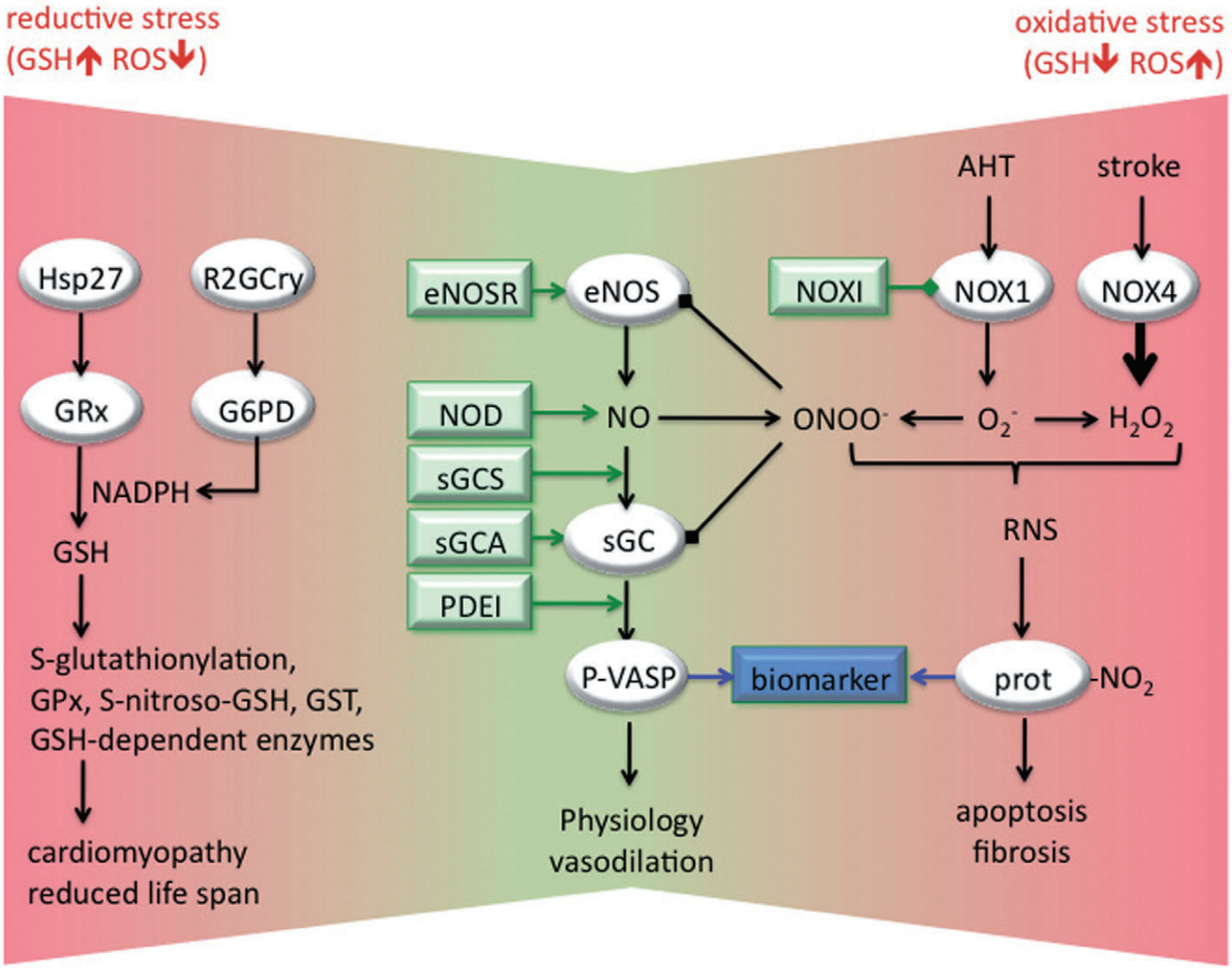


Figure 1

Balance between oxidative and reductive stress. In arterial hypertension (AHT) and stroke, physiological NO-sGC signalling and vasodilatation can be affected in three ways by NADPH (NOX)-induced oxidative stress (i.e. increased superoxide, O_2^-, and hydrogen peroxide, H_2O_2, levels): (i) scavenging of NO (with intermediate peroxynitrite, $ONOO^-$, formation); (ii) uncoupling of eNOS; and (iii) oxidation and heme-loss of NO-receptor Fe(II)sGC. Reactive oxygen species (ROS) and NO can also react to form reactive nitrogen species (RNS), which modify different cell components, including protein tyrosine nitration (prot-NO_2), correlating with cellular apoptosis and fibrosis. These pathways can be assessed by using biomarkers such as phospho-VASP (P-VASP) for physiologic NO signalling, and nitro-tyrosine for RNS chemical biology. Therapeutic options include inhibition of NADPH oxidases (NOX1 in AHT and NOX4 in stroke), eNOS recoupling (eNOSR), sGC stimulation (sGCS), sGC activation (sGCA) and phosphodiesterase (PDE) inhibition (PDEI). However, reductants or antioxidants are no therapeutic alternative. They are ineffective or even harmful, possibly via causing reductive stress, that is, unphysiological high glutathione (GSH) levels due to activation of glutathione reductase (GRx), by heat shock protein (Hsp)27 or glucose-6-phosphate dehydrogenase (G6PD)-derived NADPH. Increased GSH in turn results in glutathione peroxidase (GPx)-dependent reduction of ROS to unphysiologically low levels and leads to S-(nitroso) glutathionylation, leading eventually resulting in cardiomyopathy and reduced life span.

after NADPH is added to intact cells or even tissue segments (Dikalov *et al.*, 2007). Thus, pharmacokinetic issues may play a role when adding substrates but also pharmacodynamic factors (e.g. effects of the substrate) may influence ROS assay results.

These considerations explain why none of the available ROS assays is 'perfect'. All available assays have limitations, such as limited specificity, sensitivity, extracellular versus intracellular detection, transient versus cumulative, validity, costs, reproducibility, etc. It is therefore generally recommended to use at least two different techniques, which have to show similar results (Daiber *et al.*, 2004; Dikalov *et al.*, 2007). But this is only a rule of thumb, of course, and in some studies the results of a third assay not showing the same result may not have been included in the published paper.

Finally, to proof the presence of ROS in the system of interest is not sufficient to demonstrate any involvement of ROS in disease development and progression. Experimental

approaches should rather focus on the principle that removal of ROS by scavenging or, even better, deletion or specific inhibition of the potential relevant source can improve the condition, and that re-introduction of the ROS or its source causes the pathophysiological changes investigated (Dikalov *et al.*, 2007). Unfortunately, such data are very rarely included in publications on ROS biology.

One of the most popular probes to measure ROS is DHE. Initially, it was thought that DHE reacted with superoxide to form ethidium, which intercalates with DNA, resulting in fluorescent signals, which reflected levels of superoxide. However, it is now known that the reaction between DHE and superoxide specifically yields 2-hydroxyethidium. In parallel, ethidium can be formed in the assay, but this is not a specific superoxide product. Rather, it reflects the redox status of a cell (Fink *et al.*, 2004; Dikalov *et al.*, 2007; Zielonka *et al.*, 2009). The two products display different fluorescent spectra with an extensive overlap in fluorescence. Therefore, the commonly used fluorescence filters do not allow distinguishing between ethidium and 2-hydroxyethidium, but this is possible by separation of the two products with HPLC (Zhao *et al.*, 2005). Still, in many publications it is wrongly stated that fluorescence recorded with DHE in tissue sections reflects superoxide. Nevertheless, we believe that DHE is an excellent tool for cellular localization and semi-quantitative analysis of ROS in tissue sections. Indeed, we believe that it is of minor relevance to distinguish between and quantify individual ROS (e.g. superoxide vs. H_2O_2), as they are rapidly converted inside a living cell, and it is unknown, which ROS is the relevant type.

Oxidative stress biomarkers

Studies on ROS-dependent molecular mechanisms pointed to specific mediators that play a key role in the development of CVD. Some of these can be seen as circulating blood biomarkers in order to assess cardiovascular risk and/or to monitor the efficacy of cardiovascular drug therapy.

As an example, plasma total antioxidant status (TAC) is measured as an indicator of the general antioxidant status of an individual (Dhamrait *et al.*, 2004). There are, however, major concerns about its usefulness (Young, 2001), as for example in biological fluids the major contributor in most TAC assays is urate (>50% of the TAC activity). However, urate is of limited importance as an antioxidant *in vivo* (Young, 2001).

Isoprostanes, which have also been proposed as biomarkers, are prostaglandin-like compounds produced primarily from arachidonic acid catalysed by reactive oxygen and nitrogen species. They are classified as the 'gold standard' for the measurement of oxidative stress (Uno and Nicholls, 2010). However, most studies have used single spot measurements that can be misleading as the kinetics of isoprostanes in plasma and urine are different (Halliwell and Lee, 2010). Also, they should be standardized, but there is no agreement yet on how to do this (Halliwell and Lee, 2010).

Thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA) are the most commonly used biomarkers of lipid peroxidation (Lykkesfeldt, 2007; Niki, 2009). Again, the validity of TBARS/MDA in bodily fluids has been criticized, for example for a lack of specificity, post-sampling MDA formation, antioxidants that can interfere with the assay procedure, and MDA derived from the diet.

Oxidation of lipids such as low density lipoproteins (LDL) is suggested to play a key role in the initiation and progression of atherosclerosis (Uno and Nicholls, 2010). The heterogeneity of oxLDL results in a large diversity of biomarkers, possibly with different clinical implications. Further, lipid peroxidation can probably not be used as a universal criterion of oxidative stress (Dotan *et al.*, 2004).

As all of the above described markers have drawbacks, they are unreliable as an index of oxidative stress. However, emerging biomarkers may prove to be more reliable. For example, asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS (Boger *et al.*, 1998; Boger, 2006) is increased in conditions of oxidative stress, which may ultimately lead to endothelial dysfunction. ADMA may therefore be suitable for (pre-)clinical screening of atherosclerosis (endothelial dysfunction and systemic atherosclerosis).

The NO receptor soluble guanylate cyclase (sGC) (see Figure 1) generates cyclic GMP (cGMP) when NO binds to it. A biomarker of the NO-sGC signalling cascade is the phosphorylation of the cGMP-dependent protein kinase (cGK) substrate vasodilator-stimulated phosphoprotein (VASP) (Melichar *et al.*, 2004). P-VASP can therefore be used as a marker for NO bioavailability. VASP is already used as a biomarker to monitor the efficacy of treatment with antiplatelet drugs (Weber *et al.*, 2008). It is also a candidate biomarker to monitor the efficacy of sGC activators and stimulators in restoring the NO-sGC pathway (see also 'Repairing ROS damage' below).

By interacting with NO, NO_2^-, NO_2, etc., ROS can be modified to reactive nitrogen species (e.g. peroxynitrite, $ONOO^-$) that both oxidize or covalently modify proteins and DNA (Rizvi, 2009; Stephens *et al.*, 2009). Nitrosylation of tyrosine (NO_2-Tyr) will ultimately lead to an altered structure and function of the protein, thereby changing the signal transduction pathway involved. Thus, NO_2-Tyr can be used as a general marker of nitrosative stress.

In conclusion, oxidative stress is ill defined in quantitative terms (Dotan *et al.*, 2004) and cannot be accurately assessed with any of the current biomarkers and assays. It is more likely that a combination of methods and biomarkers is needed, and different types of ROS should be evaluated in parallel to generate patterns or indices of oxidative stress.

The ultimate pathomechanistic proof of principle though has to come from pharmacological modulation and cause–effect relationships. We will therefore discuss three approaches, antioxidants, inhibitors of ROS formation and repair of oxidative damage.

The ROS scavenging approach

Antioxidants: a near death experience for the oxidative stress hypothesis

Laboratory studies showed protective effects of antioxidants and their association with various chronic diseases, including CVD. Consequently, an impressive body of literature has implicated modifiable lifestyle factors, including the diet, in oxidative stress and CVD (Stampfer *et al.*, 2000; Lichtenstein

et al., 2006; King *et al.*, 2007; Chiuve *et al.*, 2008; Ford *et al.*, 2009; Imamura *et al.*, 2009; Mozaffarian *et al.*, 2009), and large cohort studies have shown an inverse relationship between plasma levels of antioxidants and the risk of CVD, other diseases and mortality (Riemersma *et al.*, 1991; Gale *et al.*, 1995; Singh *et al.*, 1995; Sahyoun *et al.*, 1996; Nyyssonen *et al.*, 1997; Loria *et al.*, 2000; Khaw *et al.*, 2001). It thus appeared to make sense that CVD could be prevented by supplementation of antioxidants. Due to heavy promotion by supplement manufactures (Miller and Guallar, 2009) and the lay press, today millions of consumers use antioxidant vitamin supplements, often at high doses.

Nevertheless, after many large clinical trials and meta-analyses that have studied this issue, it became obvious that antioxidant supplementation did not result in any reduction in CVD morbidity and mortality. On the contrary, some large clinical trials came to the conclusion that antioxidant supplementation can even be harmful (Vivekananthan *et al.*, 2003; Miller *et al.*, 2005b; Bjelakovic *et al.*, 2007; Dotan *et al.*, 2009; Miller and Guallar, 2009). Considering the many millions of people take antioxidant supplements, and if, as an example, high dose vitamin E supplementation increases mortality even by a small amount, such as the estimated 4% (Miller *et al.*, 2005b), these supplements would be responsible for many deaths (Miller and Guallar, 2009). The same holds true for other antioxidant regimens. It is now, for example, well established that β-carotene supplements increase the risk of cancer, CVD and mortality (Bjelakovic *et al.*, 2007). Likewise, vitamin E supplements can no longer be considered safe for the general population (Dotan *et al.*, 2009; Miller *et al.*, 2005b; Miller and Guallar, 2009).

Consequently, not only antioxidants but the entire oxidative stress theory has been questioned despite the protective effects of antioxidants in pathophysiological animal models. One likely reason for the contrasting results between animals and humans is that much higher doses of antioxidants have been used in most of the animal studies compared to those administered in clinical trials (Griendling and FitzGerald, 2003).

Despite the disappointing results of antioxidant clinical trials, we will focus on evidence that the oxidative stress hypothesis is correct, but that the redox imbalance plays a different role than previously appreciated. In addition to possibly causing reductive stress (see *Redox balance*), several other mechanisms could explain adverse effects of antioxidant supplements. These include for example: (i) Inhibition of physiological ROS functions. In the vasculature, ROS play important roles as signalling molecules that regulate inflammation, cell proliferation, migration and differentiation, as well as vascular constriction and relaxation (Williams and Griendling, 2007). Untargeted scavenging of all ROS by antioxidants is likely to interfere with the physiological functions of ROS (Ristow *et al.*, 2009; Schafer *et al.*, 2009; Ristow and Zarse, 2010). (ii) Antioxidants are not targeted to the precise localizations where ROS concentrations are elevated, as oxidative stress is not evenly distributed throughout an organism. (iii) The rates of reaction between antioxidants and ROS are often lower than the reaction rates between ROS and their targets (Gotoh and Niki, 1992; Thomson *et al.*, 1995). (iv) After their reaction with ROS, antioxidants can become radicals themselves, initiating new radical chain reactions, and thus cause harm (Vertuani *et al.*, 2004) (Bowry *et al.*, 1992). (v) The association between antioxidant plasma levels and the incidence of chronic diseases may not be due to antioxidative effects. Indeed, beneficial non-antioxidant actions of 'antioxidants' have to be considered, such as their influence on gene expression, which cannot be mimicked by supplementing single or a few antioxidants.

Nevertheless, the failure of clinical studies does not disprove the role of oxidative stress in CVD. The negative data have only shown that the antioxidant supplementation approach was apparently the wrong way to counteract CVD, and better approaches are needed.

One alternative approach that has been proposed recently is a more targeted supply of antioxidants to key subcellular locations, for example mitochondria (Dikalova *et al.*, 2010a). Interestingly, the most abundant vascular NOX isoforms, NOX4 (see *Preventing oxidative stress*), has been located in the mitochondria (Block *et al.*, 2009; Ago *et al.*, 2010; Graham *et al.*, 2010) and may thus itself represent a source of mitochondrial ROS.

As such, targeting antioxidants to mitochondria in angiotensin II (Ang II)-induced and deoxycorticosterone acetate (DOCA) salt hypertensive mice reduced blood pressure (Dikalova *et al.*, 2010a). Despite these intriguing findings, the contribution of mitochondria to oxidative stress remains controversial. On the one hand, it was suggested that mitochondria are the initiating source of ROS under some circumstances, with NADPH oxidases secondarily activated by mitochondrial ROS (Dikalova *et al.*, 2010a). On the other hand, it was proposed that NADPH oxidases stimulate mitochondrial dysfunction resulting in mitochondrial ROS release (Doughan *et al.*, 2008; Ago *et al.*, 2010). While the order remains to be determined (and may be different in different disease settings), both concepts are in line with the hypothesis of ROS-induced ROS release resulting in a vicious cycle. Nevertheless, the strategy of mitochondria-targeted antioxidants is still in its infancy. Whether this concept is translatable into human health benefits remains to be shown, keeping in mind that the translation of general antioxidant supplementation from animals to humans obviously failed. A more promising approach is to tackle oxidative stress at its roots by inhibiting the disease-relevant sources of ROS. One such source is the family of NADPH oxidases, which holds great promise for future treatment of CVD.

Preventing oxidative stress: NADPH oxidases

NADPH oxidases are enzyme complexes with a membrane-spanning catalytic NOX subunit, which depends to varying degrees on other subunits. These subunits include another membrane protein, p22phox, and cytosolic proteins. Five members of the NOX family have been identified: NOX1 to NOX5. These isoforms differ in their subunit requirements, expression patterns, subcellular localization, site of ROS release (intra- vs. extracellular), mode of activation and function. Within the vasculature, NOX1, NOX2, NOX4 and NOX5 are of relevance, keeping in mind that NOX5 is not expressed in rodents. Whereas NOX1, NOX2 and NOX4

require p22phox for their activity, NOX5 is active independently of any other subunits. Further, NOX1 and NOX2 depend on several cytosolic factors, NOX organizers and NOX activators, as well as the small GTPase Rac, which are not needed for NOX4 activity (Griendling, 2004; Cave *et al.*, 2006; Bedard and Krause, 2007; Opitz *et al.*, 2007).

The expression sites of NOX isoforms in the vasculature are not yet known due to the lack of specific and broadly validated antibodies. Nevertheless, it can be said with some certainty that NOX2 is not only present in leukocytes, but also in endothelial cells (Gorlach *et al.*, 2000; Bedard and Krause, 2007), fibroblasts (Chamseddine and Miller, 2003) and cardiac myocytes (Nabeebaccus *et al.*, 2011). NOX1 was mainly found in vascular smooth muscle cells (VSMC) of rats and mice (Bedard and Krause, 2007; Nabeebaccus *et al.*, 2011). In mice, it was also detected in endothelial cells (Sorescu *et al.*, 2004). In humans, NOX1 expression in VSMC differs depending on the vessel types. For example, no NOX1 was found in human renal arteries (Schluter *et al.*, 2010) and saphenous veins (Guzik *et al.*, 2004), but human aortic VSCM do express NOX1 (Touyz *et al.*, 2002). Importantly, in spontaneously hypertensive rats (SHR) NOX1 is induced in the endothelium, possibly in close proximity to eNOS (Wind *et al.*, 2010a). The most abundant vascular NOX isoform is NOX4 with a high expression in endothelial cells and VSMC (Cheng *et al.*, 2001). It is also present in neurons (Kleinschnitz *et al.*, 2010).

NOX5: a special case

NOX5 was the last NOX isoform to be identified (Banfi *et al.*, 2001; Cheng *et al.*, 2001), and much less is known about this NOX compared to the other isoforms. This lack of knowledge is mainly due to the absence of NOX5 in rats and mice making studies on NOX5 in these species impossible. Genetically, NOX5 is the most distinct of the NOX isoforms and exists in at least five splice variants (Cheng *et al.*, 2001). It is present in the human vasculature (BelAiba *et al.*, 2007; Guzik *et al.*, 2008; Jay *et al.*, 2008). NOX5 activity apparently does not depend on any subunits. Another important feature of NOX5 is its N-terminal EF-hand regions, which results in direct activation of NOX5 by an increase in intracellular calcium (Banfi *et al.*, 2001). NOX5 can also be activated without elevating intracellular calcium by protein kinase C-dependent phosphorylation. This modification increases the calcium sensitivity of NOX5 and thus permits a higher level of activity at resting levels of intracellular calcium (Jagnandan *et al.*, 2007). Another mode of calcium sensitization occurs via binding of calcium-activated calmodulin (Tirone and Cox, 2007). Importantly, NOX5 mRNA protein levels were increased in coronary arteries from patients with coronary artery disease (CAD) compared to patients without CAD. In correlation, calcium-dependent NADPH oxidase activity, which is very likely to reflect NOX5 activity, was increased sevenfold in CAD coronary arteries. While NOX5 was mainly localized in the endothelium in early lesions, its protein levels were up-regulated in VSMC of advanced coronary lesions and lost in the endothelium (Guzik *et al.*, 2008). These data suggest a prominent role of NOX5 in human CAD.

We believe that the complexity of the NADPH oxidase family will allow to specifically inhibit the formation of pathophysiologically relevant ROS as opposed to untargeted scavenging. Indeed, an impressive amount of data shows that NOX isoforms are up-regulated in many cardiovascular as well as other chronic diseases and their risk factors, for example atherosclerosis, heart failure, myocardial infarction, fibrosis, diabetes mellitus, aging, smoking, inflammation, as well as sepsis, cancer and neurodegeneration. We here focus on the role of NADPH oxidases in hypertension and stroke. These are two examples, where there is compelling evidence for the involvement of individual NOX isoforms that is based on the use of specific pharmacological NADPH oxidase inhibitors and/or NOX knockout mice: NOX1 in hypertension and NOX4 in stroke. With respect to the possible involvement of NADPH oxidases in other disorders, we refer to recent reviews (Cave *et al.*, 2006; Bedard and Krause, 2007; Brandes *et al.*, 2010; Lassegue and Griendling, 2010).

NADPH oxidases and hypertension

Increased ROS formation with subsequent decreases in NO bioavailability caused by scavenging of NO has been proposed to be the most important cause of impaired endothelium-dependent relaxation in hypertension (Schulz *et al.*, 2008). ROS not only alter vascular contractility but also influence vascular remodelling, another phenomenon associated with hypertension (Williams and Griendling, 2007). The diffusion-limited reaction between the ROS superoxide and NO results in the formation of peroxynitrite, which is a strong oxidant. Among other pro-oxidant effects, peroxynitrite oxidizes the NOS cofactor tetrahydrobiopterin. This results in uncoupling of NOS, which then produces ROS itself instead of NO, causing further oxidations – a vicious cycle leading to further impairment of endothelial function (Rubanyi and Vanhoutte, 1986; McIntyre *et al.*, 1999). The loss in NO-mediated vasodilatation is further exacerbated by vasoconstriction mediated by ROS themselves (Auch-Schwelk *et al.*, 1989). ROS are also involved in the compensatory vascular remodelling taking place in hypertension. This includes VSMC hypertrophy (Zhang *et al.*, 2005). However, ROS not only mediate pathological changes in the vasculature, but also in the kidney and brain that further contribute to the development of hypertension (for details see Datla and Griendling, 2010).

Interestingly, endothelium-derived H_2O_2 has been suggested as an endothelium-derived hyperpolarizing factor that causes vasodilation and cardioprotection (Shimokawa, 2010). However, other studies rather suggested that H_2O_2 is vasoconstrictive (Gao and Lee, 2005; Schluter *et al.*, 2010) in some vascular beds. These contrasting findings may be caused by divergent responses of different vascular beds, the species and the concentrations. Overall, it is currently not clear what functions H_2O_2 has with regards to vasodilatation and contraction under physiological and pathophysiological conditions.

In accordance with a role of ROS in hypertension, ROS are elevated in many animal models of hypertension, including Ang II or ET-1 infusion in rodents, SHR and DOCA salt models (Lassegue and Griendling, 2004; Datla and Griendling, 2010). In these models, NADPH oxidase activity is increased in the vascular wall and kidney (Datla and Grien-

dling, 2010; Wind *et al.*, 2010a). Further evidence for a role of NADPH oxidases in hypertension stems from the use of the peptide NADPH oxidase inhibitor gp91ds-tat, which for example attenuated Ang II-induced hypertension in mice (Rey *et al.*, 2001). However, in Dahl salt-sensitive rats, it did not improve blood pressure, but it ameliorated endothelial dysfunction (Zhou *et al.*, 2006).

A crosstalk between different sources of ROS in hypertension is likely with NADPH oxidases being the primary source of ROS that triggers ROS production by other sources. For example, p47phox-dependent NADPH oxidase-driven superoxide production results in uncoupling of eNOS in DOCA salt hypertension (Landmesser *et al.*, 2003) and overexpression of NOX1 in VSMC leads to enhanced production of ROS in response to Ang II that causes eNOS uncoupling resulting in impaired vasorelaxation (Dikalova *et al.*, 2010b). In contrast, NOX5 overexpression in the endothelium of mouse aortae paradoxically increased eNOS activity. However, it reduced NO bioavailability via inactivation of NO by ROS resulting in impaired endothelium-dependent relaxation (Zhang *et al.*, 2008). Together, these studies suggest different effects of ROS released from individual NOX isoforms in their interaction with other enzymes and molecules that may in part depend on their subcellular localizations.

Several groups reported a correlation between hypertension and NOX2 expression. For example, aortic NOX2 is elevated in stroke-prone SHR in rats exposed to aldosterone plus salt and in Ang II-infused mice (Park *et al.*, 2008; Lassegue and Griendling, 2010). Depending on the model, hypertension was improved by NOX2 deletion. While it did not prevent Ang II-induced hypertension (but decreased medial hypertrophy) (Lassegue and Clempus, 2003), deletion of NOX2 reduced hypertension in 2-kidney 1-clip (Jung *et al.*, 2004) and DOCA salt mice (Fujii *et al.*, 2006).

Nevertheless, it has become evident recently, that among the vascular NOX isoforms, NOX1 seems to play a major role in the pathology of hypertension. Deletion of NOX1 in mice results in blunted pressor response to Ang II and increased vasodilatation in response to acetylcholine (Matsuno *et al.*, 2005; Gavazzi *et al.*, 2006). Consistently, mice that overexpress NOX1 in VSMC show exacerbated hypertension and aortic vascular hypertrophy in response to Ang II infusion (Dikalova *et al.*, 2005). This genetic evidence for a major role of NOX1 obtained in mice is further supported by the observation that vascular or kidney NOX1 is up-regulated in human renin transgenic mice (Didion *et al.*, 2002), 2-kidney 1-clip (Wang *et al.*, 2007) and Dahl salt-sensitive rats (Nishiyama *et al.*, 2004). NOX1 protein is also increased in aortae of aged SHR in parallel with increased NADPH oxidase-dependent ROS formation. While NOX2 was also up-regulated, NOX4 protein levels were unchanged (Wind *et al.*, 2010a). A role for NOX4 at least for basal blood pressure regulation in mice can also be excluded as NOX4 knockout mice display normal blood pressures (Kleinschnitz *et al.*, 2010). Interestingly, NOX1 showed ectopic expression in endothelial cells of aortae from aged SHR. Finally, the impaired acetylcholine-induced relaxation of SHR aortae was significantly improved by the NADPH oxidase inhibitor VAS2870 (Wind *et al.*, 2010a).

In conclusion, animal studies suggest that NADPH oxidases, in particular NOX1-based oxidases, are promising targets for the treatment of systemic hypertension. However, confirmation of these data in hypertensive patients is warranted. As a first indication, NADPH oxidase activity was recently indentified as the major source of superoxide in renal proximal resistance arteries from elderly patients with renal tumours (Schluter *et al.*, 2010).

NADPH oxidases and ischaemic stroke

The hypothesis that ROS are involved in ischaemic stroke dates back to the 1970s (Flamm *et al.*, 1978). In the cerebral vasculature of rodents, NOX4 mRNA levels are higher than in peripheral blood vessels (Miller *et al.*, 2005a) and further induced in stroke (McCann *et al.*, 2008; Kleinschnitz *et al.*, 2010). This was confirmed on protein level and evident in human brain samples (Kleinschnitz *et al.*, 2010).

A major role of NOX4 in ischaemic stroke has been revealed recently using NOX4 knockout mice (Kleinschnitz *et al.*, 2010). These data suggest that NOX4-mediated oxidative stress leads to neuronal damage via leakage of the blood-brain barriers and neuronal apoptosis, two pathophysiological hallmarks of ischaemic stroke. The protection in NOX4 knockout mice was underlined by reduced post-stroke mortality and improved neurological functions. The genetic experiments were mimicked by pharmacological inhibition of NADPH oxidases using VAS2870 in wild-type mice within a clinically relevant time after induction of stroke. Importantly, VAS2870 had no further effect in NOX4 knockout (KO) mice (Kleinschnitz *et al.*, 2010). In this study, deletion of NOX1 or NOX2 had no impact on infarct size or functional outcomes, whereas other groups have described protective effects of NOX2 (Walder *et al.*, 1997; Chen *et al.*, 2009; Jackman *et al.*, 2009) and NOX1 (Kahles *et al.*, 2010) deficiency in ischaemic stroke. These divergent findings may be caused by differences in experimental protocols, although the exact reasons remain unclear. Nevertheless, specific pharmacological inhibition of NOX4 has the potential for becoming a new treatment strategy of ischaemic stroke, where currently only very limited treatment options exist, that is, thrombolysis with recombinant tissue plasminogen activator (rt-PA), a therapy which excludes 90% of all stroke patients due to contraindications.

NADPH oxidase inhibitors

In contrast to unspecific antioxidants, direct inhibition of the relevant source(s) of ROS in different pathologies holds great promise as innovative and mechanism-based treatments in cardiovascular and other diseases. Thus, specific inhibitors for NADPH oxidases, that are ideally also NOX isoform-specific, are in dire need. Besides their potential future therapeutic applications, such inhibitors are also essential to fully establish the role of NADPH oxidases and individual NOX isoforms in different pathologies.

Interestingly, many well-established cardiovascular drugs already interfere with NADPH oxidases although most likely by indirect mechanism.

Current drugs that interfere with NADPH oxidases

Statins, or 3-hydroxy-3-methyl-glutaryl reductase inhibitors act by inhibiting geranylgeranylation of Rac (Wassmann *et al.*, 2002; Chen *et al.*, 2008), which is an important component for the activation of some NOX isoforms (Bedard and Krause, 2007; Opitz *et al.*, 2007). This inhibition prevents the translocation of Rac to the cell membrane and results in inhibition of Rac-dependent NADPH oxidases (Wassmann *et al.*, 2002; Chen *et al.*, 2008). This may explain at least some of the pleiotropic, cholesterol-independent atheroprotective effects of statins. It was indeed reported that, for example, atorvastatin decreased aortic superoxide levels in SHR. Besides, it also decreased NOX1 and p22phox, but not NOX2 mRNA levels in vessels of treated SHR, and catalase mRNA was up-regulated (Wassmann *et al.*, 2002). Cerivastatin and atorvastatin treatments in mice improved endothelium-dependent relaxation to acetylcholine. Interestingly, withdrawal of statins attenuated endothelium-dependent relaxation compared to control animals, and the relaxation was restored by the SOD mimetic, tiron. While vascular ROS were unaffected by statin therapy, which may be due to low NADPH oxidase activity under resting conditions, ROS levels increased during withdrawal. These effects did not occur in NOX2 knockout mice. In human umbilical vein endothelial cells (HUVEC), statin treatment reduced NADPH oxidase activity, and withdrawal resulted in profound translocation of Rac to the membrane and transient increase in NADPH oxidase activity (Vecchione and Brandes, 2002). Thus, NOX2 may not only be inhibited by statins, but also play a role in rebound phenomena after withdrawal of statins, resulting in an overshoot activation or translocation of Rac.

In humans, statins have been shown to improve endothelial function (Treasure *et al.*, 1995; O'Driscoll *et al.*, 1997; Tsunekawa *et al.*, 2001). Further, pravastatin has been reported to have blood pressure lowering effects (Glorioso *et al.*, 1999; Kawano and Yano, 2006). Nevertheless, statins are definitely not specific NADPH oxidase inhibitors.

Angiotensin converting enzyme inhibitors and angiotensin receptor blockers (sartans) also have beneficial effects to which indirect inhibition of NADPH oxidases is likely to contribute, as Ang II is a potent stimulus for NADPH oxidase activity in vascular cells (Griendling and Ushio-Fukai, 2000; Wingler *et al.*, 2001; Williams and Griendling, 2007; Datla and Griendling, 2010). Indeed, the hypertensive effects of Ang II appear in part to be mediated by NADPH oxidase-derived ROS (Dikalova *et al.*, 2005; Matsuno *et al.*, 2005; Gavazzi *et al.*, 2006).

Considering these effects from established drugs and the amount of evidence for the involvement of NADPH oxidases in endothelial dysfunction, associated CVD and other disorders, inhibition of NADPH oxidases as novel therapies is a promising strategy to causally treat and prevent these conditions and/or suppress associated end-organ damage.

Old and new NADPH oxidase inhibitors

For many years, specific small molecule inhibitors were not available, and this represented a major bottleneck of research into oxidative stress. However, more recently, several novel, apparently more specific and drug-like NADPH oxidase inhibitors have been published.

The most commonly used NADPH oxidases inhibitors, diphenylene iodonium (DPI) and apocynin are unspecific (O'Donnell *et al.*, 1993; Majander *et al.*, 1994; Vejrazka *et al.*, 2005; Riganti *et al.*, 2006; Williams and Griendling, 2007; Aldieri *et al.*, 2008; Heumuller *et al.*, 2008; Schluter *et al.*, 2008; Selemidis *et al.*, 2008; Jaquet *et al.*, 2009; Tazzeo *et al.*, 2009; Brandes *et al.*, 2010; Castor *et al.*, 2010; Wind *et al.*, 2010b). Thus, their effects cannot be solely attributed to inhibition of NADPH oxidases. Also, 4-2-amino-ethyl-benzolsulfonyl-fluoride (AEBSF) is not a reliable NADPH oxidase inhibitor, as it irreversibly inactivates serine proteases (Diatchuk *et al.*, 1997) and interferes with the most commonly used assays for ROS (Wind *et al.*, 2010b). Studies performed with these compounds must therefore be interpreted with greatest caution. With respect to other rather rarely used and also unspecific inhibitors, we refer to a detailed review on NADPH oxidase inhibitors (Jaquet *et al.*, 2009).

A more reliable tool to inhibit NADPH oxidases are cell-permeable peptide-based inhibitors such as gp91ds-tat (Rey *et al.*, 2001), whereas another peptide inhibitor, the naturally occurring peptide PR-39 (Gudmundsson *et al.*, 1995; Shi *et al.*, 1996), is rather unspecific as it binds to SH3 domains of many proteins (Cai *et al.*, 2003). Despite their value in experimental studies, peptides have the disadvantage of low bioavailabilities. Therefore, they have limited potential as therapeutic agents. More details about these peptides are reviewed in Selemidis *et al.* (2008). More recently, professional screening programmes for NADPH oxidase inhibitors have resulted in the discovery of several novel small molecule, non-peptidergic NADPH oxidase inhibitors.

Interestingly, many novel inhibitors show some common structural features (Table 1), that is, the inhibitors generally are flat and lipophilic aromatic heterocyclic compounds.

S17834. This synthetic polyphenol, 6,8-diallyl 5,7-dihydroxy 2-(2-allyl 3-hydroxy 4-methoxyphenyl)1-H benzo(b)pyran-4-one, was discovered as a potential regulator of adhesion molecule expression for treating chronic venous insufficiency (Verbeuren *et al.*, 2000), a condition that may be mediated by NADPH oxidase activation. S17834 decreases NADPH oxidase activity, vascular cell adhesion molecule expression and leukocyte adhesion in HUVEC cells without affecting xanthine oxidase (XOD) activity and scavenging of superoxide (Cayatte *et al.*, 2001). In ApoE-deficient mice, S17834 (130 $mg \cdot kg^{-1} \cdot day^{-1}$ for 12 weeks) inhibited atherosclerotic lesion development (Cayatte *et al.*, 2001) as it did in diabetic (streptozotocin-treated) LDL receptor-deficient mice after 6 weeks treatment with a similar dose (Zang *et al.*, 2006). The mechanism by which S17834 inhibits NADPH oxidases has not been defined.

The VAS inhibitors. A new class of compounds that inhibit NADPH oxidases are triazolo pyrimidines, with the prototype VAS2870, 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo(4,5-d)pyrimidine (Stielow *et al.*, 2006; ten Freyhaus *et al.*, 2006; Lange *et al.*, 2009; Niethammer *et al.*, 2009; Wind *et al.*, 2010a) (Tsai and Jiang, 2010) (Kleinschnitz *et al.*, 2010) and the better water-soluble derivate, VAS3947 (Wind *et al.*, 2010b). VAS2870 stemmed from a systematic compound

Table 1

Novel small molecule NADPH oxidase inhibitors

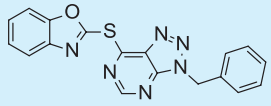
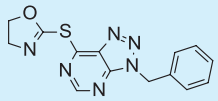
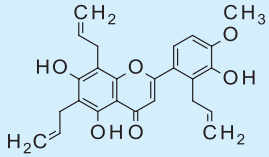
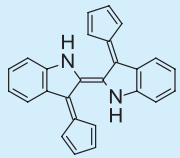
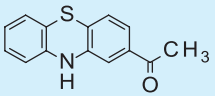
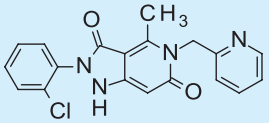
Compound	Chemical structure	NOX inhibitor potencies and NOX-related effects using *in vitro* test systems	Non-NOX-related antioxidant activity tests	NOX-related effects on pathologies *in vivo*	References
VAS2870		IC_{50} of 1–2 μM in intact human leucoplast HL-60 cells and isolated macrophages IC50 of 10.6 μM in cell free NOX2 assay 10 μM VAS2870 fully inhibits PDGF-BB induced NADPH oxidase activity in cultured rat VSMC 5 μM VAS2870 fully inhibits oxLDL induced NADPH oxidase activity in HUVEC, but has no effect on basal activity Improves endothelial-dependent relaxation in aortae from aged SHR	At 50 μM no inhibition of xanthine oxidase, no superoxide scavenging	Reduces the size of brain infarcts in a mouse model of stroke Reduces hydrogen peroxide formation in the wound margin of zebrafish	Stielow *et al.*, 2006; ten Freyhaus *et al.*, 2006; Niethammer *et al.*, 2009; Wind *et al.*, 2010a
VAS3947		IC_{50} of 1–2 μM in intact human leucoplast HL-60 cells IC_{50} of 12 μM in homogenates of CaCo2 cells and IC_{50} of 13 μM in homogenates of A7r5 cells	At 30 μM no inhibition of xanthine oxidase and eNOS, no superoxide scavenging No interference with commonly used ROS assays		Wind *et al.*, 2010b
S17384		IC_{50} for between 25 and 50 μM in intact TNF-stimulated HUVEC and in HUVEC membrane preparations	At 50 μM no inhibition of xanthine oxidase, no superoxide scavenging	Decreases aortic superoxide formation and atherosclerotic lesion area in ApoE-deficient mice Reduces aortic atherosclerotic lesion size in STZ-induced diabetic LDL receptor-deficient mice	Cayatte *et al.*, 2001; Zang *et al.*, 2006
Fulvene-5		At 5 μM, NOX2 and NOX4 (HEK293 cells) are inhibited by about 40%	No data published	Reduces haemangioma growth in a nude mouse model	Bhandarkar *et al.*, 2009

Table 1

Continued

Compound	Chemical structure	NOX inhibitor potencies and NOX-related effects using *in vitro* test systems	Non-NOX-related antioxidant activity tests	NOX-related effects on pathologies *in vivo*	References
ML171		IC_{50} in transfected HEK293 cells for NOX1, 2, 3 and 4 are 0.25, 5, 3 and 5 μM, respectively IC_{50} in HT29 cells (NOX1) is 0.13 μM	No H_2O_2 scavenging at 10 μM		Gianni *et al.*, 2010
GKT136901		K_i for NOX1 is 0.160 μM, NOX4 is 0.165 μM, and NOX2 is 1.530 μM Inhibition of high glucose-induced ROS release in MPT cells and of thrombin-induced i.c. ROS formation in HASMC	At 100 μM no major inhibition of xanthine oxidase, no superoxide scavenging, very low or no inhibition of other ROS producing or redox-sensitive enzymes	Orally bioavailable, reduces aortic lesion size and aortic CD44 and hyaluronic acid expression in ApoE-deficient mice	Sedeek *et al.*, 2010; Vendrov *et al.*, 2010

The compounds described by Borbely *et al.* (2010) are not included. NADPH oxidase inhibitors that have been proven to be unspecific and peptide-based inhibitors are not listed in this table. In addition, compounds that mediate NADPH oxidase inhibition without direct interference with NADPH oxidase inhibitors, that is, through other mechanisms as well as compounds that have not yet been described in peer-reviewed publications, but only in filed patents are not shown.

eNOS, endothelial NO synthase; HASMC, human aortic smooth muscle cells; HUVEC, human umbilical vein endothelial cells; LDL, low density lipoproteins; MPT, mouse proximal tubule cells; NOX, NADPH oxidase; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; STZ, streptozotocin; TNF, tumour necrosis factor; VSMC, vascular smooth muscle cell.

screen in HL-60 cells. At 10 μM it inhibits NADPH oxidase activity in oxLDL-exposed HUVEC (Stielow *et al.*, 2006) and platelet-derived growth factor (PDGF)-stimulated primary rat aortic VSMC (ten Freyhaus *et al.*, 2006). VAS2870 (50 μM) also inhibits the stimulation of vasculogenesis of mouse embryonic stem cells upon treatment with PDGF-BB (Lange *et al.*, 2009), and it inhibits wound margin H_2O_2 production without obvious toxicity in zebrafish larvae (Niethammer *et al.*, 2009). In addition, VAS2870 does not interact with ROS in an antioxidant manner, nor does it interfere with XOD (ten Freyhaus *et al.*, 2006). VAS3947 has been developed by *in silico* optimization of VAS2870 and has strikingly similar properties compared to VAS2870. For example, the IC_{50} values for NADPH oxidase activity of phorbol 12-myristate-13-acetate (PMA)-stimulated HL-60 cells, of PMA-stimulated whole blood and of freshly isolated human lymphocytes stimulated with PMA are essentially the same for both compounds, approximately 2 μM (Wind *et al.*, 2010b; P. Scheurer, K. Wingler, unpubl. data). VAS3947 and VAS2870 both effectively inhibit ROS production in aortae of SHR as assessed by *in situ* DHE staining (Wind *et al.*, 2010a,b). VAS3947 neither interferes with the flavoprotein, XOD, nor with the flavoheme protein, eNOS, nor with any of the ROS detection assays and had no significant antioxidant activity. Rather, VAS3947 effectively inhibits NADPH oxidase activity in three cellular models expressing different patterns of all known NOX isoforms (Wind *et al.*, 2010b). Therefore, at least *in vitro*, triazolo pyrimidines are new and specific pharmacological tools for inhibiting NADPH oxidases. The potential to inhibit NADPH oxidases is a class effect of triazolo pyrimidines, and these compounds act in a variety of cell types and tissues of phagocytic as well as non-phagocytic origin with similar efficacy. The mechanism of action of triazolo pyrimidines such as VAS3947 and VAS2870 is unclear. In human leucocytes, VAS2870 does not inhibit translocation of p47phox to the membrane (ten Freyhaus *et al.*, 2006), but may still interfere with oxidase assembly once the translocation has occurred. This assumption is supported by experiments showing that VAS2870 inhibits NOX2 activity in a cell free system when added before (IC_{50} of 10 μM) (ten Freyhaus *et al.*, 2006), but not after complex formation and stimulation of the oxidase (P. Scheurer, K. Wingler, unpubl. obs.). Nevertheless, future studies are required to clarify the precise mechanism of action, pharmacokinetics and *in vivo* efficacy of triazolo pyrimidines.

Excitingly, VAS2870 was recently applied *in vivo* for the first time to mice that had undergone transient middle cerebral artery occlusions, a model of ischaemic stroke. Intrathecal treatment with VAS2870 within a therapeutically relevant time window, that is, 2 h after reperfusion protected mice from brain damage (Kleinschnitz *et al.*, 2010).

GKT136901. This drug-like small molecule, 2-(2-chlorophenyl)-4-methyl-5-(pyridin-2-ylmethyl)-1H-pyrazolo [4,3-c]pyridine-3,5(2H,5H)-dione, was recently introduced as a NOX1/4 inhibitor. It was investigated with respect to NADPH oxidase activities in cell free assays with isolated membranes from polymorphonuclear cells (high levels of NOX2) and from cells overexpressing NOX1 or NOX4. The compound (10 μM) also inhibited NADPH oxidase activity, p38MAP kinase activation as well as TGF-β1/2 and fibronectin induction in mouse proximal tubular cells incubated with high glucose. It is a potent compound with a Ki of 165 nM for NOX4 and of 160 nM for NOX1, whereas the Ki for NOX2 is 1530 nM. It did not display any significant inhibition of XOD. It was further valuated *in vitro* in a pharmacological profile including 135 target proteins at a concentration of 10 μM. Only very low or no inhibition for other ROS producing enzymes, redox-sensitive enzymes and other proteins was observed (Sedeek *et al.*, 2010), pointing to a high specificity of GKT136901. In human aortic smooth muscle cells, GKT136901 (30 μM) inhibited intracellular ROS formation and thrombin-induced CD44 and HAS2 mRNA and protein levels, without affecting NOX1 and NOX4 expression (Vendrov *et al.*, 2010). GKT136901 is bioavailable following oral administration. Ten $mg \cdot kg^{-1}$ of the compound was delivered through oral gavage once daily, on 5 days per week over a period of 12 weeks to wild-type and various knockout mice (ApoE KO, p47phox KO, CD44 KO and ApoE/p47phox double KO), which were fed either a standard or a Western diet. This resulted in a decrease of aortic lesion areas and aortic ROS production in ApoE mice fed the Western diet. Further, plasma 8-isoprostane levels, expression of CD44, HA and of the monocyte/macrophage marker CD11b were attenuated in aortic lesions. GKT136901 did not have any significant effect on body weight, plasma total cholesterol or triglyceride levels (Vendrov *et al.*, 2010). The data available for GKT136901 are indeed promising. Additional studies are now warranted to assess the pharmacokinetic properties and *in vivo* actions in other models.

ML171. Several phenothiazines have been identified as NOX1 inhibitors by high-throughput screening using a HT29 cell-based assay (Gianni *et al.*, 2010). Phenothiazine scaffolds are found in various antipsychotic drugs, for example chlorpromazine, promazine and trifluoperazine (Mitchell, 2006). Based on structure–activity relationship studies to identify more potent NOX1 inhibitors, 2-acetylphenothiazine (ML171), which is not used as an antipsychotic drug, was selected for further analysis. ML171 has an IC_{50} for NOX1 in the nanomolar range, that is, 0.129 μM in HT29 cells and 0.25 μM in a HEK293-NOX1 reconstituted cell system. IC_{50} values for NOX2, NOX3 and NOX4 in respective HEK293 reconstituted cell systems were 5 μM, 3 μM and 5 μM, respectively, while IC_{50} values for XOD were 5.5 μM. The inhibition in the NOX1-based HEK293 cell system was overcome by increasing levels of NOX1 expression, but not by increasing NoxA1 and NoxO1. A possible side effect of using ML171 as NOX1 inhibitors in the clinic is the potential antipsychotic effect due to the presence of the phenothiazine structure. However, SAR analysis indicated that several phenothiazines with antipsychotic effect are unlikely to alter NOX1-dependent ROS generation (Gianni *et al.*, 2010), although this is not proven. In addition, ML171 did not significantly bind to a large battery of human or rodent G-protein coupled receptors (GPCR), channels and transporters expressed in the central nervous system, did not significantly bind most of the receptors tested in the binding assays with the exception of serotonin (5-HT2B and 5-HT2C), and adrenergic (alpha2C) receptors (% of inhibition >60%). Nevertheless, secondary concentration–response analysis revealed Ki values in the micromolar range, which is suggestive of low affinity of ML171

for these GPCR (5-HT2 receptor subtypes Ki = 0.56 to 3.0 μM, and alpha 2 receptor subtypes Ki = 2.7–6.9 μM). Therefore, the authors concluded that ML171 does not inhibit NOX2 function in the immune system nor likely exert unwanted antipsychotic effects. Finally, ML171 blocked NOX1-dependent extracellular matrix-degrading, actin-rich cellular structures (invadopodia) in colon cancer cells (Gianni *et al.*, 2010). However, in this assay 10 μM ML171 was used, a concentration well above the reported IC_{50} for NOX2, 3 and 4, as well as XOD. Furthermore, the authors did not include NOX5 in their assays, and no *in vivo* data are yet available for this compound.

Fulvene-5. This recently described NADPH oxidase inhibitor was identified using a structure-based approach. Fulvenes are highly water-soluble aromatic ring structures. Fulvene-5 showed inhibitory activity against NOX2 and NOX4 in stably transfected HEK293 cells, where 5 μM resulted in about 40% decrease of ROS production. It also inhibited haemangioma growth in mice that were treated with Fulvene-5 for 2 weeks, without displaying any apparent toxicological effects (Bhandarkar *et al.*, 2009). Unfortunately, no data are published on the IC_{50} values of this compound, on its specificity and the activity towards other NOX isoforms, as well as on its pharmacokinetics.

In summary, these novel compounds and compound classes have interesting profiles and are already valuable tools for research. However, more detailed knowledge about their *in vivo* efficacy is warranted, although first and promising *in vivo* data have been published for some of them. Generally, long-term effects of NADPH oxidase inhibition are not yet established. One obvious problem may arise from inhibition of NOX2-mediated oxidative burst and associated immunological dysfunctions.

Repairing ROS damage

Clearly, reduction of oxidative stress has considerable pharmacological and therapeutic potential. However, taking the normal development time for new drugs into account, an assessment of their clinical benefits lies in the more distant future. Despite these limitations, a surprising plethora of pharmacological options has emerged in recent years and has already advanced in late clinical development stages or entered the market.

Inhibiting phosphodiesterases

Inhibition of phosphodiesterases (PDE), in particular PDE type 5, augments NO-cGMP signalling irrespective of whether it was pathophysiologically reduced beforehand or not. Its first indication was erectile dysfunction, an early marker of CVD (Thompson *et al.*, 2005), where NO signalling may indeed be dysfunctional. In the more recent indication for PDE5 inhibitors, pulmonary hypertension, this does not seem to be the case (Kirsch *et al.*, 2008). Nevertheless, PDE5 inhibitors are effective in pulmonary hypertension (Wilkins *et al.*, 2008; Galie *et al.*, 2009).

Stimulating sGC

For the same indication, another approach has been developed also augmenting NO-cGMP signalling, sGC stimulation. This compound class allosterically enhances the NO-induced cGMP formation by Fe(II)sGC, so that low, submaximal concentrations of NO are potentiated and exert the same cGMP formation as higher concentrations of NO. In addition, these compounds have a small direct stimulating effect on sGC. In principle this compound class is applicable to all disease states where reduced bioavailability of NO, for example by oxidative stress, plays a pathomechanistic role. The NO levels will remain unchanged and low, but their effects will be augmented. The original compound, YC-1 had still some off-target effects (Galle *et al.*, 1999; Li *et al.*, 2008). However, the successor compounds from Bayer, BAY 41-2272 and BAY 63-2521/riociguat (Mittendorf *et al.*, 2009) were devoid of this at therapeutically relevant concentrations (Bischoff and Stasch, 2004). Other indications for this compound class may include systemic hypertension (Zanfolin *et al.*, 2006).

Activating oxidized and apo-sGC

Another principle, sounding confusingly similar, is sGC activation. However, mechanistically this is a profoundly different principle. Under conditions of oxidative stress, the NO receptor Fe(II)sGC can be oxidized to Fe(III)sGC and eventually looses its heme. It then becomes ubiquitinylated and degraded (Meurer *et al.*, 2009). Heme-free apo-sGC is essentially unresponsive to NO, but can bind sGC activators that occupy the empty sGC heme binding site and re-activate apo-sGC to the same V_{max} as Fe(II)sGC in the presence of NO (Evgenov *et al.*, 2006; Stasch *et al.*, 2006). The first compound of this class, BAY 58-2667/cinaciguat, does this by also preventing sGC ubiquitinylation and thereby preventing sGC degradation. Later compounds such as HMR-1766/ataciguat (Zhou *et al.*, 2008) are devoid of this effect (Hoffmann *et al.*, 2009). Cinaciguat is currently developed for acute heart failure, an indication that may benefit from an unusual vasodilatory profile, that is, preference for microvasculature over large conducting blood vessels and diseased over healthy blood vessels. Thus, sGC activators are the first vasodilator compound class that specifically dilate diseased blood vessels making steel phenomena highly unlikely. Other development targets include peripheral artery disease and chronic pain (Schmidt *et al.*, 2009).

Re-coupling uncoupled eNOS

Finally, a third target of oxidative stress is uncoupled eNOS, a process that involves oxidation of tetrahydrobiopterin (Reif *et al.*, 1999; Kotsonis *et al.*, 2000), an essential NOS cofactor, and accumulation of ADMA (Luo *et al.*, 2010), a competitive inhibitor at the enzyme's substrate binding site for L-arginine (Cardounel *et al.*, 2007). Uncoupling means that oxygen is activated but no longer transferred onto L-arginine. Thus, uncoupled eNOS releases ROS, similar to NADPH oxidases. Substitution or regeneration of tetrahydrobiopterin alone or in combination with supplementation of L-arginine can recouple eNOS and restore NO synthesis (Settergren *et al.*, 2009).

Viewpoint

In conclusion, there is now evidence for a direct pathomechanistic role of NADPH oxidase-dependent oxidative

stress causing disease, in particular in ischaemic stroke (Kleinschnitz *et al.*, 2010), hypertension (Matsuno *et al.*, 2005; Gavazzi *et al.*, 2006; Wind *et al.*, 2010a) and heart failure (Kuroda *et al.*, 2010). The alternative approach, to apply antioxidants, has failed and may even cause harm by leading to reductive stress. The detection of oxidative stress both in the experimental setting and as a clinical biomarker is a great challenge and will only become relevant when robust and meaningful measures (patterns, indices) become available. Specific detection of single ROS species (e.g. O_2^- vs. H_2O_2 or peroxynitrite) has limited relevance because in most cases these ROS interact and enter secondary reactions. More robust assays that cover a broad range of ROS (e.g. the DHE tissue stain, nitro-tyrosine detection, etc.) may provide more meaningful markers. The key is to establish cause–effect relations for oxidative stress, specific inhibitors of ROS sources and KO mice. Until these become available clinically, pharmacological principles that repair the consequences of oxidative stress, for example recouple eNOS and re-activate apo-sGC, will bridge this gap.

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Conflict of interest

H.H.H.W.S. declares that he holds shares in vasopharm GmbH (Würzburg, Germany), which pharmaceutically develops NADPH oxidase inhibitors. H.H.H.W.S. and K.W. are inventors of a patent on VAS2870 and VAS3947, which is owned by vasopharm GmbH (Würzburg, Germany). K.W. is a former employee of vasopharm GmbH (Würzburg, Germany). H.H.H.W.S. and K.W. receive funding from Servier, which develops S17834.

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