1. Introduction

1.1. Reactive oxygen and nitrogen species formation, redox biology and oxidative stress in health and disease

Excessive formation or insufficient break-down of reactive oxygen and nitrogen species (ROS and RNS) plays an important role for the development and progression of many diseases and drug-induced complications [1,2]. In the long run, this will lead to accumulation of oxidative damage in biomolecules and impaired cellular redox regulation, a condition that is termed oxidative stress [3,4]. Many cardiovascular, neurodegenerative, and inflammatory diseases as well as cancer have been demonstrated to be associated with or even triggered by oxidative stress [5–8]. The latter is a well-established hallmark of cardiovascular disease [9] as supported by phenotypic changes in animal models upon genetic manipulation of enzymes involved in the synthesis or detoxification of ROS and RNS [1,10,11].

These preclinical data and the resulting concept of the detrimental role of ROS and RNS stimulated large scale clinical studies to test the efficacy of antioxidants (namely vitamins C and E) by oral treatment of patients (e.g., HOPE, HOPE-TOO; for review see [1,12,13]). It was disappointing to realize that these antioxidant trials revealed in most cases no beneficial effects or even detrimental outcomes (e.g., for vitamin E) [12–16]. In contrast, several small cohort trials using in most cases acute (short-term) and high dose administration (e.g., via infusion) of antioxidants such as vitamin C demonstrated a highly beneficial action in various diseases including arterial hypertension, diabetes mellitus and in chronic smokers (reviewed in [12,17]).

Potential explanations for this obvious discrepancy could be that either the achievable dose of oral antioxidant therapy was not high enough (e.g., by insufficient delivery to sites of oxidative stress and/or non-competitive rate constants for the reaction of ROS and RNS with these classical antioxidants), that high concentration of these antioxidants per se may generate pro-oxidant tocopherol and ascorbyl radicals or that systemic therapy with non-specific antioxidants interferes with redox signaling pathways controlled by ROS and RNS (reviewed in [1,12,13]).

Therefore, more promising antioxidant strategies might comprise...
the use of antioxidants with tissue- or cell organelle-specificity (e.g. mitochondria-targeted compounds such as mitoQ) [18], activators of endogenous antioxidant pathways (e.g. Nrf2/HO-1) [19] or source specific inhibitors (e.g. for different Nox isoforms) [20], or even repair the resulting oxidative damage (e.g. activators and stimulators of oxidized soluble guanylyl cyclase) thus leaving important cellular redox signaling mechanisms intact [13,21].

The failure of classical antioxidants to fight successfully oxidative stress associated diseases stresses the point that we have to learn more about the sources of ROS and RNS, their interaction, discrimination between beneficial (physiological) redox signaling and detrimental (pathophysiological) oxidative damage pathways. Especially the development of new redox drugs requires specific knowledge of the reactive species being involved as well as spatial and temporal information on ROS and RNS formation. In the subsequent paragraphs we will discuss different approaches to identify and quantify ROS and RNS formation and their role in the pathophysiology of the various diseases.

1.2. Basic characterization of oxidative stress and redox processes for pharmacological, preclinical and clinical outcome studies

In order to establish that a certain disease is associated with excessive ROS and RNS formation (oxidative stress) it is not necessary to reveal the exact identity of these species and may even not require identification of the sources of ROS and RNS formation. In this case it will be sufficient to employ ROS and RNS detection assays like a redox biomarker, similar to the established footprints of nitro-oxidative stress including 3-nitrotyrosine- or 4-hydroxynonenal-positive proteins or 8-oxo-G-positive RNA, for the qualitative assessment of oxidative stress.

This approach can be used e.g. to screen for new drugs to treat a certain disease just by following the change of phenotype via assessment of oxidative stress parameters. As an example, one can detect vascular ROS and RNS formation by dihydroethidium (DHE)-dependent fluorescence microtopography in angiotensin-II infused, hypertensive animals, which is associated with a certain cardiovascular phenotype such as high blood pressure and endothelial dysfunction [22,23]. This ROS and RNS detection assay can then be used to test novel cardiovascular drugs for additional antioxidant pleiotropic effects e.g. in the regulation of specific genes being involved in redox homeostasis [22,23].

At this level of investigation it is not essential to know the exact identity of the reactive species being formed. It is just important to know whether the measured parameter and the phenotype of the investigated experimental animal are getting changed by drug therapy or the genetic manipulation of genes being involved in redox homeostasis. Of course it is important that the used assay is specific for oxidative stress detection. This can be verified for example by correlation studies between vascular function and other known markers of oxidative stress (e.g. 4-hydroxynonenal, 8-oxo-G) or other widely accepted ROS and RNS detection assays (e.g. HPLC-based quantification of 2-hydroxyethidium as a specific marker for superoxide formation). Also expected responses of the ROS and RNS detection assay to specific inhibitors (e.g. VAS2870 for Nox enzymes, PEG-SOD as a superoxide scavenger) or genetic manipulations of genes involved in redox homeostasis (e.g. deletion or overexpression of Nox and SOD isoforms) may foster the credibility of a particular assay.

If all of these controls were done and yielded the expected results, the assay could be criticized by others as being not specific for a certain reactive species or only generating observational, confirmatory results (although this concern can be disputed by using the appropriate inhibitors or genetically modified cells or animals), but not as leading to false-positive signals. Such observational/descriptive data on oxidative stress as a general biomarker can be followed-up by experts in redox biochemistry who have all the necessary tools at hand (e.g. cutting edge ROS and RNS detection assays) and can characterize the ROS and RNS generated in the respective animal models and cellular systems.

1.3. Advanced characterization of oxidative stress and redox processes for drug development and investigations of disease mechanisms

It is a different situation for drug development. There it is definitely necessary to know which ROS or RNS species is being formed and from which source, allowing to either develop the drug in the direction of a direct ROS or RNS scavenger with optimized reactivity for the identified reactive species (e.g. a superoxide dismutase mimetic for superoxide formation), or to inhibit the formation up-stream (e.g. by inhibition of a certain NADPH oxidase isoform). As an example for requirement of specific assays for drug development we would like to mention L-012 ECL, which was suggested as a useful tool for the determination of NADPH oxidase activity in whole blood or isolated leukocytes [24]. L-012 ECL was later used for screening for potential NADPH oxidase inhibitors in isolated immune cells but produced high numbers of false-positive hits. Later it turned out that the ECL signal in these assays mainly depends on peroxidase-dependent oxidation of the dye [25] and screening assays could produce positive hits with peroxidase inhibitors or any antioxidant that interfere with the redox cycle of the peroxidase (compound 0, I, II). Nevertheless, L-012 ECL in isolated leukocytes or even whole blood can be used for a first screening for NADPH oxidase inhibitors since besides the false-positive hits it will also identify the positive hits and thereby allow to minimize the number of drug candidates in the pipeline. Especially L-012 ECL in whole blood represents a high throughput screening method that detects the oxidative burst of leukocytes in response to phorbol esters and endotoxins, which is absent in whole blood of gp91phox deficient mice [26,27]. "Real" NADPH oxidase inhibitors must then be identified by subsequent more specific assays. For the elucidation of an exact disease mechanism it may be also recommended to obtain spatial and temporal information of ROS and RNS formation as possible with the emerging genetically encoded fluorescence reporter assays (e.g. HyPer) in isolated cells or other techniques for in vivo imaging (e.g. L-band EPR). However, these advanced methods are limited by other restrictions as discussed below.

2. Usefulness of traditional RONS detection assays

Given the wide distribution and obviously successful use of the "old", traditional assays for ROS and RNS detection, we will take the role of the devil's advocate and present some results elaborated by us and other groups. There are also a number of useful review articles published including guidelines how to use the traditional (and the new) assays for RONS detection, highlighting the specific pitfalls and drawbacks of them [28–32].

2.1. Dihydroethidium oxidative fluorescence microtopography (DHE cryo staining)

The initial description of the dihydroethidium oxidative fluorescence microtopography (DHE cryo staining) as a new assay to assess vascular ROS formation was published by Miller and colleagues using the atherosclerosis model Watanabe Heritable Hyperlipidemic (WHHL) rabbits [33]. The authors showed that ex vivo incubations of the cryo sections with polyethylene-glycolated superoxide dismutase (PEG-SOD) abolished the DHE fluorescence signal throughout the vascular wall and adenosinal transfection of the endothelial cell layer with Cu,Zn-SOD abolished the DHE fluorescence signal in the endothelial cell layer only. Based on these observations, Miller and colleagues concluded that DHE cryo staining detects vascular superoxide formation. It should be noted that DHE is not oxidized by ROS that "accumulated" before or during the storage but comes from de novo formation by active enzyme complexes (e.g. uncoupled eNOS or NADPH oxidases) even after freezing and thawing cycle for which we provide references below. Our group is using the DHE cryo staining
assay now for more than 15 years. One of the coauthors, Thomas Münzel, and David Harrison were pioneers in using this method (for review see [34]). Now we realize that the recent use of this assay provokes quite reproducible reviewer comments, secures special editorial attention and requires laborious control experiments when submitting a manuscript, despite the fact that we are not aware of any research report that provides direct disproof of the DHE cryo staining technique. The only specific recommendation we could detect in a scientific statement from the American Heart Association is that DHE microscopy should not be used in the absence of DNA (e.g. in platelets) since oxidized DHE products need to intercalate with the DNA for optimal fluorescence but in general no other alarming information regarding the DHE cryo staining method was found in this article, besides that DHE fluorescence in general, if not combined with HPLC, may be not specific for superoxide [35]. Therefore, we will here provide some results obtained with this assay supporting that this assay may be still a valid method to detect superoxide in vascular tissue.

Fig. 1. Detection of eNOS uncoupling in hypertensive animals (AT-II infusion model) by oxidative fluorescence microtopography. (A) To determine eNOS-dependent ROS formation, vessels were preincubated with the NOS inhibitor L-NAME (500 µM, lower panel), embedded in Tissue Tek resin, frozen, cryo-sectioned, and stained with DHE (1 µM) [23]. It should be noted that DHE does not react with "accumulated" ROS (most likely superoxide) in the cryo-sections, which would have been decomposed during storage, but DHE is oxidized by de novo formed ROS coming from uncoupled eNOS or NADPH oxidase after freezing and thawing. For detailed methodology see [10,22,36,59]. (B) Densitometric data are presented as bar graphs. (C) eNOS uncoupling was assessed by densitometric quantification of DHE staining in the endothelial cell layer which was extracted from the whole microscope image. A fixed area was used for densitometric quantification and the procedure is shown for one representative endothelial cell layer of AT-II treatment group. The method of densitometric quantification of endothelial DHE staining was adopted from a published protocol [68](A-C) Stainings were selected from unused pictures and graphs were drafted de novo from original data published in Schuhmacher et al., Hypertension 2010 [23]. Aortic endothelial DHE staining correlated well with endothelial dysfunction measured by ACh-dependent relaxation using isometric tension recording [62](D), impaired calcium ionophore-stimulated NO formation determined by EPR [62](E) and eNOS S-glutathionylation quantified by IP and Western blot analysis [63](F). (D-E) From Hausding et al., Basic Res. Cardiol. 2013 [62]. With permission of Springer-Verlag Berlin Heidelberg. Copyright © 2013. (F) From Kröller-Schön et al., Antioxid. Redox Signal. 2014 [63]. With permission of Mary Ann Liebert, Inc. Copyright © 2014. The scheme summarizes these positive correlates of eNOS uncoupling: endothelial dysfunction, impaired NO formation and eNOS S-glutathionylation as shown here, as well as increased asymmetric dimethyl-L-arginine (ADMA) levels, oxidative disruption of the zinc-sulfur-complex in the dimer binding interface of eNOS and oxidative depletion of tetrahydrobiopterin (BH4) as shown elsewhere (G). Adverse phosphorylation (Thr495, Tyr657) and S-nitros(yl)ation of eNOS were discussed to be involved in eNOS uncoupling but final evidence is still missing. All of these eNOS modifications and modulators of eNOS activity have been discussed in detail as potential redox switches leading to eNOS uncoupling or at least dysfunction [11,17,143].
tolerance in rat [38], mouse [39], rabbits [40] and human [41]; isosorbide-5-mononitrate-induced endothelial dysfunction [26]; cycloxygenase inhibitor-induced NADPH oxidase activation in rat [42]; atherosclerosis in Watanabe Heritable Hyperlipidemic (WHHL) rabbits [40,43], ApoE knockout [44] and high fat diet fed mice (Steven et al., in revision in Cardiovasc. Res.; hypertension in rat [22] and mouse [23,27]; sepsis in rat [45,46]; psoriasis in mouse [47]; congestive heart failure and alcohol cardiomyopathy in mouse [48]; aging process in mice [49]. We also applied DHE staining in various cell culture systems: hyperglycemic endothelial cells [50]; acetaldehyde challenged cardiomyocytes [51]; oxidative burst in phorbol ester stimulated human neutrophils [52]. These are only selected references since in total we have successfully used the DHE (cryo) staining assay in more than 70 original publications with all kinds of pharmacological interventions of genetic manipulation of redox pathways (for summary see [1,10,34]). The DHE (cryo) staining intensity always nicely correlated with the severity of the respective phenotype, was reduced by pharmacological therapy [22,23,36,43,45,48,53] or genetic deletion of the Nox subunit p47phox [54] and aggravated by genetelic deletion of protective antioxidant enzymes such as heme oxygenase-1, manganese superoxide dismutase or glutathione peroxidase-1 [49,55,56] and eliminated by ex vivo incubation with specific superoxide quenchers such as PEG-SOD [53].

It would be a quite surprising coincidence if in all these models, interventions and studies DHE (cryo) staining always would have identified the diseased group where higher oxidative stress levels were shown by other widely accepted methods for ROS and RNS detection, in particular since in all these models reduction in superoxide levels went along with an increase in vascular NO bioavailability as assessed by EPR measurements in mouse/rat aorta [57,58]. We would also like to stress that we routinely provided correlations with additional biomarkers of oxidative stress and more advanced and specific methods for ROS and RNS detection along with the DHE (cryo) staining data. We demonstrated that the DHE (cryo) staining signal is sensitive to pre-incubation with the NADPH oxidase inhibitor apocynin [59], VAS2870 [49] or PEG-SOD [60], whereas PEG-catalase had only minor effects [60]. Blockade of the DHE (cryo) staining signal by superoxide dismutase preparations was also shown by others [61]. According to our recent observations the DHE (cryo) signal is markedly increased in aorta from angiotensin-II infused wildtype mice but almost absent in aorta from angiotensin-II infused gp91phox deficient mice (Oelze et al. unpublished). In addition, experiments in mice with angiotensin-II infusion revealed that deletion of macrophages almost completely wiped out vascular superoxide signals as detected by DHE cryo staining, strongly reduced vascular NADPH oxidase activity, improved vascular (endothelial) function and normalized blood pressure, suggesting that the phagocytic and not the vascular NADPH oxidase is the key player in causing oxidative stress and adverse vascular phenotype in the vessel in response to angiotensin-II infusion [27].

Besides the reliability of the assay, it has some more features. DHE cryo staining is one of the rare assays that can be reliably used for detection of an uncoupled eNOS (for review see [10]). For this purpose only the DHE staining in the endothelial cell layer is quantified as shown in Fig. 1 and described previously [23]. As demonstrated in hypertensive mice, endothelial DHE staining of cryo-sectioned aorta was increased in response to angiotensin-II infusion, which was suppressed by the eNOS inhibitor L-NAME by blocking eNOS-derived superoxide formation. The DHE staining in the endothelium of control tissue is increased by L-NAME, most likely by blocking the function of intact eNOS thereby removing nitric oxide which is a sink for superoxide (due to the very fast reaction to peroxynitrite) and leaving more superoxide for detection. Of note, in our hands L-NAME did not significantly modulate the DHE staining intensity in the media or adventitia, underlining the specificity for NOS-derived superoxide. Importantly, endothelial DHE staining and the modulation by L-NAME nicely correlate with other markers of eNOS uncoupling and dysfunction, which are endothelial dysfunction and impaired NO bioavailability [49,62,63] as well as eNOS S-glutathionylation quantified by IP and Western blot analysis [26,38,49,63,64] (Fig. 1). eNOS S-glutathionylation is an established marker of eNOS uncoupling and eNOS-derived superoxide formation as demonstrated by Zweier and colleagues using electron paramagnetic resonance (EPR) spectroscopy and DHE staining [65].

Moreover, endothelial DHE staining inversely correlated with vascular tetrahydrobiopterin (BH4) levels in spontaneously hypertensive rats, which was rescued by inhibition of protein kinase C with midostaurin [66] or in diabetic rats, which was normalized by atorvastatin therapy [67]. Likewise endothelial DHE staining was increased and vascular BH4 levels were decreased in diabetic mice, all of which was normalized by transgenic overexpression of GTP-cyclohydrolase, the rate-limiting enzyme in BH4 synthesis [68]. As put forward by Zweier and colleagues the monomer/dimer ratio is a marker of eNOS uncoupling, which was accompanied by eNOS-derived superoxide formation as measured by SOD-inhibitable cytchrome c assay [69]. Thus, in our hands the eNOS monomer/dimer ratio was high in diabetic rats and normalized by atorvastatin therapy, which showed positive correlation with endothelial DHE staining [67]. Also asymmetric dimethylarginine (ADMA) levels in plasma were correlated with endothelial DHE staining in hypertensive mice, all of which was normalized by transgenic overexpression of dimethylarginine dimethylaminohydrolase (DDAH), the degrading enzyme of ADMA [70]. In addition, adverse phosphorylation of eNOS at Thr495 and Tyr657 was reported to be associated with increased eNOS S-glutathionylation, endothelial dysfunction and increased DHE staining in animal models of aging [49] and nitrate tolerance [38].

Besides these correlations with other markers of eNOS uncoupling, endothelial DHE staining in hypertensive mice is stereospecifically blocked by L-NAME but not by D-NAME, which is known to not affect eNOS activity [69]. The BH4 precursor sepiapterin corrects endothelial dysfunction and reduces DHE staining in aged glutathione peroxidase-1 deficient mice [49]. The effects of L-NAME on endothelial DHE staining also correlated with L-NAME modulation of other superoxide specific assays such as lucigenin ECL in intact aortic ring segments or HPLC-based quantification of 2-hydroxyethidium in cardiac tissue segments. Diabetic rats showed significant increase in aortic lucigenin ECL and an appreciable decrease by L-NAME, whereas lucigenin ECL was increased in aortic ring segments from control or telmisartan-treated diabetic rats when incubated with L-NAME [36]. L-NAME decreased the 2-hydroxyethidium signal in HPLC measurements in heart tissue from ApoE knockout mice but increased the signal in resveratrol treated mice, which correlated well with improved BH4/ BH2 levels in resveratrol treated ApoE knockout mice [71]. Furthermore, L-NAME increased the aortic 2-hydroxyethidium content in wild-type mice, whereas L-NAME decreased this indicator of aortic superoxide formation in angiotensin-II infused mice or Nox1 overexpressing mice [72]. Previous reports have shown that lucigenin ECL in aortic segments from nitrate tolerant rabbits largely depends on endothelial superoxide formation since denudation of the ring segments (removal of endothelial cell layer) decreased the lucigenin ECL signal [73]. Finally we would like to mention another important feature of the DHE cryo staining assay: It can be used to inform us about the topography of superoxide production within the vessel: endothelium vs. smooth muscle vs. adventitia. For example adventitial DHE cryo staining correlated well with markers of inflammation (CD68) and 3-nitrotyrosine [49]).

Several groups use the DHE (cryo) staining technique successfully. Zweier and colleagues, who are well experienced using EPR spectroscopy for detection of eNOS uncoupling, recently used endothelial DHE (cryo) staining in vascular biopsies of patients with obstructive sleep apnea before and after continuous positive airway pressure (CPAP), a non-pharmacological therapy for intermittent hypoxia during sleep
apnea [74]. The authors reported an improvement of flow-mediated dilation, a measure of endothelial function, in the patients after CPAP, which nicely correlated with increased nitric oxide bioavailability in the microvascular cryo sections (measured by a copper-based fluorescent probe, CuFL) and decreased endothelial superoxide production (measured by endothelial DHE staining). Beneficial effects of CPAP were mimicked by incubation of the cryo sections from sleep apnea patients with BH4, which decreased endothelial superoxide formation and increased nitric oxide levels more efficiently in the diseased group with almost no change upon CPAP. Likewise, incubation of the cryo sections from sleep apnea patients with L-NAME decreased endothelial superoxide formation and nitric oxide levels more efficiently in the diseased group with only minor changes upon CPAP. Laher and coworkers reported similar impairment of endothelial function (measured by acetylcholine-dependent relaxation) by intermittent hypoxia and diabetes with a dramatic aggravation when both risk factors were present [75]. These functional data were well correlated by a similar degree of eNOS uncoupling (measured by endothelial DHE cryo staining) intermittent hypoxia or diabetes groups with a substantial further increase in the presence of both risk factors. This pattern also correlated well with other parameters for oxidative stress, inflammation and eNOS dysfunction. Endothelial DHE staining was also increased in aortic cryo sections from animals with constantly high blood glucose levels and even higher acute blood glucose fluctuations, all of which correlated with vascular 8-isoprostaglandin levels, membranous p47phox content and malondialdehyde as well as 3-nitrotyrosine positive proteins [76].

In a recent review an excellent colocalization of vascular DHE staining and malondialdehyde as well as 3-nitrotyrosine positive proteins [76]. Lucigenin ECL was also reported for hindlimb tissues after ischemia and reperfusion [77]. In perivascular microvascular cryo sections (measured by a copper-based chemiluminescence probe for superoxide, although under certain conditions it may undergo redox cycling [86,87], e.g. in the absence of cells, the presence of oxidoreductases such as xanthine oxidase and NADH (the presence of hypoxanthine will largely prevent redox cycling by lucigenin) and high lucigenin concentrations (> 10 µM) as identified by EPR spectroscopy [24,34,88]. In isolated human neutrophils the oxidative burst is detected by lucigenin ECL and typical inhibitors of NADPH oxidase, inhibitors of NADPH oxidase activation (e.g. PKC inhibitors, calcium chelators) or SOD completely inhibit this signal and, importantly, HPLC-based measurement of 2-hydroxyethidium showed a comparable pattern regarding all inhibitors and stimulators employed [63]. Of note, the presence of lucigenin (5, 50, 100 and 250 µM) decreased the superoxide signal in phorbol ester-stimulated human neutrophils measured by HPLC-based determination of 2-hydroxyethidium (see suppl. Figure S2F in [63]) or EPR-based quantification of DFMPO-superoxide adduct [52]. Lucigenin can be successfully used for the detection of eNOS uncoupling in intact aortic ring segments in the presence and absence of L-NAME in hypertensive rats (see above) [36], hyperlipidemic rabbits [43] and diabetic rats [37]. Lucigenin ECL is also used for two decades to detect superoxide formation from NADPH oxidase in membrane fractions of aortic tissues [89,90] and even longer, since 1984, in particulate fractions of activated neutrophils [91]. After the initial discovery of superoxide producing NADPH oxidase in isolated leukocytes (oxidative burst) by Babior and colleagues (measured by cytochrome c reduction which was blocked by superoxide dismutase) [92], the same authors reported on a vital oxidative burst from particulate fractions (27,000 g preparations) from homogenates of zymosan-activated human neutrophils in the presence of NADPH (measured by cytochrome c reduction, prevented by superoxide dismutase), which was absent in particulate fractions from neutrophils from patients with chronic granulomatous disease [93,94]. Later it was shown that translocation of the Nox subunits p47phox and p67phox from the cytosol to the membrane is an essential step for detection of NADPH-triggered superoxide formation by particulate fractions of phorbol ester-stimulated human neutrophils, which again was absent in neutrophils from patients with chronic granulomatous disease [95], a process that is accompanied by Rac-2 translocation from the cytosol to the membrane [96]. The identity of the membrane component of NADPH Oxidase was then identified as cytochrome b558 and preparations of solubilized membrane fractions displayed NADPH-dependent superoxide formation (measured by cytochrome c reduction and inhibition by superoxide dismutase) [97], which was even reproducible in frozen liposomal and cytosolic samples (detailed personal communication with Edgar Pick). Detailed information on the stability and activity of NADPH oxidase complexes in liposomal and cytosolic preparations, even after freezing and thawing, is provided in a state-of-the-art review by Edgar Pick on “Cell-Free NADPH Oxidase Activation Assays: “In Vitro Veritas”” [98]. Likewise, NADPH-dependent superoxide formation was measured by EPR in membrane fractions from frozen renal transplant tissues of patients with allograft rejection and ischemic kidney damage [99].

Since that time the measurement of NADPH oxidase activity by lucigenin ECL is also well established in membrane fractions of aortic tissue e.g. from hypertensive and nitrate tolerant rats [89,90], being confirmed by EPR spectroscopy in angiotensin-II stimulated rat smooth muscle cells [100]. We used the method for measuring NADPH oxidase activity in cardiac membrane fractions and reported increased activity in heart membranes from hypertensive rats associated with increased membrane content of p67phox and Rac-1, all of which was inhibited by the 3rd generation beta-blocker nebivolol (Fig. 2) [22]. Also in cardiac membrane fractions of diabetic rats we observed increased NADPH-stimulated lucigenin ECL signals, which was associated with increased membrane content of p47phox, p67phox and Rac-1 and increased total cardiac superoxide formation as measured by HPLC-based quantification of 2-hydroxyethidium, all of which was normalized by therapy with the AT1-receptor-blocking telmisartan [36].

We also established that increased cardiac membrane NADPH oxidase activity in diabetic animals was normalized by the organic nitrate pentaeethriyl tetranitrate with antioxidant properties, which was positively correlated with reductions in cardiac 3-nitrotyrosine and malondialdehyde content [64]. In hypertensive mice cardiac membrane NADPH oxidase activity was increased as measured by NADPH-stimulated lucigenin ECL signals and confirmed by HPLC-based
quantification of 2-hydroxyethidium in the same membrane fractions [23]. These are just a few examples from our previous studies performed within the last 2 decades. Importantly, membrane fractions did not yield an appreciable chemiluminescence signal when NADH was used as a stimulus, compatible with a superoxide signal from NADPH oxidase. We also emphasize that the membrane fractions prepared by our centrifugation technique do not contain mitochondria or larger fragments of broken mitochondria since mitochondrial constituents were removed by an additional 20,000 g centrifugation step followed by a 100,000 g centrifugation of the supernatant to obtain the membrane fractions. Thus, this membrane preparation technique is clearly different to the widely used method to prepare particulate fractions that were generated by removal of cell nuclei and debris followed by a 27,000 g to 60,000 g centrifugation step. These particulate fractions likely contain mitochondria and broken mitochondria, which may account for the reported NADH signals (reviewed in [101]). The contamination by mitochondrial constituents may not interfere with the assay in particulate fractions from NADPH oxidase rich homogenates (e.g. neutrophils and other phagocytes) but may generate worrisome signals in mitochondria rich tissues (e.g. heart or liver).

Concerning the above described observation of increased NADPH oxidase activity in membrane fractions of hypertensive rats and normalization by nebivolol in vivo therapy [22], we would like to present also in vitro experimental data supporting the usefulness of lucigenin ECL assays in cardiac membrane fractions. The NADPH-stimulated lucigenin (5 µM) ECL signal in cardiac membrane fractions from hypertensive rats (angiotensin-II infusion model) was concentration-dependently decreased by in vitro incubation with the novel highly selective beta1-receptor blocker nebivolol and a third generation beta-blocker with additional alpha adrenoceptor antagonizing capacities, carvedilol, whereas older beta-blockers such as atenolol and metoprolol had no significant effects on superoxide signals (Fig. 2) [22]. In response to incubation with these beta-blockers and first measurement of NADPH oxidase activity by lucigenin ECL, the same membrane preparations were subjected to another ultracentrifugation step after incubation with the beta-blockers. The cytosolic fraction (supernatant) was discarded after the 100,000 g centrifugation step and the membrane pellet was used for Western blotting. It turned out that nebivolol and carvedilol that suppressed the lucigenin ECL signal had partially dislocated the regulatory subunits p67phox and Rac-1 (D). The detailed in vitro protocol is provided in (E). All details on the membrane NADPH oxidase assay are provided in [22]. Graphs were drafted de novo from original data published in Oelze et al., Hypertension 2006 [22].

![Fig. 2. Detection of NADPH oxidase activity in hypertensive animals (AT-II infusion model) by lucigenin enhanced chemiluminescence (ECL) in cardiac membrane fractions.](image_url)
together with Noxa1 and Noxo1 [22]. The potent NADPH oxidase inhibitory properties of nebulovol were also confirmed in endothox- or phorbol ester-stimulated human leukocytes (measured by L-012 ECL and DEPMPO EPR spectroscopy) [43] as well as whole blood oxidative burst (measured by L-012 ECL) [45,52].

Among the numerous studies using the NADPH oxidase assay in membrane or particulate fractions we want to present a selection of some important examples. Sorescu and coworkers observed NADPH-stimulated DEPMPO-superoxide signals by EPR spectroscopy in particulate fractions of vascular smooth muscle cells [102]. NADH yielded lower signals and the eNOS inhibitor L-NNA as well as modulators of mitochondrial ROS formation had no effect on the signals. The authors of the study also showed that only the NADPH but not the NADH-stimulated EPR signal in these membrane fractions is sensitive to the inhibitor of Flavin-dependent oxidoreductases diphenyl iodonium [102]. Finally the lucigenin ECL signal (at 5 and 50 µM) was more pronounced with NADPH, whereas the signal obtained with 500 µM lucigenin was higher with NADH mainly because of the redox cycling issue. Dikalov and colleagues measured NADPH-stimulated 3-carboxyproxyl (CP)-radical signals by EPR spectroscopy in particulate fractions of vascular smooth muscle cells [100]. The signal was suppressed by SOD, increased by prior angiotensin-II incubation of the cells and normalized by siRNA against Nox1 but not Nox4. The authors also quantified NADPH-dependent hydrogen peroxide formation in these particulate fractions by peroxidase–acetamidophenol–mediated cooxidation of CPH, which was suppressed in the presence of catalase. Again, angiotensin-II pretreatment of the cells increased the H$_2$O$_2$ signal and was abolished by siRNA against Nox1 and also Nox4. Guzik and coworkers determined NADPH-triggered hydrogen peroxide generation in human coronary membrane fractions by peroxidase–acetamidophenol–mediated cooxidation 1-hydroxy-4-phosphono-oxy-2,2,2,6 tetraethylpiperidine (TEMPO), which was sensitive to calcium [103]. This NADPH/calcium-stimulated H$_2$O$_2$ signal was substantially increased in membrane fractions from coronary artery disease patients, which also contained high amounts of Nox5 protein. In cultured human endothelial cells siRNA against Nox5 largely suppressed the NADPH/calcium-stimulated H$_2$O$_2$ signal. Lee and coworkers reported on NADPH-dependent and SOD-inhibitable superoxide signals (measured by CPH EPR) in membrane fractions of vascular smooth muscle cells [104]. The platelet-derived growth factor stimulated signal was significantly decreased in membrane fractions of cells from Nox1 deficient mice and increased when Nox1 overexpressing cells were used. Dikalova and colleagues observed increased NADPH-dependent and SOD-inhibitable superoxide signals (measured by CPH EPR) in aortic membrane fractions from Nox1 overexpressing mice [72]. Angiotensin-II infusion of mice further increased the superoxide signal in the membrane fractions of wildtype or Nox1 overexpressing mice.

The reason why we highlight the previous use of the NADPH oxidase membrane assays in general and the lucigenin ECL-based NADPH oxidase membrane assays in particular is that their use is more and more under criticism by reviewers and editors. The use of these assays in future studies is further complicated by a recent report by Zielonka and colleagues in a letter to the editor [107] (for rebuttal letter by the authors see [108]), their results leave us with substantial concerns regarding an assay we have used since more than 20 years. Clarification of the credibility of the NADPH-triggered lucigenin ECL assay in membrane fractions requires further detailed investigations in the well characterized angiotensin-II infusion model (e.g. the importance of the presence of dithiothreitol or other reducing agents during the homogenization and centrifugation steps, the buffer that is used for the lucigenin ECL assay since HEPES and Tris have been reported to interfere with reactive oxygen and nitrogen species detection, the use of superoxide scavengers, inhibitors for NADPH oxidase activity, eNOS and P450 enzymes, studies with membrane fractions from gp91phox and eNOS knockout mice as well as confirmation of lucigenin ECL signals by other assays such as HPLC-based 2-hydroxyethylidium measurements or EPR-based techniques). In addition, we will compare the results of the lucigenin ECL assay with other established markers of oxidative stress such as 4-hydroxynonenal or 8-oxo-dG, and most importantly provide data on the membrane content of the cytosolic regulatory subunits of Nox2 (Nox1), p47$^{phox}$, p67$^{phox}$ and Rac-1 (in our opinion an essential proof for the successful generation of functional NADPH oxidase in membrane fractions), all of which was not included in the initial work by Rezende and coworkers [105].

2.3. L-012-based whole blood chemiluminescence assay (L-012 oxidative burst)

Our last example addresses the L-012 ECL assay in whole blood that we have originally reported as a NADPH oxidase assay [24]. Initially, L-012 was proposed as a specific probe for cellular superoxide formation [109,110] and was shown to detect ROS from activated neutrophils [111]. We and others have demonstrated that L-012 more likely detects peroxynitrite since superoxide-derived ECL was much less pronounced as compared to signals generated with authentic peroxynitrite or simultaneously generated nitric oxide and superoxide [24,52]. Based on these initial reports L-012 ECL was used by others for screening for potential NADPH oxidase inhibitors produced high numbers of false-positive hits. As already mentioned above Zielonka and colleagues have demonstrated peroxidase-dependent oxidation of the dye and superoxide formation as a by-product of redox cycling explaining suppression of L-012 signals by SOD [25]. Nevertheless, L-012 ECL in isolated leukocytes or even whole blood can be used for measuring endotoxin or phorbol ester-stimulated oxidative burst, which largely depends on NADPH oxidase derived hydrogen peroxide formation, as proven by the absence of the L-012 ECL signal in whole blood of gp91$^{phox}$ deficient mice [26,27].

As for the other “old”, traditional assays we will present some examples for the in our hands successful use of the L-012 ECL signal. We employed the L-012 ECL assay for measuring mitochondrial peroxynitrite formation in nitrate tolerance induced by nitroglycerin in rats [39,112–117] or mice [56,118,119]. In these publications L-012 ECL signals correlated well with 3-nitrotyrosine levels in mitochondria of various tissues upon in vitro and in vivo nitroglycerin treatment,
with ROS and RNS detection by other assays such as HPLC-based 2-hydroxyethidium measurement or dihydrorhodamine fluorescence, inhibition of the redox-sensitive enzyme mitochondrial aldehyde dehydrogenase (ALDH-2) and other markers of oxidative stress such as products of lipid peroxidation or 8-oxo-G and was improved by various antioxidant therapies (reviewed in [120]). We also repeatedly used L-012 ECL for determination of whole blood oxidative burst in diabetic rats [53,121], nitrate-tolerant rats [39,122], hypertensive mice [27,63] and septic rats [45,46]. In these studies L-012 ECL signals perfectly correlated with 3-nitrotyrosine, malondialdehyde or 4-hydroxynonenal levels in serum, plasma and various tissues, all of which was normalized by various antioxidant therapies and NAPDH oxidase inhibitors such as VAS2870 or apocynin as well as calcium chelators such as BAPTA-AM. As already mentioned above, the signal was also absent in whole blood of gp91phox deficient mice with arterial hypertension or isosorbide-5-mononitrate-induced nitrate tolerance [26,27].

In some studies the oxidative burst signal was also measured by other techniques such as amplex red or luminol / peroxidase, HPLC-based 2-hydroxyethidium and EPR-based spin trapping assays. Finally, we also employed the L-012 ECL assay in human blood samples of patients with peripheral arterial occlusive disease (PAOD) displaying a proinflammatory phenotype of their immune cells that also determined the stage of the disease, intermittent claudication or critical limb ischemia [123]. The soluble form of the triggering receptor expressed on myeloid cells-1 (sTREM-1), a parameter for the activation state of certain immune cells, correlated well with the L-012 ECL signal, all of which inversely correlated with the walking distance of the PAOD patients (more or less than 300 m) and also characterized those patients with critical limb ischemia, the end stage of the disease [124,125]. Home-based exercise not only improved the pain-free walking distance in the PAOD patients but also decreased the pro-inflammatory phenotype and ROS levels in whole blood as measured by the L-012 ECL assay [125]. The beneficial effects of physical activity in PAOD patients was also supported by a follow-up study revealing that exercise training influences the distribution and levels of circulating angiogenic cells and proangiogenic Tie-2 expressing monocytes [126]. Non-supervised exercise training, although still improving the walking distance, was less effective in the influence of proangiogenic cells and inflammatory burden than supervised exercise training.

Here, we would like to present one specific example for the use of the L-012 ECL assay in septic or more precisely endotoxic rats in more detail [46]. Oxidative burst was measured by the L-012 ECL assay in whole blood of endotoxic rats as compared to healthy animals, which was normalized by dipeptidyl peptidase-4 (DPP-4) inhibition by linagliptin or glucagon-like peptide-1 (GLP-1) supplementation by liraglutide (Fig. 3A). The signal pattern of oxidative burst was mirrored by EPR-based quantification of nitrosyl-iron hemoglobin (Hb-NO) in whole blood, a surrogate parameter for inducible nitric oxide synthase (iNOS) activity (Fig. 3B). The formation of superoxide by phagocytic NAPDH oxidase during oxidative burst and nitric oxide by inducible nitric oxide synthase in whole blood of endotoxic animals implies the generation of peroxynitrite that we detected by its footprint 3-nitrotyrosine-positive proteins in plasma (Fig. 3C). This observation was in good accordance with the fact that peroxynitrite formation in immune cells is a response to invading pathogens [127]. Increased peroxynitrite formation and/or hydrogen peroxide formation/peroxidase activity was also traced by whole body in vivo imaging upon L-012 injection using a bioluminometer (IVIS Spectrum Imager, PerkinElmer, Waltham, MA, USA), which especially increased the ECL signal in the intestine and gut of lipopolysaccharide treated mice (Fig. 3D). Since these interconnected parameters also predicted the beneficial effects of the mentioned drugs on the prognosis and survival of endotoxic mice (Fig. 3E), we are convinced that the L-012 ECL assay in whole blood can be used as a diagnostic as well as a prognostic parameter in various disease models or even patients. Linagliptin and liraglutide therapy as well as genetic dipeptidyl peptidase-4 deficiency significantly improved the survival of endotoxic mice.

3. State-of-the-art and cutting-edge ROS and RNS detection assays – advantages, weaknesses and limitations

Oxidative stress is a major trigger of endothelial dysfunction and manifest cardiovascular disease [17]. Therefore, the precise determination of reactive oxygen and nitrogen species formation is of great importance for the evaluation of disease mechanisms and potential therapeutic drug targets. A major determinant of vascular dysfunction and cardiovascular disease development decreased vascular "NO bioavailability as a consequence of increased nitric oxide ("NO inactivation by the free radical superoxide (O2-) leading to the formation of the highly reactive intermediate peroxynitrite (ONOO-). Superoxide and peroxynitrite formation correlate positively with the degree of vascular dysfunction and the severity of cardiovascular disease, whereas vascular nitric oxide bioavailability correlates negatively with these parameters. Therefore, the measurement of these reactive oxygen and nitrogen species, nitric oxide, superoxide and peroxynitrite, is of outmost importance for the characterization of the underlying oxidative stress pathophysiology of cardiovascular disease. The first evidence for superoxide being an antagonist of nitric oxide (formerly known as the endothelium-derived relaxing factor = EDRF) was provided by Gryglewski and coworkers (Fig. 4) [128]. These authors transferred "NO from bradykinin-stimulated cultured endothelial cells ("NO containing perfusate) to an organ bath with endothelium-denuded aortic rings. They observed that the longer the distance between the endothelial cell culture and the organ bath was, the more inactivation of "NO (EDRF) was evident. This inactivation was prevented by addition of superoxide dismutase to the cell culture suggesting that superoxide is a direct antagonist of "NO. The reaction of "NO and superoxide yields peroxynitrite, a toxic, highly reactive biological oxidant [129–131], with important meaning for the initiation and perpetuation of cardiovascular and neurodegenerative disease, e.g. by selective oxidative damage of cellular structures such as the inactivation of MnSOD by a selective nitration mechanism [132]. Patients with established coronary artery disease and higher burden of oxidative stress (assessed via improvement of endothelial dysfunction by vitamin C) have a worse prognosis and are at higher risk for cardiovascular events [133]. In addition, in animals, endothelial (vascular) function correlates well with mitochondrial and overall vascular reactive oxygen species formation during the aging process [49,134] and NAPDH oxidase activity in the setting of type 1 diabetes mellitus [64].

A major draw-back in oxidative stress research is that many established and frequently used assays for the detection of reactive oxygen and nitrogen species in biological samples (mainly chemiluminescence-based assays) were almost all controversially discussed during the last decade. E.g. the lucigenin-based assay has been accused to cause redox cycling on its own and should be not used because of generating artificially high signals [87], although this is not always the case and clearly depends on the lucigenin concentration (250 vs. 5 µM) [24]. Especially by using lower lucigenin concentrations there is a close correlation between lucigenin signals and superoxide production assessed by EPR measurements. L-012-based screening for NAPDH oxidase activity was discarded due to redox cycling in the presence of peroxidases [25], although in our hands it seems well suited for oxidative burst measurements of leukocytes in whole blood [63]. In addition, recently, the lucigenin-based NAPDH oxidase assay in cardiac membrane fractions was called into question and it was reported that this assay does not measure NAPDH oxidase activity since one observed unchanged NAPDH oxidase activity in Nox1-Nox2-Nox4 triple knockout mice [105].

These examples illustrate that accurate, state-of-the-art assays for the detection and quantification of reactive oxygen and nitrogen species in biological samples are of outmost importance, although we believe that the traditional chemiluminescence and fluorescence assays have a
Fig. 3. Superoxide, nitric oxide and peroxynitrite formation in lipopolysaccharide (LPS)-induced endotoxic shock. L-012 enhanced chemiluminescence (ECL) was used for phorbol ester (PDBu)-triggered oxidative burst measurement in whole blood (A), which mainly originates from NADPH oxidase-derived superoxide in granulocytes or more correctly its degradation product hydrogen peroxide in the presence of peroxidases, as known for all other luminol derivatives [25]. Since L-012 also generates appreciable chemiluminescence upon reaction with peroxynitrite, this species could contribute to the overall chemiluminescence signal as well [24]. The L-012 signal nicely correlates with nitrosyl-iron hemoglobin (Hb-NO) levels (B), a surrogate parameter of inducible nitric oxide synthase (iNOS) activity in leukocytes, as measured by electron paramagnetic resonance (EPR).
spectroscopy in whole blood of LPS-treated rats [46]. Representative EPR spectra are shown below the quantification bar graph. Both together, Nox-derived superoxide burst and INOS-derived nitric oxide, yield peroxynitrite, which in turn leads to protein tyrosine nitration as detected by dot blot analysis using a specific antibody against protein-bound 3-nitrotyrosine (C). Alternatively, nitrate markers can also originate from a (myelo)peroxidase/H2O2/nitrite-dependent pathway, which largely depends on the nature of invading pathogens [163]. Likewise, L-012 ECL can be also used for the detection of oxidative burst (and thereby inflamed tissues) in living animals using in vivo luminescence imaging devices (D; Steven et al. unpublished; L-012 dose in mice: 100 mg/kg injected i.p. in 200 µl DMSO 5 min before sacrifice, then 10 min illumination and signal recorded with an IVIS Spectrum Imager, PerkinElmer, Waltham, MA, USA). All of these parameters nicely reflected the severe inflammatory phenotype induced by LPS treatment and the beneficial anti-inflammatory and antioxidant effects of the dipeptidyl peptidase 4 inhibitors (DPP4i) linagliptin (Lina) and sitagliptin (Sita) as well as the glucagon-like peptide 1 analogue (GLP1a) lixagliptin (Lira) [46]. L-012 ECL also had prognostic value in LPS-triggered endotoxic shock as demonstrated by the survival curves (E) [46]. From Steven et al., Basic Res. Cardiol. 2015 [46]. With permission of Springer-Verlag Berlin Heidelberg. Copyright © 2015.

Especially, HPLC-based detection of DHE oxidation products is powerful since the separation of the 2-hydroxyethidium products allows specific quantification of superoxide formation. We used electron paramagnetic resonance (EPR) spectroscopy for the detection of 'NO formation in biological samples for more than 2 decades [57]. So far, this technique is the most specific detection method for whole blood and aortic 'NO formation [46,63]. However, also these previously gold-standard methods need to be updated from time to time with the latest available devices (e.g. much more sensitive electrochemical detection of the DHE products) or new DHE analogs allowing site-specific detection of superoxide (e.g. mito-SOX for mitochondrial superoxide and hydropropidine for extracellular superoxide) or the most advanced new spin traps providing higher stability in biological samples and thereby allow the EPR-based detection of superoxide and/or peroxynitrite-derived free radicals in cells and tissues (some candidates might be found in [32,135–137]).

High performance liquid chromatography (HPLC)-based detection of superoxide formation in the extracellular space, cytosol and mitochondria can be established by hydropropidine, a positively-charged water-soluble analogue of dihydroethidium (DHE) for extracellular superoxide formation [138], DHE for cytosolic superoxide formation and mitoSOX, a mitochondria-targeted DHE analogue for mitochondrial superoxide formation (Fig. 4) [138–140]. HPLC-based assay for the detection of peroxynitrite (e.g. by detection of nitrophenyl products) and protein-bound hydroperoxides (e.g. by conversion of coumarin boronic acid to its hydroxyl product) can be established by...
using specific boronate probes [30]. Also fluorescein boronate probe
was reported as a suitable assay for quantification of cell-derived
peroxynitrite in endothelial cells and parasite-activated macrophages
[141]. Alternatively, our own new assay based on the conversion of
salicylaldehyde to 2-nitrophenol can be used for the detection of
peroxynitrite in biological samples (in order to obtain the necessary
sensitivity it may be required to use mass spectrometry for product
quantification) [142]. HPLC-based detection of extracellular hydrogen
peroxide from cells and tissues can be established by the conversion of
Amplex® red to resorufin in the presence of horseradish peroxidase
(HRP), as already used in our group for isolated aortic ring segments
and isolated immune cells [63]. The knowledge of superoxide forma-
tion in each compartment is essential, not only since subcellular
sources contribute differently to various disease but there is also a
vital crosstalk between reactive oxygen species from different subcell-
ular sources [143]. Upon successful set-up of HPLC-based assays,
these methods can be transferred to fluorescence plate reader-based
detection of hydrogen peroxide by Amplex® red/horseradish perox-
idase (AR/HRP), peroxynitrite by coumarin-7-boronic acid (CBA),
oxidative nitrosation by diaminofluorescein or diaminohorodamine
(DAF, DAR)-based probes [144,145] and superoxide by DHE and
analogues [146] in order to establish high throughput techniques.

Electron spin resonance (EPR)-based detection of nitric oxide by
EPR methods (e.g. nitric oxide formation in tissues and cells by
nitrosyl-Fe(DETC)2 spin trapping or in whole blood by nitrosyl-iron
hemoglobin, Hb-NO) is the gold standard for nitric oxide detection in
biological samples [46,62,63]. In addition, whole blood Hb-NO and
aortic NO-Fe(DETC)2 EPR spectroscopy for 15N-NO, and discrimina-
tion between endogenous sources for nitric oxide synthesis (15N-NO,
triplet signal) and nitric oxide synthesis by exogenous delivery of 15N-
labeled substrates (e.g. 15N-L-arginine or 15N-nitrite/nitrate) for nitric
oxide synthesis (15N-NO, doublet signal) may provide important
mechanistic insights [147,148]. However, the use of fluorescent probes
for the time-dependent and location-specific formation of nitric oxide
might be straightforward, although these dyes need proper character-
ization in various biological systems to be sure about their specificity.
For example HPLC-based detection of nitric oxide formation by
diaminofluorescein-diacetate (DAF-FM DA) represents an important
tool to assess nitric oxide formation in different cells and tissues,
that can be combined with fluorescence microscopy and even in vivo
imaging techniques [149]. Also the use of a copper-based fluorescent
probe (CuFL) seems to be an attractive approach for in vivo imaging
of nitric oxide formation [150,151]. Currently new spin traps for super-
oxide with higher sensitivity and stability in biological samples, e.g. in
the presence of antioxidants, than currently used methods that
are based on the classical spin traps DMPO, DEPMPO or spin probes CP-
H, CM-H (although the spin probes provide no superior information
over HPLC-based superoxide detection with DHE since they lack
specificity for a certain reactive species), are evaluated with respect
to their use in biological samples. The use of classical spin traps such as
DMPO or DEPMPO in biological samples is limited by their instability
in the presence of cellular antioxidants but they are suitable for
detection of superoxide in isolated immune cells [63]. Candidates for
new spin traps are Mito-DIPPMPO and Mito10-DEPMPO for mito-
ochondrial superoxide formation [136] or FDMPO, DPPMPO,
DBPMPO, DEHPMPO and DIPPMPO with superior half-life of HOO-
and HO-spin adducts in biological samples [152].

The cutting edge and future techniques comprise ROS-induced
formation of microbubbles that are detected by ultrasound and can
be used in living animals or even humans (reviewed in [17]). A study in
mice used ultrasound to measure the reaction of ROS with liposome-
encapsulated allylhydrazine, a liquid compound, that yields nitrogen
and propylene gas that is detected by ultrasound methods [153]. Also
L-band electron paramagnetic resonance (EPR) spectroscopy in com-
bination with the most advanced spin traps represents a cutting edge
technique for the in vivo detection of ROS and RNS (reviewed in [17]).

Combined with the respective spin trap, these techniques have the
potential for the specific detection of vascular ROS and RNS formation
in isolated tissues and whole animals [135,154]. Also combination of
different techniques such as immuno-spin trapping as reported for
trapping of thyl radical in proteins or other radicals in DNA by DMPO
and formation of stable spin-adducts with subsequent Western blotting
against DMPO-bound proteins or DNA molecules are already used for
the detection of ROS and RNS formation, or more precisely their
footprints in biological samples [155]. Delivery of chemiluminescent or
fluorescent nanoparticles or nanoparticles loaded with specific ROS
and RNS detection probes might provide the next step in oxidative
stress and redox biology research (reviewed in [155] and for drug
delivery in [156]). Also the delivery of specific dyes and/or ROS and RNS
scavengers bound to specific antibodies, as already done for site-
specific drug delivery [17,157], in order to target specific cellular
structures, organelles or sites of inflammation might be a strategy that
will become relevant in the future. Genetically encoded fluorescent
enzymatic probes (e.g. HyPer for hydrogen peroxide detection) for the
highly specific and timely as well as spatially resolved monitoring of
ROS and RNS formation represent one of the most advanced cutting
edge tools that is already used in daily routine in isolated cells
[158,159]. Likewise, combination of redox biosensors like glutaredox-
in-1 with reporter enzymes like green fluorescent protein in constructs,
that can be genetically encoded by transfection to specific cells, organs
or even whole animals, allow sensing of intracellular redox state, even
in different cellular compartments (by targeting to specific organelles)
and provide the basis for the development of new techniques like “redox histology” [160]. Major limitations of all cutting-edge (future)
assays may be that they require the most advanced spin traps, fluorescent dyes, constructs for transfection of cells that are not
available for a broad scientific community. Moreover, some of these
assays require large and expensive instrumentation and/or highly
sophisticated techniques that are not available in all laboratories and
cannot be applied in the daily routine. Finally, some of these assays
work well in model systems but are not yet validated in a broad range
of biological samples (e.g. tissues) and for some spin traps and dyes the
stability and specificity are not yet characterized in various biological
samples (e.g. some fluorescent flash probes that were routinely used
for cellular ROS detection showed substantial dependence on the pH [161]
and accordingly could lead to large deviations when used in different
tissues or cellular compartments with different pH).

4. Conclusions and future strategies

According to the recent recommendations in the guidelines of the
American Heart Association none of the above described assays is
among the first or second line assays recommended for the detection
of ROS and RNS in cardiovascular tissues [35], although still widely used
by a broad community. With the present review we want to present
evidence that the “old”, traditional assays may still be used for the
detection of ROS and RNS in cardiovascular samples, when either
controlled by additional assays and/or redox biomarkers, or by
appropriate inhibitors of ROS and RNS sources or scavengers of these
species. With the present review we want to stimulate a scientific
discussion concerning these assays. The discussion resembles pretty
much the previous discussion concerning the validity of the lucigenin
assay and redox cycling issues appearing when used in higher
concentrations. Our group will start to compare the traditional assays
in specific animal models that are well characterized with state-of-the-
art techniques as well as accepted redox biomarkers since we consider
it important to have definite proof on whether the traditional assays
have a meaning or not. All of this empiric data at least warrant that the
traditional assays are properly refuted or confirmed by state-of-the-art
techniques. We think they have a meaning since there is too much
correlation between results generated with these assays and disease
phenotypes, prognosis of animals and probably also patients.
Conflict of interest

None.

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