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Review

Electron paramagnetic resonance (EPR) spin trapping of biological nitric oxide☆

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Abstract

Nitric oxide (NO) is a free radical species with multiple physiological functions. Because of low concentrations and short half-life of NO, its direct measurement in living tissues remains a difficult task. Electron paramagnetic resonance (EPR) spin trapping is probably one of the best suitable platforms for development of new methods for quantification of biological NO. The most reliable EPR-based approaches developed so far are based on the reaction of NO with various iron complexes, both intrinsic and exogenously applied. This review is focused on the current state and perspectives of EPR spin trapping for experimental and clinical NO biology.

Keywords: Reviews; Nitrosyl–iron complexes; Electron paramagnetic resonance

Contents

1. Introduction

Nitric oxide (NO) is synthesized from L-arginine by a family of enzymes called nitric oxide synthases (NOS): neuronal NOS (NOS I), inducible NOS (NOS II) and endothelial NOS (NOS III) [1,2]. There is a general consensus that NO has important and

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diverse physiological functions, but how this complex activity is coordinated in vivo, remains poorly understood [3]. Therefore, development of reliable analytical methods for the monitoring of NO levels in living tissues is an important strategy of current NO biology. In this regard, electron paramagnetic resonance (EPR) spin trapping appears to be of particular interest because it allows direct detection of free radicals in intact biological systems [4]. This review is focused on the current state and perspectives of the EPR spin trapping approach for detection and quantification of both free NO and some important iron–NO derivatives in the living systems (cultured cells and experimental animals so far).

2. Basic principles of EPR spin trapping

The methodology of EPR spin trapping was introduced by E.G. Janzen about 30 years ago (for review see ref. [5]). EPR spin trapping can be defined as a process whereby short-lived radical species are intercepted by a special compound called a spin trap resulting in stabilization of the radical for sufficient time to be detected/characterized by EPR spectroscopy. The reaction principle of spin trapping is as follows:

$$R^* + ST \rightarrow R\text{-}SA^* \quad (1)$$

where R^* is a radical intermediate, ST is the spin trap and R-SA^* is a radical spin trap adduct. EPR spin trapping can provide specific information on the radicals generated both in chemical and biological situations. As a general rule, the amount of spin adduct formed is proportional to the intensity of the EPR signal (in contrast to photometry, there is no extinction coefficient). EPR spectroscopy is a method, first discovered in 1944 by E.K. Zavoiski, that employs an external magnetic field simultaneously with microwave irradiation (conventionally being 9 GHz, X-band). A characteristic feature of EPR is that one can use non-transparent and non-modified samples. Because free radicals contain unpaired electrons, which behave like small magnets and thereby interact with the nucleus and electrons of neighboring atoms, they can be unequivocally characterized by EPR. The general principle of EPR (Fig. 1) can be described by the formula:

$$\Delta E = h\upsilon = g\beta H \quad (2)$$

where ΔE is the energy of resonant absorption, h is Planck's constant, υ is microwave frequency, β is Bohr magneton, a constant related to electron charge and mass (equal to $4.66858\,cm^{-1}\,G^{-1}$), H is magnetic field at which resonance occur, and g is a spectroscopic factor (tensor) which is a characteristic of a given paramagnetic center. The g factor can provide important information on the electron density distribution and geometry of the paramagnetic system. Accordingly, there are three principal g factors: g_x, g_y and g_z. When $g_x = g_y = g_z$, the signal is called isotropic and indicative of the spherical symmetry (characteristic for organic radicals or rapidly rotating molecules in solution). When $g_x = g_y$ $(=g_\perp) \neq g_z$ $(=g_{||})$, the system is of axial symmetry, and when $g_x \neq g_y \neq g_z$, the system is of rhombic symmetry. Other important EPR parameters are the shape and width of the spectral line as well as the hyperfine and super-

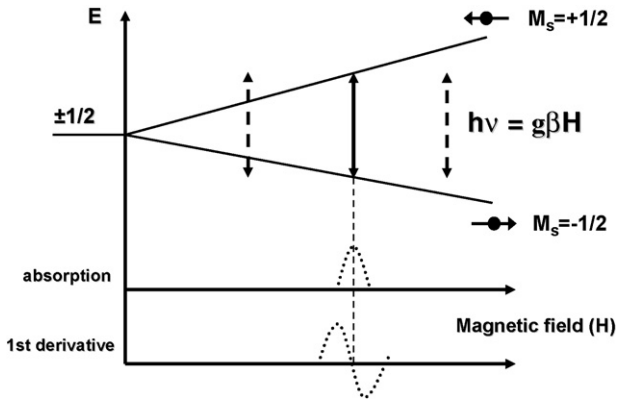

Fig. 1. The general principle of electron paramagnetic resonance (EPR) spectroscopy. In the simplest paramagnetic system, a free electron can exist in one of two possible spin states (Ms +1/2 and Ms −1/2); in the absence of magnetic field both states have identical energy. The external magnetic field (H) interacts with electron magnetic moments resulting in appearance of the energy difference between spin states and dividing electrons into two groups (formally, with magnetic moments being either parallel or anti-parallel to H). When the energy difference between the spin states of paramagnetic system becomes equal to the microwave energy ($h\upsilon$), the absorption occurs. The details of spectral line bear important information on the interactions of unpaired electrons; therefore, in order to allow phase sensitive detection, the magnetic field is modulated (with modulation coils) resulting in the derivative spectrum outcome.

hyperfine structure (splitting) of the spectra. The area under absorption curve (double integral of the 1st derivation curve) is proportional to the amount of a given paramagnetic centers (spins) present in the resonator cavity; thus, EPR spectroscopy is a truly quantitative method.

It should be acknowledged that the quantum mechanics of both conventional and advanced EPR spectroscopy is rather complex; however, many biologists and chemists throughout the world successfully use the technique and receive useful information on free radicals without getting too deep in the theory. The number of biomedical applications of EPR is constantly increasing and with appearance on the market of an inexpensive, tabletop EPR machines (MS 200 from Magnettech or *e-scan* from Bruker), the method is expected to become common in many biomedical laboratories. Both of these spectrometers have comparable sensitivity and reproducibility; the potential limitation of *e-scan* is that it does not operate at low magnetic fields; therefore, the study of high spin paramagnetic centers, such as Met-Hb or Fe^{III}–transferrin is impossible. For more detailed information on the EPR method and, in particular, with regard to the EPR spectroscopy of biologically relevant nitrosylated compounds, the reader is referred to the monograph by Henry et al. [6].

3. Organic NO traps and NO probes

Classical nitrone/nitroso spin traps such as 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), α-phenyl-*N*-*tert*-butylnitrone (PBN) or 3,5-dibromo-4-nitrosobenzene sulfonate (DBNBS), which are widely used for detection of oxygen-derived free radicals, do not form stable and/or characteristic NO adducts, neither in solution nor in biological systems [7,8]. More recently

developed spin trapping agents, such as NO cheletopic traps (NOCTs) [9] and *aci* anions of nitroalkanes ($RHC=NO_2^-$) [10], can form the true NO spin-adducts exhibiting characteristic EPR spectra in solution, but they fail to detect biological NO, mainly due to the low rate of reaction with NO and/or rapid reductive degradation. The stable free radical nitronyl nitroxides are widely used for the detection of NO in vitro. The EPR spectra of nitronyl nitroxides consist of five lines with the relative intensities of 1:2:3:2:1 which is attributed to an interaction of the unpaired electron with two equivalent nitrogens ($A_N = 0.8$ mT). In neutral solutions, nitronyl nitroxides react with NO in a stoichiometric (1:1) manner (rate constant of about 10^4 M^{-1} s^{-1}) to produce imino nitroxides and $^{\bullet}NO_2$. The later species have a distinctly different EPR signal consisting of seven lines with $A_N^1 \sim 0.4$ mT and $A_N^2 \sim 1$ mT due to an electron interaction with two nonequivalent nitrogens [11,12]. Nitronyl nitroxides are often referred to as NO spin traps; however, because NO adducts are not formed during the reaction, the term "paramagnetic NO probe" would be more appropriate. The common compounds of this family are lipophilic 2-phenyl-4,4,5,5-tetramethylimidazoline-1oxyl 3-oxide (PTIO) and its water-soluble analog carboxy-PTIO [13]. Among the disadvantages of nitronyl nitroxides are (1) reductive problem (2) production of the harmful free radical $^{\bullet}NO_2$ and partial overlapping signals from both the reacted and unreacted probe [11–13].

4. Intrinsic NO (spin) traps

Although NO is a free radical, it cannot be measured directly using EPR spectroscopy because its signal is too broad. However, EPR assessment of NO production is possible via the formation of a stable paramagnetic NO adduct that has a distinguished EPR spectrum. The most reliable approach developed so far has been to use metal complexes, especially Fe^{II}-complexes that are known to avidly trap NO (for review see ref. [14]). The interaction of NO with putative Fe^{II}-containing proteins resulting in the formation of various types of nitrosyl–iron complexes is an important element in the biological function of NO [15]. In many cases, these nitrosyl–iron complexes are readily detectable by EPR and, therefore, a wide variety of iron-complexes ubiquitously present in cells and tissues can be regarded as potential targets for NO and intrinsic NO (spin) traps. Additionally, EPR spectroscopy can provide some important clues relating to the physiological NO targets. Interestingly, early pioneering EPR experiments performed between 1965 and 1969 in the laboratories of Commoner [16], Bliumenfel'd [17], and Emanuel [18] with mammal cells had demonstrated the EPR signals of nitrosyl–iron complexes. In fact, these EPR findings provided the first hints about the possibility of NO biosynthesis in mammals; however, they were not appropriately appreciated by the scientific community. Some of the interesting NO-related findings from EPR method are summarized in Table 1.

4.1. Tissue nonheme iron

It has been known for years that NO readily reacts with iron (especially Fe^{II} ions) in solution, and forms various types of paramagnetic nitrosyl–iron complexes with characteristic EPR signals, depending on solvent, pH and anionic ligands present [19]. When cysteine (pH > 7) was present in excess, the EPR signal recorded at room temperature was characterized by a *g*-value of 2.03 and 13-component superhyperfine splitting which was attributed to the interaction of unpaired electron with two ^{14}N nuclei of the nitroso groups and with four protons of methylene groups of two cysteine ligands. These EPR data facilitated the assignation of the nitrosyl–iron complexes formed in the presence of Fe^{II}, cysteine and NO as the mononuclear *iron–dinitrosyl* complexes with thiolate ligands [19]; these complexes are now widely referred to as DNIC. Further studies [17,20] showed that the EPR signal of low molecular weight DNIC–cysteine, when recorded at liquid nitrogen temperature (77 K), turned out to be anisotropic ($g_{\perp(\text{perpendicular})} = 2.04$; $g_{||(\text{parallel})} = 2.015$, $g_{(\text{average})} = 2.03$) and identical to the EPR "signal-2.03" that had been early discovered in the rat liver exposed to carcinogens [16] and in cultured yeasts [21]. This analysis of the EPR spectra of DNIC-cysteine solutions recorded in either liquid or frozen (immobilized) states, now allows us to attribute the EPR signal with $g = 2.03$ found in many inflammatory NO overproducing tissues [22–26] as being indicative of *iron–dinitrosyls* (DNIC) (Fig. 2). The content of DNIC in inflammatory tissues can reach rather high levels; thus, in rat aortas exposed to lipopolysaccharide (LPS) it was reported to be about 5 nmol g^{-1} wet tissue [25], while in the parasite-infected rabbit liver it can be as high as 100 nmol g^{-1} wet tissue [20]. However, DNIC target protein(s) and the potential functional role of DNIC species in inflammatory tissue remain to be established. This is likely to be a difficult

Table 1
Some important results in NO biology obtained by EPR method

Result	References
Demonstration of O_2-dependent conformation change of nitrosyl hemoglobin (R/T transition)	Kosaka et al. [48], Kon [49]
Discovery of biological dinitrosyl–iron complexes in mammal cells and tissues. First clues on NO synthesis in mammals.	Vithayathil et al. [16], Vanin et al. [17]
Demonstration of source of atoms composing biological NO (guanidine nitrogen of L-arginine and molecular oxygen)	Kotake et al. [68], Kubrina et al. [91]
Demonstration of metabolic fate of NO in blood via interaction with oxy- and deoxy-hemoglobin (humans)	Wennmalm et al. [40]
Evidence of circulating nitrosyl–hemoglobin levels as indicator of endothelial function (rodents)	Kirima et al. [51], Dikalov and Fink [56]
Demonstration of NO overproduction in septic shock (rodents)	Westenberger et al. [54], Paya et al. [55]
Demonstration of adventitia as potent source of NO in inflammatory blood vessels (rodents)	Kleschyov et al. [25]
EPR imaging of NO production in vivo (rodents)	Kuppusamy et al. [92], Yoshimura et al. [93]

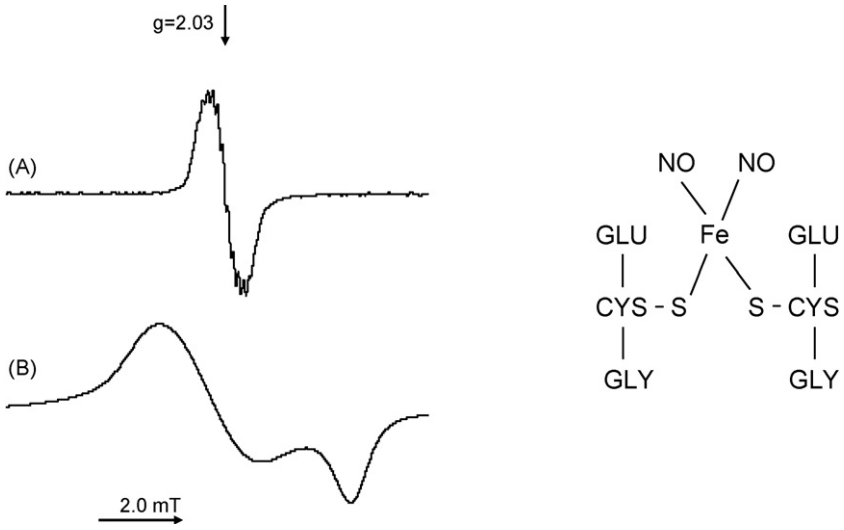

Fig. 2. Electron paramagnetic resonance (EPR) spectra (X-band) and the structure of dinitrosyl–iron complex with reduced glutathione (DNIC-glutathione). EPR spectra of the DNIC-glutathione solution (1 mM; Fe:glutathione molar ratio is 1:20) were recorded either at (A) room temperature (isotropic EPR signal) or (B) liquid nitrogen temperature (axial symmetry EPR signal). The instrument (MS200, Magtettech) parameters were 20 mW microwave power, 10 mT sweep width, 0.03 mT amplitude modulation, 100 kHz modulation frequency, 60 s sweep time.

task because DNICs are prone to intensive ligand-exchange reactions [14], and it is possible that the DNIC origin can be different from the DNIC target(s). Recent model experiments suggest that low molecular weight DNICs are not trivial NO donors but signaling species with a special physiological function that cannot be reproduced either by NO or iron complexes by themselves [27–30].

4.2. Tissue heme proteins

Most heme proteins present in mammalian tissues can interact with NO, leading to the formation of nitrosyl–heme complexes which can potentially be detected and characterized by EPR spectroscopy. Additionally, heme–NO complexes can be formed as a result of reductive nitrosylation from heme and nitrite at low oxygen tension [6]. Due to the high affinity of NO for heme groups, NO has been widely used as a paramagnetic probe to characterize the oxygen binding sites of many purified heme proteins; the list of these proteins include myoglobin [31], cytochrome C [32], peroxidase [33], catalase [34], cyclooxygenase [35], P-450 monooxygenases [36] and many other enzymes. The nitrosylation of the protein heme group sometimes has a functional effect (inhibition or activation of the protein activity), the best known example being the activation of soluble (NO-sensitive) guanylyl cyclase (sGC) [37]. The EPR characteristics of different heme–NO proteins are unique. At the same time they have some common features: usually signals have three principal g-values (around 2.07, 2.00, 1.98) and distinct three-line superhyperfine splitting, the extent of which is mainly determined by the coordination type of the heme–NO moiety (hexa- or penta-) [38]. The prominent EPR signals of heme–NO species can be observed in most types of mammal tissue exposed to elevated levels of NO. However, it is usually impossible to discriminate between the individual nitrosylated-heme proteins due to mutual overlapping of their EPR signals. The dominant heme–NO EPR signal often belongs to the major heme-protein expressed in a given tissue (i.e. myoglobin in the heart [39]).

4.3. Hemoglobin (Hb)

Intra-erythrocytic Hb plays a key role in the regulation of intravascular NO bioactivity. In the early days of NO research, the circulating Hb has been considered solely as a sink for cardiovascular NO [40]. Recent studies have revealed that intra-erythrocytic Hb can not only destroy, but also preserve, and then deliver the NO-related bioactivity to hypoxic tissues [41]. The molecular mechanism of this novel function of Hb is not completely understood and is controversially discussed in the literature [41–45] (recently reviewed in ref. [46]). The interaction of NO with Hb results in generation of several paramagnetic Hb/NO derivatives that can be recognized by EPR spectroscopy in blood samples; therefore, the utilization of this method can be of particular importance [6,44,47]. There are two principal reactions of NO within red blood cells and both of them can be directly traced by EPR. The first reaction is related to the "sink" pathway: NO rapidly ($k = 5 \times 10^7\ M^{-1}\ s^{-1}$) and irreversible reacts with oxy-Hb forming nitrate and Met-Hb which has distinguished EPR signal with a g-value of 6 [40,48]. However, the generation of Met-Hb cannot be considered as being NO specific. The second reaction: NO at almost equally rapid rate ($k = 2 \times 10^7\ M^{-1}\ s^{-1}$) binds to deoxy-$Hb^{II}$ forming a stable EPR detectable Hb^{II}–NO.

An important contribution to the EPR spectroscopy of Hb–NO derivatives had been made by H. Kon [49]. It is important to emphasize that the EPR signals attributed to Hb^{II}–NO are different depending on whether NO binds to the α- or β-heme/subunit, as well as on the Hb conformation state. There are three general spectral species in the Hb–NO preparation: α-heme–NO which can be either (1) hexa- or (2)

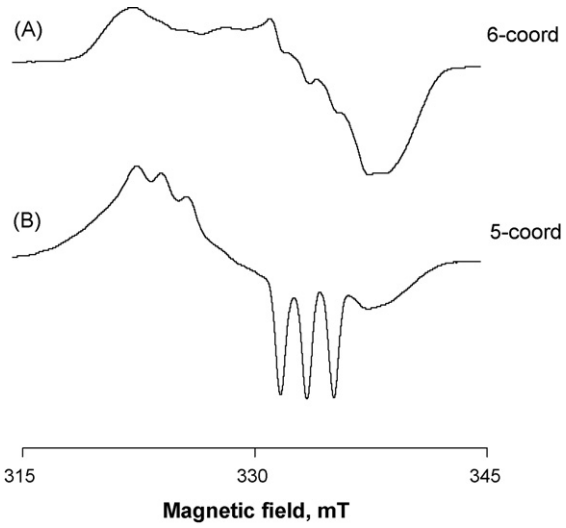

Fig. 3. Electron paramagnetic resonance (EPR) spectral transition of Hb–α-heme–NO from 6-coordinated (6-coord) species in arterial blood (A) to 5-coordinated (5-coord) species in venous blood (B) obtained from endotoxin-treated rats. The spectral transition indicates the breakage of the bond between Fe and proximal ligand (histidine) during the R/T conformation change of Hb in arteriovenous cycle. EPR spectra (X-band) were recorded at liquid nitrogen temperature (77 K). The instrument (MS200, Magnettech) parameters were 10 mW microwave power, 30 mT sweep width, 0.6 mT amplitude modulation, 100 kHz modulation frequency, 60 s sweep time, 3 number scans.

penta-coordinated, and (3) β-heme–NO which is always hexa-coordinated [6,44,48–50]. While the association rate of NO to α-heme and β-heme is the same, the dissociation rate is faster for β-heme than α-heme so that, in equilibrium, α-heme–NO usually dominates. Accordingly, rat erythrocytes exposed to NO, first produced a mixed EPR signal. However, in time, the EPR signal responsible for β-heme–NO disappeared while the α-heme–NO EPR signal persisted [48]. This relatively stable Hb–α-heme–NO species can be observed in the circulation in vivo (at least in rodent models [48,51]). The EPR signal of Hb–α-heme–NO demonstrates a spectacular transition in arteriovenous cycle, reflecting the oxygen-dependent R/T conformation change of Hb [48]. When the Hb is in the R-state, α-heme–NO is hexa-coordinated and the hyperfine structure of the EPR signal is not pronounced (Fig. 3A). In the T-state, strain is placed on the proximal histidine bond, leading to its cleavage and generation of penta-coordinated α-heme–NO; these species yield pronounced three-line hyperfine structure (Fig. 3B). Different preparations of deoxy-Fe^{II}-Hb have been used as NO spin trapping tools in experiments on the isolated cells and organelles [52,53]. Ex vivo EPR assessment of the intra-erythrocytic Hb–NO has been used as a means of proving NO (over) production in rodent models of septic and hemorrhagic shock [47,48,54,55]. More recently, this technique has been used as an index of systemic NO production in rats [51,56]. It should not be forgotten, however, that Hb–NO can be formed from nitrite, as a result of reductive nitrosylation process [6] and/or at low pH values [57]. Therefore, as in the case of other heme proteins, the existence of the EPR signal of Hb–NO/heme–NO should be interpreted with caution—it is indicative of heme–NO levels, but not necessarily of NO levels. The later notion does not diminish the value of these EPR studies, especially with regard to the modern view on the nitrite/heme–NO system as an intravascular reservoir of NO bioactivity [44,58,59]. The recent demonstration of functional NOS III within red cells [60] also emphasizes the importance of Hb–NO/EPR studies. In analytical methodology language: the EPR assessment of heme–NO levels can be regarded as being quantitative, but related to NO levels in a semi-quantitative manner.

Quantification of the Hb–NO EPR signal can be done by comparison with the EPR signal of a stable paramagnetic compound (i.e. solutions of $CuSO_4$, nitroxyl radical) of known concentration; the EPR signals should be recorded under the same conditions and double integration of the signals should be performed [6]. A method for direct monitoring of circulating Hb–NO in humans would be of utmost importance for clinical science. Unfortunately, current EPR techniques do not allow measurement of basal Hb–NO in human blood, mainly due to low concentrations (<200 nM) and overlapping of the Hb–NO EPR signal by other intrinsic blood EPR signals (mainly from Cu^{2+}-containing proteins) [44]. Several groups tried to improve the recovery of Hb–NO in blood using the procedure of subtraction of the EPR signal of Hb–NO-depleted blood [51,61]. Recently, a regression-based spectral analysis technique facilitated detection of Hb–NO in humans during NO inhalation, but not of basal Hb–NO; additionally, the Hb–NO content was split into components species: β-heme–NO (6-coordinated), α-heme–NO (5-coordinated) and α-heme–NO (6-coordinated) [44]. It appears that future efforts should be directed at improving the digital resolution of EPR spectra as well as on the creation of novel computer programs. The combination of EPR spectroscopy with some preparative methods (i.e. isolation/concentration of Hb fraction) might be also very helpful.

5. Iron-dithiocarbamate(s) as NO spin traps

The efficiency of NO spin trapping can be sharply increased by the exposure of tissue to some exogenous Fe–S-containing complexes; of which various Fe-RR′-dithiocarbamate complexes are best studied (where R/R′ can be methyl-, ethyl-, glucamine- and other substitutes). This NO spin trapping approach has been used in numerous applications both in vivo and in vitro and has been comprehensively reviewed [14,56,62–65]; for detailed protocols the reader is referred to these publications. Here we concentrate on the general principle of the approach and our personal view on its potential perspectives.

5.1. Physicochemical biology of NO–Fe-dithiocarbamate(s)

Ternary complexes of the general formula NO–Fe(S_2CN-RR′)$_2$ have been known for a long time, while their EPR characteristics were first described in1960s by J. Gibson [66] and B. A. Goodman et al. [67]. At ambient temperature the solution of NO–Fe(S_2CN-RR′)$_2$ (with ^{56}Fe and ^{14}NO isotopes) is characterized by the isotropic EPR signal at a *g*-value of 2.035 and triplet superhyperfine structure (A_N) of about 1.3 mT. In the

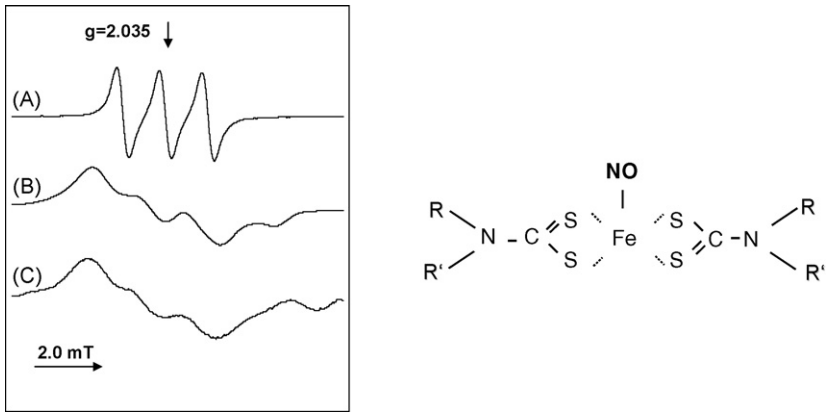

Fig. 4. Electron paramagnetic resonance (EPR) spectra and the general structure of mononitrosyl–iron complexes with RR′-dithiocarbamates. EPR spectra (X-band) of the water-soluble NO–Fe(MGD)$_2$ standard recorded at room temperature (A) or at liquid nitrogen temperature (B) and of the lipophilic NO–Fe(DETC)$_2$ formed in the mouse aorta during incubation (37 °C; 1 h) with acetylcholine (1 μM) and colloid Fe(DETC)$_2$ (100 μM) (C). The instrument (MS200, Magnettech) parameters were 10 mW microwave power, 10 mT sweep width, 1.0 mT amplitude modulation, 100 kHz modulation frequency, 60 s sweep time. R- and R′- can be methyl-, ethyl-, glucamine-, or other substitutes.

frozen state (77 K) the solution exhibits the EPR signal with axially symmetric g-tensor of about $g_\perp = 2.04$ and $g_{||} = 2.02$ and triplet structure at $g_\perp$ (Fig. 4). The triplet structure of the signal originates from the interaction of unpaired electron with the ^{14}N nucleus of NO ligand. However, if ^{14}NO is replaced by ^{15}NO the resulting EPR spectrum will be characterized by doublet superhyperfine structure and if ^{57}Fe is incorporated into the complex, the corresponding EPR signal will acquire additional poorly resolved splitting [14,67,68]. On the basis of EPR and crystal-structure analysis of NO–Fe(S$_2$CN-RR′)$_2$, it can be concluded that the complex has C_{2V} symmetry with a Fe–N–O bond angle of about 174°; the NO ligand is present in these complexes in the form of nitrosonium cation (NO^+) and the unpaired electron is located primarily on the d_z2 orbital of iron (electron configuration d^7, i.e. formally Fe^{1+}) [66,67].

The complexes are known to be redox-active as they can be readily oxidized into diamagnetic (electron configuration d^6) species by halogens or $^{\bullet}NO_2$ and reduced back into paramagnetic state by reducing agents [69]; this property should be kept in mind while dealing with biological NO. The properties of RR′ groups (polarity and size) within dithiocarbamates determine the tissue compartmentalization both of the trap and corresponding NO adducts; however, the reactivity toward NO/Fe–NO may also be affected. For example, the highly lipophilc Fe-diethyldithiocarbamate (DETC) partitions into the hydrophobic interior of membranes and the NO–Fe(DETC)$_2$ EPR signal is always associated with tissue [70,71]. In contrast, the water soluble Fe-*N*-methyl-D-glucamine dithiocarbamate (MGD) does not enter cells and the corresponding NO adduct can be found exclusively in extracellular fluid [72].

It is important to realize that exactly the same NO–Fe-dithiocarbamate complexes can be formed either by the Fe-dithiocarbamate interaction with "free" NO or due to the interception of pre-existing Fe–NO groups by "free" dithiocarbamate. The rate constants of the reactions between NO and Fe-dithiocarbamates are very high (1.1×10^8 M^{-1} s^{-1} for Fe-proline-dithiocarbamate [73] and 4.4×10^8 M^{-1} s^{-1} for Fe-*N*-dithiocarboxy-sarcosine [74]). This near-diffusion rate limited reaction ensures the exceptionally high efficiency of Fe-dithiocarbamate NO spin trapping in biological systems (can approach 100%). The reaction of interception of Fe–NO groups (i.e. from DNIC species or nitroprusside) by "free dithiocarbamate" is also very fast and very efficient [30,70], although the rate constants are not yet determined. When dealing with biological samples, the following chemistry should be also taken into account: (1) Endogenous copper can compete with iron for dithiocarbamate ligand resulting in the formation of complexes whose EPR signal partially overlaps the NO–Fe(S$_2$CN-RR′)$_2$ signal [14]. (2) Water-soluble Fe^{II}-MGD has been reported to be able to reduce nitrite to NO at physiological pH, and thus potentially cause artefacts [75]; however, because of the low rate constant (1–5 M^{-2} s^{-1}) the significant role of this reaction at physiological nitrite levels is unlikely. Interestingly, when the colloid formulation of Fe(DETC)$_2$ is used for NO measurement in isolated blood vessels, even high concentrations of nitrite (100 μM) do not interfere with the assay [64]. (3) Fe^{II}-MGD was reported to react with *S*-nitrosoglutathione to produce NO–Fe(MGD)$_2$ [76]. A similar reaction was observed when colloidal Fe(DETC)$_2$ was added to blood plasma supplemented with *S*-nitrosoglutathione [64].

5.2. Methodological perspectives

Here, we will consider the methodological aspects of the NO spin trapping approach utilizing Fe-dithiocarbamates and, in particular, those related to experiments with isolated blood vessels. Several lines of evidence suggest that a significant proportion of NO produced by NOS III in vascular endothelium undergoes multiple reactions and does not reach its major target, sGC in the vasculature and platelets. The degree of NO scavenging is especially high under the conditions of oxidative stress, which is known to be associated with most of cardiovascular diseases. Therefore, an analytical approach allowing quantification of the total NO production (related to NOS activity) and the proportion of NO that escapes the consumption within blood vessels (related to NO bioavailability) is important. The accumu-

lated information on NO–Fe-dithiocarbamate biochemistry can provide the necessary framework for EPR assessment of these important NO parameters; however, the exact protocols should be adjusted according to specific experimental conditions.

5.2.1. Total NO production

The ideal trap should overcompete the intravascular NO reactions; therefore it should react with NO rapidly and be present at sufficiently high concentrations close to the site of NO production. Additionally, both the trap and the corresponding NO adduct should be stable in the intracellular environment and should not interfere with the tissue redox state or NOS activity. Currently, the lipophilic $Fe(DETC)_2$ trap, especially its colloidal formulation apparently most closely meets the above criteria [64,71,77]. For the following reasons: (1) NOS III is a membrane bound enzyme and both NO and $Fe(DETC)_2$ tend to partition into the hydrophobic cellular compartments. (2) Due to the high rate of reaction between NO and $Fe(DETC)_2$, the concentrations of the trap necessary to compete efficiently with the intracellular targets (even under conditions of oxidative stress) are 50–200 μM. (3) The assay is resistant to the elevated levels of extracellular superoxide and nitrite. (4) NOS III agonists (acetylcholine, calcium ionophore) increase the NO–$Fe(DETC)_2$ EPR signal in a concentration dependent manner (calculated rate of NO production 1–10 pmol cm^{-2} min). (5) The linear increase of the signal over 1 h indicates the constant rate of NO production and high stability of the (reduced) NO adducts in healthy vascular tissue. (6) Post-incubation treatment with dithionite does not increase the NO–$Fe(DETC)_2$ signal in the control intact blood vessels, indicating the absence of EPR silent complexes and the sufficient reducing capacity in healthy vessels.

Recent studies suggest that peroxynitrite can react with Fe-dithiocarbamate(s), forming an intermediate that can be rapidly converted into paramagnetic NO–$Fe(DETC)_2$ in the presence of reducing agents [78]. Another finding is the demonstration of the possibility of oxidized Fe^{III}-dithiocarbamates to trap NO, forming an EPR silent complex, which then can be reduced by endogenous reducing agents [64,74]. The capacity of the blood vessel to reduce/keep NO–$Fe(DETC)_2$ in the paramagnetic state may be an important determinant of the "NO recovery" by this EPR method. Thus, it might be speculated that in intact blood vessels the accumulated NO–$Fe(DETC)_2$ may reflect the majority of the vascular NO produced, including some part of reactive NO species like peroxynitrite. While this statement requires solid experimental confirmation, it is consistent with our recent results showing that vascular NO levels, as determined by colloid $Fe(DETC)_2$ assay, may not be compromised despite significant NADPH oxidase-dependent vascular oxidative stress [79]. In contrast, the formation of NO–$Fe(DETC)_2$ in blood vessels can be decreased when NOS III is uncoupled and produces less NO [80]. Several groups have used the colloid $Fe(DETC)_2$ approach to measure NO levels in cultured vascular cell in which oxidative stress is usually higher than in intact tissue [81–84]. In the model of severe intracellular oxidative stress (challenge with geldanamycin) the NO–$Fe(DETC)_2$ signal was decreased down to 50% and this was prevented by cell permeable superoxide dismutase [81]. However, taking into account the redox properties of NO–$Fe(DETC)_2$, this result may not necessarily reflect the true levels of NO bioavailability. To discriminate between the two possible reasons of the decreased NO signal (reducing capacity versus superoxide), the post-incubation treatment with a reducing agent, such as sodium dithionite is recommended [69]. One more reason to argue against the view that the colloidal $Fe(DETC)_2$ assay reflects truly the NO bioavailability in intact blood vessels is that the cellular sources of NO production and superoxide production are often spatially dissociated.

In contrast to classical spin trapping agents, which provide only qualitative or at the best semi-quantitative assessment of free radicals due to low rates of reactions and instability of adducts, the use of colloidal $Fe(DETC)_2$ allows quantification of NO. A special feature of this assay is that NO accumulates in tissue in the form of stable NO–$Fe(DETC)_2$, which is directly measured by EPR. Using this assay, it is impossible to judge the steady state concentrations of NO, but rather about the rate of NO production. The limit of detection (LOD) of absolute amount of NO–$Fe(DETC)_2$ in the sample is variable and depends on the sensitivity of EPR spectrometer as well as on the presence of other tissue paramagnetic centers. For a modern EPR spectrometer and rat aortic rings, the LOD has been reported to be about 6 pmol NO–$Fe(DETC)_2$ per sample (<200 mg wet weight) [25]. However in practice, the amount of NO–$Fe(DETC)_2$ accumulates in tissues to much higher values (100–300 pmol per sample). EPR measurements using the Magnettech instrument (X-band) under cryogenic conditions (77 K) are quite reproducible, i.e. the intra-assay coefficient of variations for a frozen sample is 5.2% [85].

For quantification of the absolute amount of NO–$Fe(DETC)_2$ formed in tissues, a frozen aqueous solution of NO–$Fe(MGD)_2$ of known concentration and of the same volume is usually used [71]. Both NO–$Fe(DETC)_2$ and NO–$Fe(MGD)_2$ have identical EPR signals; however, the preparation of the water-soluble standard is much easier. Because the intensity of EPR signal is always proportional to the amount of NO–$Fe(DETC)_2$ present in the sample (there is no extinction coefficient), the construction of a calibration curve is not necessary. Recent studies have demonstrated that NO–$Fe(DETC)_2$ generated in vascular tissue can be quantified by EPR not only at liquid nitrogen temperature, but also at ambient temperature if extraction of NO–$Fe(DETC)_2$ by ethyl acetate is employed [86].

5.2.2. NO bioavailability

The exact protocol(s) for accurate evaluation of this parameter in blood vessels is not yet fully elucidated. It is conceivable that the major requirement for this assay would be physical separation of the NO-trap/probe from the blood vessel/NO producing cells. The trap of choice could be a water-soluble Fe^{II}-dithiocarbamate or the $Fe(DETC)_2$-loaded effector cells [62]. To prevent the oxidation of extracellular Fe^{II}-dithiocarbamate, the addition of low concentrations of mild reducing agents such as dithioerythritol can be used [73]. Alternatively, the post-incubation treatment of the medium with dithionite can also be utilized; however, this should be done with care as dithionite can reduce nitrite to NO, especially at low pH values. Recently, to avoid the potential interference with cellular

biochemistry, a teflon membrane has been employed to maintain separation between the NO trapping solution and the NO producing cells [87]; the limitation of this technique is the necessity to strictly control the distance between cell surface and NO probe.

5.2.3. S-Nitrosothiols

While potentially possible, the exact protocols for measurement of *S*-nitrosothiols in biological samples by EPR have not yet been fully elucidated. The possible combination of some preparative methods and utilization of several dithiocarbamates may be required.

5.2.4. Trapping Fe–NO

It was hypothesized previously that an interaction of NO with intrinsic cellular/tissue metals (Fe^{n+}, Cu^{n+}, etc.) is an ubiquitous means of modifying/coordinating the local NO (re)activity [88]. However, the methodology that would allow tracing such intermediates in living tissues is lacking. Since various dithiocarbamates can efficiently "extract" Fe–NO groups from most (if not all) known nitrosyl–nonheme iron complexes [14,25,30], they can be used as tools for quantification of the NO–Fe complexes formed in tissue under specified conditions.

5.2.5. Trapping nitroxyl (NO^-)

It was hypothesized previously that under certain conditions the immediate product of NOS is not the neutral radical NO, but rather nitroxyl anion (NO^-) [89]. The methodology that can prove or disprove this intriguing hypothesis is lacking. To discriminate between NO^- and NO-radical one can potentially use an Fe^{III}(ferri)-dithiocarbamate complex which forms the paramagnetic complex with the former but not with the latter [90]. Basic requirements for such types of experiments in intact tissues would be utilization of a water-soluble Fe^{III}-dithiocarbamate (1:3 molar ratio) and the absence of reducing agents in the medium. Unfortunately the available water-soluble Fe-dithiocarbamates form NO adducts which are prone to rapid oxidation. Elaboration of novel dithiocarbamates that form the more stable nitrosyl–iron complexes would certainly increase the chances of detecting NO^- production.

6. Conclusion

Novel NO traps/formulations should be engineered for a targeted assessment of NO production and NO bioavailability in intact functional tissues both in vitro and in vivo. Recent data suggest that Hb–NO serves as a circulating reservoir of bioactive NO. Hb–NO levels (basal and stimulated) may be an important surrogate measure of endothelial function. Despite its great potential, the conventional EPR approach does not allow measurement Hb–NO levels in human blood, mainly due to low the Hb–NO concentrations and overlap of the Hb–NO EPR spectrum by other signals from intrinsic blood constituents. One possibility to solve this problem might be to couple an EPR approach with an appropriated preparative method. Various dithiocarbamate(s) with particular pharmacokinetic profiles have been described in the literature; however, only few of them have been tested and are currently used for NO spin trapping in the biological settings. This does not preclude the possibility that novel dithiocarbamates could help to improve protocols that already exist for biological NO/Fe–NO spin trapping.

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