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Nitrosylhemoglobin, an unequivocal index of nitric oxide release from nitroaspirin: in vitro and in vivo studies in the rat by ESR spectroscopy

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Abstract

Electron spin resonance (ESR) spectroscopy was applied for the unequivocal detection/quantitation of nitric oxide (NO) as nitrosylhemoglobin (HbFe(II)NO) released from nitroaspirin, benzoic acid,2-(acetyloxy)-3-[(nitrooxy)methyl]phenyl ester (NCX-4016; NO-ASA), the lead of a new class of nonsteroidal anti-inflammatory drugs. In both in vitro and in vivo experiments, the paramagnetic complex was detected at 100 K in the venous blood of the rat (microwave power, 20 mW) and characterized by a three-line hyperfine structure with coupling constants (A_x and A_z) of 17 G at $g_x = 2.066$ and $g_z = 2.009$. The kinetics of NO release from the drug were first determined in vitro by incubating rat blood with 1 mM NO-ASA and confirmed by the two-line hyperfine structure obtained with the labeled compound (^{15}N-NO-ASA). In in vivo studies, the hematic levels of HbFe(II)NO were determined after oral (p.o.) and intraperitoneal (i.p.) administration of the drug (100 and 200 mg kg^{-1}). In p.o. treated animals, the complex was detectable at 1 h post-dosing and its formation was maximal at 4–6 h, where the antithrombotic activity peaks. In i.p. treated animals, HbFe(II)NO complex peaks at the second hour to decline thereafter: in these animals, the ESR technique was applied to also detect nitrosylmyoglobin as an index of NO diffusion/compartmentalization in myocardial tissue. The results of this study emphasize the great potentiality of ESR spectroscopy for the study of the release, the metabolic fate and distribution of NO from nitrovasodilators.

Keywords: Nitroaspirin; Nitric oxide release; Nitrosylhemoglobin; Rat blood; Electron spin resonance spectroscopy; In vitro and in vivo studies

1. Introduction

Nitroaspirin, benzoic acid,2-(acetyloxy)-3-[(nitrooxy)methyl]phenyl ester (NO-ASA), is the lead compound of a new class of nitric oxide (NO)-releasing nonsteroidal-anti-inflammatory drugs,

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which has been shown: (a) to possess anti-inflammatory activity comparable with that of the parent compound but with markedly reduced gastrolesivity; (b) to inhibit platelet activation, thrombus formation; and (c) to be effective in preventing pre-neoplastic lesions in the colon of rats: all these effects presumably due to an NO release-mediated mechanism [1–6]. However, until now, only indirect evidence supports the delivery of bioactive NO from this molecule. In in vitro models, they are based on the stimulation of cGMP levels in platelets and on the dose-dependent inhibition of thrombin-induced aggregation of platelets pretreated with aspirin [3]: while in vivo in the rat, they are based on the determination of nitrites/nitrates (NO*x*) levels in both plasma and serum after oral administration [2,4,5]. Anyway, the estimate of NO via its oxidation products is not an unequivocal index of bioactive NO release: it only gives a rough measure of the metabolic products arising from denitration of the nitroester function, or of the reaction of NO with oxyhemoglobin, products that will only in part be converted to bioactive NO. Nitrite, under physiological acidic conditions, forms nitrous acid that spontaneously decomposes to NO, but nitrates are eliminated unchanged in urines [7–9]. Since the half-life of nitrite in human blood is 110 s [10], the measurement of plasma nitrite ex vivo without preventing its rapid conversion to nitrate during blood sampling is unlikely to be a reproducible and accurate measure of released NO. In addition, in man, there exists a great intra- and inter-individual variance of circulating NO*x*, since this parameter is influenced by a large number of factors including diet (about 50% of circulating nitrate depends directly on oral nitrate intake), inhalation of atmospheric gaseous nitrogen compounds (NO*x*), urea cycle, drug metabolism (denitrifying liver enzymes), bacterial synthesis within the bowl, or endogenous reduction [10].

Recently, the group of Stamler et al. [11] and that of Gladwin et al. [7] reported that NO, once released, avidly binds to deoxy-hemoglobin (2.6×10^7 M^{-1} s^{-1}), with formation of nitrosylhemoglobin (HbFe(II)NO), where NO is maintained in a bioactive state. This has led to the possibility to directly measure NO formation via its coordinate binding to Hb [12]: the paramagnetic nature of the complex gives rise to a unique electron spin resonance (ESR) spectrum with a characteristic three-line hyperfine structure due to the significant delocalization of the unpaired electron onto the ^{14}N-NO. For this reason, the ESR technique has been recently proposed as a tool to detect NO production in rat blood following i.p. treatment with two widely used vasodilating agents nitroglycerin [13,14] and isosorbide 5-mononitrate [15], as well as in patients receiving intravenous infusion of nitroglycerin [16].

At the light of these premises, the aim of this work was to investigate the potentiality of the ESR technique for the detection and quantitation of NO release as nitrosylhemoglobin (HbFe(II)NO) from NO-ASA in rat blood. The study was first carried out in vitro, after incubation of NO-ASA or of its ^{15}N-labeled analog with rat blood, and the kinetics of NO release were compared with those of the widespread used nitrovasodilator isosorbide 5-mononitrate. Subsequently, in in vivo experiments, the hematic levels of HbFe(II)NO were determined after acute intraperitoneal (i.p.) and oral (p.o.) administration of the drug (100 and 200 mg kg^{-1}), and the time-course of the complex formation followed until 24 h post-dosing. In addition, in i.p. treated animals, the ESR technique was applied to detect nitrosylmyoglobin directly in myocardial tissue.

2. Materials and methods

2.1. Chemicals

Benzoic acid, 2-(acetyloxy)-3-[(nitrooxy)methyl]phenyl ester (NCX4016; NO-ASA), the ^{15}N-labeled derivative (^{15}N-NO-ASA) and isosorbide 5-mononitrate were kindly provided by NICOX S.A. (Paris, France). Polyethylene glycol (PEG400; average molecular weight, 400) and vanadium (III) chloride were purchased from Sigma (Sigma-Aldrich S.r.l., Milan, Italy); spermine-NONOate, N-[4-[-1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine, was from Molecular Probes (Società Italiana Chimici, Rome, Italy).

2.2. Animals

Male Wistar rats (Charles River, Calco, LC, Italy; 300 ± 20 g body weight) were maintained in compliance with the policy on animal care expressed in the National Research Council guidelines (NRC 1985). Food laboratory chow and drinking water were available ad libitum; animals were fasted overnight before the drugs treatment. For blood sampling, the animals were anesthetized with intraperitoneal sodium pentobarbital (60 mg kg^{-1}) and venous blood withdrawn from abdominal vein by heparinized syringes.

2.3. In vitro experiments

Venous rat blood (3 ml) was deoxygenated for 30 min with a gas mixture containing 95% N_2 and 5% CO_2 in a sealing glass apparatus specifically designed by us, equipped with inlet and outlet purge valves and a water warming jacket to maintain controlled temperature. Blood samples were incubated at 37 ± 1 °C in anaerobic conditions under gentle stirring. NO-ASA, ^{15}N-NO-ASA and isosorbide-5-mononitrate were dissolved in PEG400 and added to blood samples through a rubber septum at a final concentration of 1 mM. The blank was prepared by adding the vehicle (final content, 0.2%, v/v). At fixed times, 300 μl aliquots were taken by a N_2 degassed syringe, immediately transferred to ESR quartz tubes (3 mm i.d.) and kept in liquid nitrogen until the ESR analyses.

Spermine-NONOate (used as spontaneous NO donor) was prepared in 0.01 M NaOH and added to rat blood for a 30 min period in the experimental conditions before described.

2.4. In vivo experiments

The test drugs were dissolved in PEG400 (150 mg ml^{-1}) immediately before administration. In a first set of experiments, rats ($n = 4$ for each time point) were i.p. treated with the vehicle, NO-ASA or ^{15}N-NO-ASA at dosages of 100 and 200 mg kg^{-1}. Blood samples from anesthetized (vehicle and treated animals) were taken at 1, 2 and 4 h post-administration from the abdominal vein and sampled for ESR analyses as already described. The hearts from control and treated rats were excised and immediately perfused with cold saline to remove the residual blood in the myocardial tissue. The left ventricle was then isolated using a scalpel, cut in slices (4–5 mm thick), transferred into ESR quartz tubes (4 mm i.d.) and frozen in liquid nitrogen until determination of nitrosylmyoglobin (the heart tissue was always kept at 4 °C during mincing). A second group of animals ($n = 4$ for each time point) was orally treated (by gavage) with the vehicle or the drugs (100 or 200 mg kg^{-1}) and sacrificed 1, 2, 4, 6, 12 and 24 h post-administration. Plasma NO*x* levels were determined by a chemiluminescence-based assay with a Sievers Instruments Model 280 Nitric Oxide Analyzer (NOA™; Sievers, Boulder, CO, USA; Sensor Medics, Milan, Italy) according to Ref. [17].

2.5. ESR measurements of nitrosylhemoglobin and nitrosylmyoglobin

ESR spectra were recorded at 100 K with a Bruker EMX spectrometer (X band) equipped with a high sensitivity cylindrical cavity (ER4119HS; Bruker) in the following instrumental conditions: microwave frequency, 9.316 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 5 G; number of scans, 20; resolution, 1024 points; sweep time, 21 s; center field, 3500 G; sweep width, 5000 G. To better analyse the hyperfine structure of the HbFe(II)NO signal, the center field was set at 3200 G with a sweep width of 1200 G. The spectra were analysed by a Bruker WINEPR system (version 2.11) and the relative measurements of *g* factors have been performed using the stable free radical 2,2-diphenyl-1-picrylhydrazyl as the reference standard ($g = 2.0037 \pm 0.0002$; powder). The concentration of HbFe(II)NO, expressed as micromolar, was determined by double integration of the signal using $CuSO_4$-ethylenediamine tetraacetic acid (EDTA) as reference standard, as described by Kosaka et al. [18]. Stock solutions of 10 mM $CuSO_4$ were prepared in 50 mM sodium phosphate buffer (pH 7.4) containing 20 mM EDTA. Working standard solutions were pre-

pared by dilution with 50 mM sodium phosphate buffer in the range 0.1–10 μM $CuSO_4$ and stored in liquid nitrogen for the whole time of the experiment. The calibration curve was determined on five levels of concentration (three repetitions for each level); the precision of the method was determined by measuring the repeatability and reproducibility for the analyte at three concentration levels (0.5, 5, and 10 μM) and the relative standard deviations (RSDs) were calculated.

The ESR analyses of nitrosylmyoglobin were carried out at 100 K using the same instrumental conditions, except for the center field, set at 3300 G.

3. Results

3.1. In vitro experiments

All the experiments were performed in venous blood under anaerobic conditions, from the beginning of the reaction to the ESR analysis, to prevent the oxidative degradation of NO and of the radical adduct HbFe(II)NO, and to favor the formation of the pentacoordinate nitrosyl complex, analytically more diagnostic (three-line hyperfine structure) with respect to the hexacoordinate derivative, the dominant species in oxygenated blood [19].

Fig. 1 shows the ESR spectrum of native rat blood recorded at 100 K, characterized by four well detectable signals attributable to the paramagnetic species present in plasma and erythrocytes: met-hemoglobin (met-Hb $g = 5.714$), transferrin ($g = 4.110$), ceruloplasmin ($g = 2.054$) and a semiquinone radical ($g = 2.000$) [18–20]. On magnifying the ceruloplasmin region (Fig. 2a), the signal of the endogenous HbFe(II)NO adduct can also be detected: of the three lines of the hyperfine structure, one is overlapped by the semiquinone radical, but the other two at $g = 2.019$ and 2.009 perfectly match those of the HbFe(II)NO complex (Fig. 2b) generated by anaerobic incubation (30 min) of native rat blood with the reference compound spermine-NONOate (1 mM), a polyamine/NO adduct that spontaneously decomposes at 37 °C and pH 7.4, with a known rate constant for decomposition [21]. The ESR spectrum shown in Fig. 2b was unequivocally attributed to the pentacoordinate complex HbFe(II)NO on the basis of the two sets of three lines at $g_x = 2.066$ and $g_z = 2.009$ and of the hyperfine coupling constants (A_x and $A_z = 17$ G). The signal at $g = 1.986$ is relative to the hexacoordinated complex (R form).

Spermine-NONOate was also used to set and optimise the instrumental conditions in terms of sensitivity and reproducibility. Signal averaging (multiple scans) was used to improve the signal-to-noise ratio and the best results were obtained using 20 scans, a resolution of 1024 data points and a microwave power of 20 mW. ESR spectra of HbFe(II)NO reported in the literature are generally recorded at 5–10 mW microwave power: power saturation experiments performed between 0.2 and 20 mW (data not shown) indicate a strict linear correlation between the intensity of the signal and the square root of the microwave power, allowing one to record ESR spectra at the highest sensitivity. The concentration of HbFe(II)NO was determined by double integration of the signal, using Cu^{2+}-EDTA as the reference standard, since the double-integrated area of HbFe(II)NO has been demonstrated to match

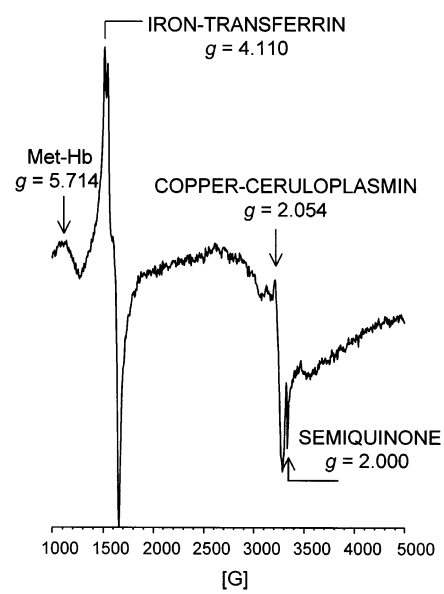

Fig. 1. ESR spectrum of venous blood from control rats recorded at 100 K.

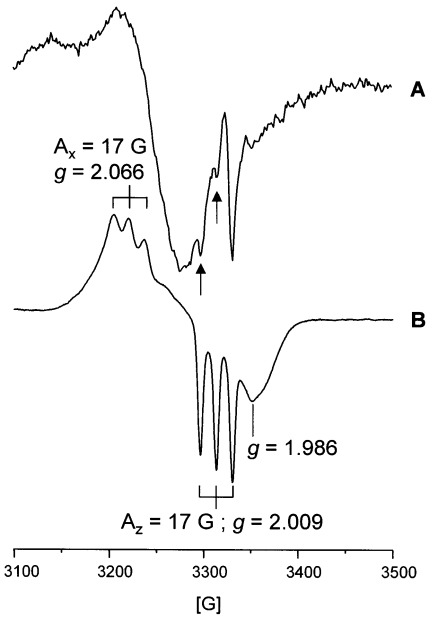

Fig. 2. Nitrosylhemoglobin ESR signal in native (A) and spermine-NONOate-incubated venous blood (B). Arrows indicate two of the three lines of the HbFe(II)NO signal at $g = 2.019$ and 2.009.

with that of $CuSO_4$-EDTA [18]. The equation of the calibration curve (range, 0.5–10 μM) was $y = 4.211 \pm 0.05764x - 0.1884 \pm 0.2896$ ($r^2 = 0.9976$; S.E. of estimate $S_{y,x} = 0.846$) and the calculated limits of detection and quantitation were 0.2 and 0.5 μM, respectively. The intraday repeatability, expressed as RSDs (three concentration levels; six replications each) were 6.6, 4.7 and 4.2% for 0.5, 5 and 10 μM, respectively. The interday repeatability (3 days, $n = 9$) at the same concentrations were 9.3, 7.8 and 6.4%, respectively.

When NO-ASA was incubated with rat blood at 37 °C under anaerobic conditions, the typical three lines at $g \approx 2$ of HbFe(II)NO (Fig. 3) appeared already after 15 min of incubation and increased in a time-dependent manner up to 120 min, the last observation time (concomitantly, there was a parallel increase in the peak at $g =$ 1.986 due to the hexacoordinate nitrosyl complex). The NO release by NO-ASA was further confirmed by incubation of blood with the ^{15}N-labeled derivative: the ESR spectra relative to all the observation times (120 min in Fig. 3) exhibit a signal with the typical two-line hyperfine structure (coupling constant, 21 G) due to the nuclear spin of ^{15}N-NO ($I = 1/2$) [12]. No differences were observed between blood samples (15–120 min) incubated with the vehicle and control blood (data not shown).

Fig. 4 shows the time-course of HbFe(II)NO formation from NO-ASA, ranging from 8.7 ± 2.4 μM after 15 min of incubation to 65.6 ± 7.5 μM at 120 min; NO-ASA gives a kinetics fairly comparable with that of the slow NO releaser, isosorbide-5-mononitrate, a drug widely used to chronically treat angina (8.9 ± 1.5 μM at 15 min and 57.1 ± 7.0 μM at 120 min). These results demonstrate that rat blood is able to metabolize NO-ASA to bioactive NO, which owing to the high affinity for Hb gives the paramagnetic adduct HbFe(II)NO.

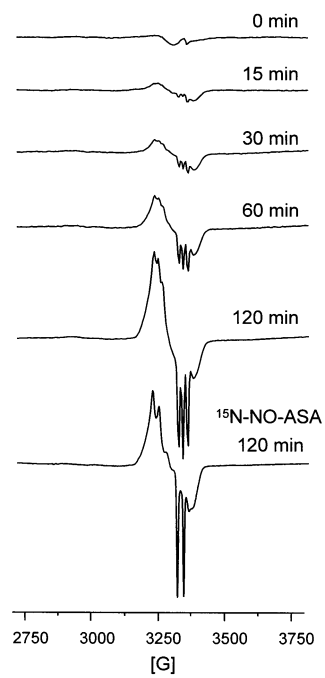

Fig. 3. ESR spectra of rat blood incubated with NO-ASA and ^{15}N-NO-ASA. Venous rat blood samples (3 ml) were incubated for 120 min with 1 mM NO-ASA or ^{15}N-NO-ASA under anaerobic conditions and 0.3 ml aliquots for each incubation time were immediately transferred to ESR quartz tubes (3 mm i.d.) and kept in liquid nitrogen until the ESR analyses.

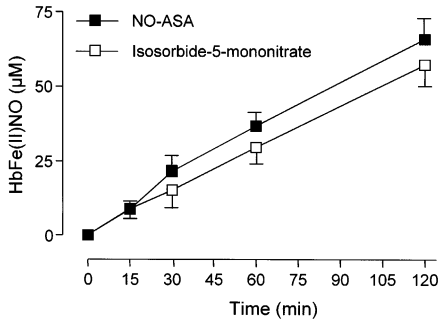

Fig. 4. Kinetics of HbFe(II)NO formation in blood in vitro from NO-ASA and isosorbide 5-mononitrate. Values are the means ± S.D. of five independent experiments.

3.2. In vivo experiments

A well-resolved ESR signal of HbFe(II)NO was detected already 1 h after the i.p. administration of 100 mg kg^{-1} NO-ASA (Fig. 5), which significantly increases at the higher dose: the double integration of the signals gives hematic

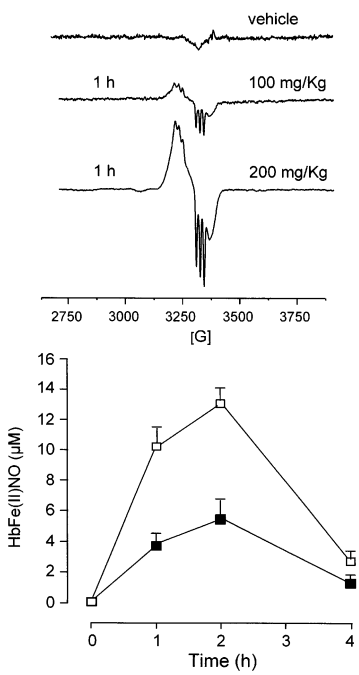

Fig. 5. ESR spectra and kinetics of HbFe(II)NO formation in vivo following i.p. administration of 100 mg kg^{-1} (■) and 200 mg kg^{-1} (□) NO-ASA. Values are the means ± S.D. relative to four animals (three repetitions for each time point).

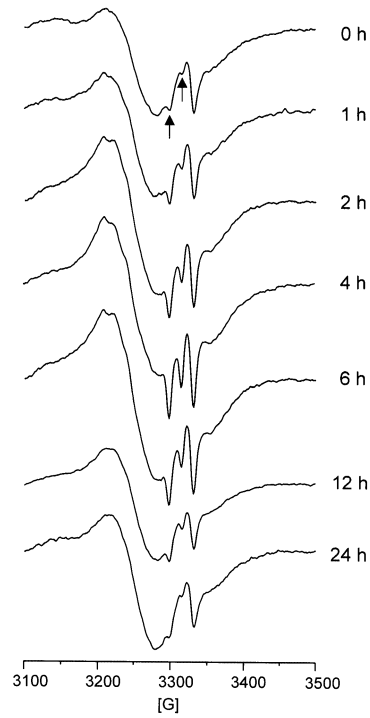

Fig. 6. ESR spectra of blood samples from rats orally treated with 200 mg kg^{-1} NO-ASA. Arrows indicate two of the three lines of the HbFe(II)NO signal at $g = 2.019$ and 2.009.

HbFe(II)NO concentrations of 3.7 ± 0.6 and 10.2 ± 1.3 μM, respectively (the ESR spectra of blood from animals treated with the vehicle were super-imposable to those from controls). At the second hour, HbFe(II)NO levels increased to 5.5 ± 1.2 μM (100 mg kg^{-1}) and to 12.9 ± 0.9 μM (200 mg kg^{-1}), and decreased thereafter (fourth hour) to 1.3 ± 0.7 μM (100 mg kg^{-1}) and 2.7 ± 0.8 μM (200 mg kg^{-1}), suggesting a rapid transfer of NO to hemoglobin β-chain cysteine 93 via S-nitrosation (S-NOHb-βCys^{93}) [22] or to other redox-activated thiols in red blood cells (GSH).

When NO-ASA was administered p.o. at the dose of 200 mg kg^{-1} (Fig. 6), a time-dependent increase of the two lines at 2.019 and 2.009, and concomitantly of the signal at $g = 1.998$ due to the rise of the third line of the triplet, was observed, to indicate an increase of HbFe(II)NO over the basal levels. To better resolve and quantitate the HbFe(II)NO signal, from each ESR spectrum was subtracted the corresponding spectrum

from vehicle-treated rats: in this way, the difference spectra (Fig. 7 is relative to the sixth-hour samples) clearly evidence the typical hyperfine structure of the nitrosyl complex that can be easily integrated. The kinetics of HbFe(II)NO formation in rat blood after oral treatment with 200 and 100 mg kg^{-1} are shown in Fig. 8a. One hour after administration, HbFe(II)NO was detectable only in animals treated with 200 mg kg^{-1} (0.55 ± 0.10 μM), while with the lower dose was still below the limit of detection. With both the dosages, the peak of HbFe(II)NO formation was observed between 4 and 6 h post-administration (1.78 ± 0.33 and 1.62 ± 0.24 μM at 200 mg kg^{-1}; 0.68 ± 0.14 and 0.73 ± 0.18 μM at 100 mg kg^{-1}); the levels significantly decreased at the twelfth hour, and fell below the limit of quantitation at the eighteenth hour.

The kinetics of HbFe(II)NO formation in blood of animals given 200 mg kg^{-1} p.o. strictly parallel to that of NO*x* in plasma determined by the chemiluminescence-based assay (Fig. 8b): nitrite/nitrate levels increase almost linearly up to 4 h post-administration, peak between 4 and 6 h (141.2 ± 13.6 and 160.2 ± 13.5 μM), and significantly decline thereafter, to fall to the basal values (50.5 ± 12.1 μM) at the eighteenth hour.

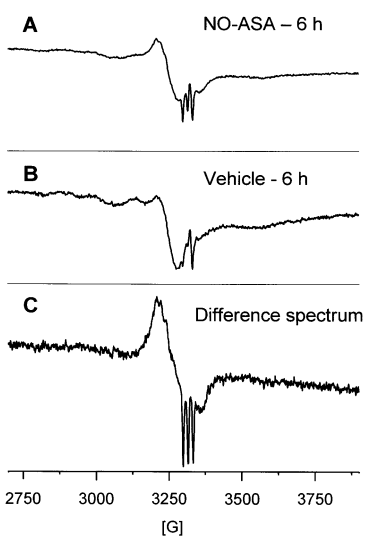

Fig. 7. ESR spectra of blood from vehicle (A) and NO-ASA treated (B) rats at the sixth hour post-administration (p.o.). The difference spectrum of nitrosylhemoglobin (C) was obtained by substracting (B) from (A). Results are representative of at least four determinations.

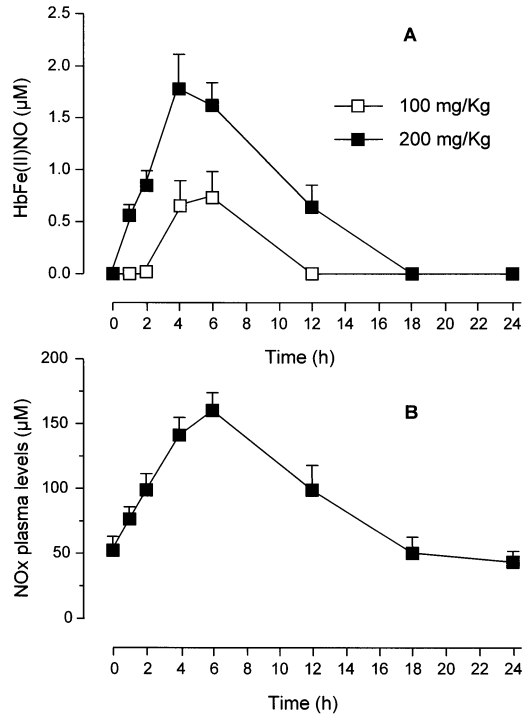

Fig. 8. Kinetics of HbFe(II)NO formation in vivo following oral administration of 100 and 200 mg kg^{-1} NO-ASA (A) and of plasma NO*x* in rats treated with 200 mg kg^{-1} (B).

When ^{15}N-NO-ASA was i.p. or p.o. administered to rats (200 mg kg^{-1}), the blood samples relative to all the observation times evidenced the spectra of HbFe(II)^{15}N-NO, with the typical two-line hyperfine structure due to the nuclear spin of ^{15}N-NO. This demonstrates that measured NO is generated from the metabolism of NO-ASA and concomitantly excludes any involvement of the drug or of its metabolites in the activation of endogenous NO-synthase. Fig. 9 shows two representative ESR spectra of blood samples relative to 1 h for i.p. and 6 h post-administration for p.o. treatment.

Finally, we also analysed, in all blood samples from i.p. or p.o. treated rats, the ESR signal in the $g = 6$ region attributed to met-Hb species (data not shown). We never found an increase in the signal intensity over the basal value at all the

observation times, even at the peak of Hb-NO formation (4–6 h p.o.; 2 h i.p.). This excludes any unphysiological oxidative interaction of NO with Hb, due to a massive release of NO from the molecule and confirms its character of slow NO releaser.

3.3. Nitrosylmyoglobin detection in NO-ASA-treated animals

In Fig. 10a is shown the typical ESR spectrum of the myocardial tissue from control rats, characterized by the signals of a semiquinone radical ($g = 2.005$) and of the reduced iron–sulfur centers ($g = 2.02$, $g = 1.94$). The assignments were further confirmed by the power saturation experiments (data not shown) performed according to Nakanishi et al. [23]. One hour after the i.p. dose of NO-ASA (100 mg kg^{-1}; Fig. 10b), the ESR spectrum of the myocardial tissue shows two additional signals at $g = 2.08$ and $g = 2.04$ attributable to the nitrosylmyoglobin complex [24]; both the signals increased in the myocardial samples of rats treated with 200 mg kg^{-1} (Fig. 10c). In addition, a concomitant increase of the signals at $g = 2.02$ and $g = 1.99$ was also observed. One hour after the i.p. doses of 100 and 200 mg kg^{-1}, the height of the signal at $g = 2.08$ (expressed in arbitrary units) increased to 2321 ± 395 and 5000 ± 821 a.u., respectively, and then reduced to 1023 ± 143 and 1234 ± 197 a.u. at the fourth hour post-administration.

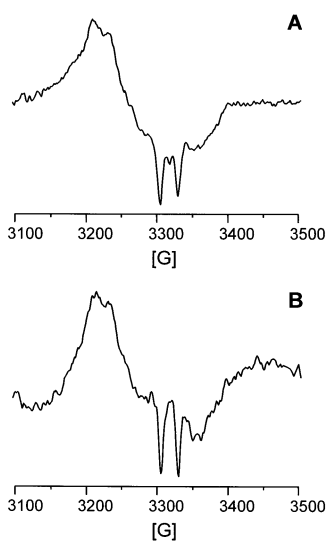

Fig. 9. ESR spectra of $HbFe(II)^{15}N$-NO in blood samples from rats treated with 200 mg kg^{-1} ^{15}N-NO-ASA. (A) 1 h after i.p. administration; (B) 6 h after oral administration.

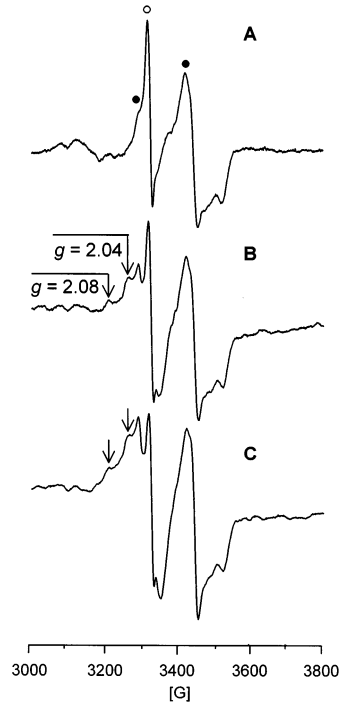

Fig. 10. ESR spectra of myocardial tissue from rats i.p. treated with NO-ASA. (A) Vehicle; (B) 1 h after treatment with 100 mg kg^{-1}; (C) 1 h after treatment with 200 mg kg^{-1}. Signals of reduced iron–sulfur centers (●) and of a semiquinone radical (○). Arrows indicate the two signals relative to nitrosylmyoglobin at $g = 2.08$ and 2.04.

4. Discussion

The first aim of this work was to achieve unequivocal evidence that the newly developed drug NO-ASA, when incubated with rat blood or when acutely administered by i.p. or oral route, is able to release NO. This has been clearly demonstrated by ESR spectroscopy determining NO in radical form as HbFe(II)NO and this analytical evidence provides strong support that the reduced gastrole-

sivity of the drug in the animal and the other pharmacological effects are due to the release of this important vasorelaxing mediator.

In this context, it is the first time to our knowledge that the ESR technique is successful in monitoring NO from a NO donor after oral administration, i.e. after extensive hepatic first pass. Until now it has been applied for the detection/quantitation of HbFe(II)NO from some prototype vasodilators used in therapy to treat/prevent angina (nitroglycerin, isosorbide mono- and dinitrate) following administration routes (i.p., i.v., sublingual) [13–16], which ensure potentially immediate effects.

From an analytical point of view, HbFe(II)NO is an ideal marker of NO formation since the high specificity of ESR technique furnishes a signal that is a real fingerprint for the complex. The second important advantage is that the determination of NO can be made directly on the biological matrix, without any sample manipulation. This means to greatly cut the analysis time and to ensure a greater reproducibility. In addition, nitrosylhemoglobin is characterized by a relatively high stability: the half-life in blood at 37 °C is 40 min, which increases at least to 30 days when the samples are immediately stored in liquid nitrogen. The long-lasting conservation of blood makes it possible to collect and process a large number of biological samples until the ESR analysis. In our conditions, the instrumental sensitivity was significantly improved in respect to the currently reported limit of detection, approximately 1 μM [16] and this was achieved using a high-sensitivity cylindrical cavity, accumulating 20 scans, setting the resolution to 1024 data points, using a 20 mW microwave power, performing the experiments at 100 K and using a blood volume of 300 μl (studies are in progress to further improve the limit of detection by performing ESR analysis on isolated erythrocytes).

Alternative methods for nitric oxide detection, such as mass spectrometry or chemiluminescence, enable one to measure even lower amounts of NO [25]. The first approach, based on NO trapping as nitrosothioproline, extraction and then derivatization, although time-consuming, is well suited to measure exhaled NO but not NO in biological matrices. The second method, the most sensitive one, is highly reliable for estimation of exhaled NO and of the NO oxidation products (nitrite/nitrate) in biological samples (i.e. plasma, tissues) after proper reduction to NO. Anyway, as previously mentioned, chemiluminescence analysis of nitrite/nitrate can overestimate the effective amount of bioactive NO. This is confirmed by our results that show plasma NO*x* levels in animals treated p.o. with a 200 mg kg^{-1} peak, as HbFe(II)NO, between 4 and 6 h post administration, but their concentrations are greatly higher (85–100 μM versus approximately 2 μM). Although we have not measured in NO-ASA-treated animals the plasma levels of other bioactive long-lasting forms of NO storage (S-nitrosothiols; RS-NO), it has been already demonstrated that total RS-NO following administration of a spontaneous NO-donor can reach maximal plasma concentrations ranging from 2 to 3 μM [26].

New microelectrode techniques, which detect NO by amperometric means [25], may be useful to measure NO production in in vitro systems (single cells, both in culture and in situ), but their specificity and reliability in ex vivo specimens still needs to be validated (no routine assays).

Other basic information stems from this work and highlights the great potentiality of the ESR technique: (a) to follow the kinetics of release of NO from different drugs in different biological matrices (blood, liver tissue, cell cultures) in vitro, thus to predict their behavior in vivo and the potential NO-mediated adverse effects; (b) to study the metabolic fate and distribution of NO after different routes of administration, in order to delineate the pharmaco-toxicological profile of a NO-donor in preclinical studies, i.e. to clarify which tissue acts as reservoir for bioactive NO, as demonstrated, for example, by the ability of ESR to specifically detect the formation of nitrosylmyoglobin in myocardial tissue as an index of effective compartmentalization of NO inside the heart; (c) to monitor the blood levels of nitrosylhemoglobin in humans following chronic treatment with nitrovasodilators to provide effective levels of NO; and (d) to check the presence of toxic met-hemoglobin.

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