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ELECTRON SPIN RESONANCE AND CHEMILUMINESCENCE ANALYSES TO ELUCIDATE THE VASODILATING MECHANISM OF SODIUM NITROPRUSSIDE

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Abstract

Aim of the study was to elucidate the vasodilating mechanism of sodium nitroprusside (SNP). To do this, SNP was i.v. infused in pigs (1.67 μmoles/Kg) and the following paramagnetic metabolites identified by ESR: i) nitrosylhemoglobin (HbFe(II)NO) as an index of the bioconservative pathway; ii) transferrin; iii) $[Fe^{(II)}(CN)_5NO]^{3-}$ and $[Fe^{(II)}(CN)_4NO]^{2-}$, the reduced penta- and tetra-coordinated intermediates of SNP; iii) methemoglobin (Met-Hb). The results indicate that: a) ≈17 % of the dose is converted to HbFe(II)NO at the end of infusion; b) NO administered as SNP does not undergo bioinactivation (oxidative metabolism) since no significant increase of met-Hb was observed b) the equilibrium involving the paramagnetic species of SNP is shifted towards HbFe(II)NO, since a significant increase of transferrin but no detection of the reduced paramagnetic intermediates of SNP was observed. The results obtained indicate that the hemodynamic effect induced by SNP is not mediated by HbFe(II)NO, at least under physiological conditions; hence a direct release of NO form SNP in the vascular target should be considered. To demonstrate this mechanism, endothelial cells were incubated with SNP and the release of NO determined by a novel chemiluminescence method. The results indicate that the endothelium is able to metabolize SNP, with the formation of stoichiometric amounts of NO. In conclusion, SNP is rapidly metabolized to HbFe(II)NO but the pharmacological response is mediated by a direct mechanism of NO release of the parent compound at the cellular target.

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Introduction

Sodium nitroprusside or sodium pentacyanonitrosylferrate $Na_2[Fe^{(III)}(CN)_5NO]$ (SNP) is a potent, rapid-acting vasodilator which is widely used clinically in hypertensive emergencies, heart failure and for controlled hypotension during surgery. Like other nitrovasodilators, SNP it thought to induce vasodilation at the vascular smooth muscle cell through a putative common intermediate, nitric oxide (NO), which by activating guanylate cyclase, catalyzes cyclic GMP accumulation and the pathways resulting in smooth muscle relaxation and the inhibition of platelets aggregation and adhesion (Murad, 1986; Ignarro, 1989).

SNP spontaneously releases NO both thermally and photochemically (Bates et al., 1991), but it is stable in the dark and in aqueous in vitro physiological media (Rao et al., 1991). Hence, energy absorption induces electron transfer from the Fe^{2+} center to the NO^+ ligand, resulting in the weakening of the Fe-NO bond and subsequent release of NO (Bates et al., 1991). SNP also decomposes to NO in an aqueous environment in the presence of biological reductants, such as thiols, which have been shown to improve the hypotensive potency of SNP (Rao et al., 1991), and ascorbic acid, whose mechanism of NO release has been elucidated (Smith and Dasgupta, 2001). Although the *in vivo* mechanism of NO release is hypothesized to involve formation of S-nitrosothiols (by reaction with GSH or cysteine) (Ignarro et al., 1981), the molecular mechanism of NO release is far to be fully elucidated. According to several *in vitro* studies, carried out in homogenous phase or in cell systems, the SNP bioactivation process would involve the following key steps (Williams, 2003):

i) reduction of Fe^{3+} to Fe^{2+} by reducing agents (ascorbic acid and deoxyhemoglobin);

ii) formation of a reduced penta-coordinated intermediate $[Fe^{(II)}(CN)_5NO]^{3-}$, in equilibrium (by loss of CN^-), with the unstable tetra-coordinated form $[Fe^{(II)}(CN)_4NO]^{2-}$;

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iii) decomposition of $[Fe^{(II)}(CN)_4NO]^{2-}$ and release of NO.

iv) reaction of NO with Hb to give nitrosylhemoglobin (HbFe(II)NO)

This mechanism has been confirmed in erythrocyte lysates, where formation of the paramagnetic species HbFe(II)NO, generated by transfer of NO to Hb *via* the tetra-coordinated intermediate, has been selectively detected by ESR (Electron Spin Resonance Spectroscopy) (Wilcox et al., 1990). The reductive mechanism in SNP bioactivation has been further confirmed in different cell system models. In particular, Rochelle et al. (Rochelle et al., 1994), by incubating SNP with porcine endothelial cells and by using ESR spectroscopy to identify the paramagnetic metabolic species of the drug, demonstrated the formation of the penta-coordinated intermediate and of nitrosylated thiol species (nonheme iron-nitrosyl-sulfur complex, Fe-NOSR), generated by reaction of the tetra-coordinated intermediate with membrane thiols. Using a sensitive and specific redox chemiluminescence assay for NO, Kowaluk et al. (Kowaluk et al., 1992) reported that SNP is readily metabolised to NO in subcellular fractions of bovine coronary arterial smooth muscle, and that the dominant site of metabolism is in the membrane fraction. This led to the isolation of a small membrane-bound protein or enzyme that requires electrogenic cofactors (NADPH, NADH) and cysteine to convert SNP to NO. Hence, although *in vitro* studies indicate that activation of SNP in mammalian tissues can occur in both erythrocytes, with formation of HbFe(II)NO, and in cell systems, with formation of nitrosylated species, no definitive *in vivo* experimental evidences support this mechanistic hypothesis. At the light of the emerging role of HbFe(II)NO in the transport and delivery of NO (Gow et al., 1999; Gross et al., 1999; Stamler et al., 1997; Stamler, 2003), the first aim of this work was to shed some light, in an *in vivo* model based on SNP infusion in pigs, on the haematic bio-activation process of SNP and on the following drug action mechanisms: a) the reductive metabolism of SNP, leading to the formation of the penta- and tetra-coordinated species and b) the vasodilating role of HbFe(II)NO, formed from

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the tetra-coordinated form by nitrosylation of haemoglobin. In addition, to gain a deeper insight into the molecular mechanism of SNP at cellular level, we also examined the potential of endothelial cells to metabolize SNP to NO, using a sensitive and selective chemiluminometric method.

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Materials and methods

Materials. Argon (6.0) and NO were kindly provided by Sapio Industrie Srl (Caponago, Milan, Italy). Hemoglobin from bovine blood, sodium nitroprusside, $K_3Fe(CN)_6$, $CuSO_4$, ethylenediaminetetraacetic acid disodium salt (EDTA), Dulbecco's phosphate buffered saline (PBS), Dulbecco's modified Eagle Medium (DMEM) were purchased from Sigma (Sigma-Aldrich S.r.l., Milan, Italy); penicillin and streptomycin, HAT media supplement, L-glutamine were from Invitrogen (Gibco San Giuliano Milanese, Milan, Italy) and fetal calf serum from Celbio (Milan, Italy); spermine NO-NOate and calcein AM from Molecular Probes (Space Import-Export, Milan, Italy).

Surgery. Eight Large White pigs of either sex, weighing 23.50±2.66 kg (SD), were used, about 3-month-old (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy). When housed in the animal facility, they were fed a standard diet with free access to water and were deprived of food 24 h before the experiment. All experiments were conducted in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals (Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Sezione di Biochimica e Fisiologia, Università degli Studi di Milano).

The animals were sedated with Domitor (medetomidine, Pfizer, Italy) at 0.03 mg/kg i.m. and Zoletil 100 (tiletamine-zolazepam, Virbac Srl**,** Italy) at 4 mg/kg i.m., and anesthetized with 15 mg/kg of thiopental sodium (Farmitalia, Carlo Erba, Italy) injected into the auricular vein. A steady depth of anesthesia was maintained during the experimental protocol by continuous infusion of a dilute solution of thiopental sodium (9 mg/kg/h). The animals were tied in the supine position on a heated operating table, tracheostomized, and intubated with an endotracheal tube inserted into the lower portion of the extrathoracic trachea. They were then paralyzed with pancuronium bromide (0.2 mg/kg i.v., Organon Teknika B.V., Boxtel,

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Netherlands) and mechanically ventilated with room air using a 900C Servo ventilator (Siemens-Elema, Sweden). Supplementary paralyzing agent was administered when necessary. The baseline ventilator settings consisted of a fixed tidal volume of 0.210±0.05L and a constant inspiratory flow of 0.350±0.04 L/s. The ratio of inspiratory to total breathing cycle duration was 0.33±0.01. The humidifer was omitted from the inspiratory line; the equipment dead space was 29.5 mL (Albertini and Clement, 1995).

Polyethylene catheters were inserted into the right femoral artery to monitor systemic arterial pressure and into the right femoral vein for drug administration. A balloon-tipped catheter (Pediatric Swan-Ganz 5F) was introduced into the right jugular vein and allowed to float through the right heart to the pulmonary artery, so that pulmonary arterial pressure and pulmonary capillary wedge pressure (Pw) could be measured. The left jugular vein was surgically isolated for infusion of SNP. Systemic and pulmonary arterial pressures were recorded by connecting the catheters to a fluid-filled capacitance manometer (4-422 Bell & Howell). Cardiac output (CO) was measured by a thermodilution technique (Cardiac Output Computer 701, Italy). All signals were calibrated independently and recorded simultaneously on a six-channel pen recorder (Polygraph mod. 8K40, Nec San-ei Instruments, Japan). Arterial blood samples were analyzed with a blood gas analyzer (Instrumentation Laboratory system 1302, Italy). The following parameters were evaluated: PO_2, PCO_2, pH, $\%O_2Hb$; systemic (SVR) and pulmonary (PVR) vascular resistances were calculated as MAP/CO and (MPAP-Pw)/CO and expressed as mmHg/L/min.

Experimental protocol. Pigs were divided into two groups: group 1 (n = 4) received only saline solution; group 2 (n = 4) received SNP (2.00 mM) in physiological solution, prepared immediately before the use and protected by the light for the all experiment. The solutions were infused through the jugular vein at a flow rate of 0.83 ml/min for 24 min, equivalent to 1.67 μmol/Kg by a syringe pump (KD Scientific, model KDS200, MA, USA).

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For HbFe(II)NO and Met-Hb analyses, 5-ml blood samples were drawn from the right femoral vein according to the experimental protocol reported in Fig. 1: after anesthesia (T_{00}); after surgery and immediately before SNP infusion (T_0); after infusion of 816 nmol/Kg (half-infusion, T_{HI}), immediately at the end of infusion (T_{EI}), and after SNP infusion (in minutes): 5 (T_5), 15 (T_{15}), 30 (T_{30}), 60 (T_{60}), 90 (T_{90}), 120 (T_{120}), and 180 (T_{180}). At the same times 2-ml samples were taken from the right femoral artery for blood gas analysis. The hemodynamic parameters (MAP, MPAP, and heart rate) were recorded continuously, while CO, SVR, and PVR were measured at T_0, T_{EI}, T_{15}, T_{30}, T_{60},T_{90}, T_{120}, T_{150}, and T_{180}.

ESR analyses of HbFe(II)NO, Met-Hb and transferrin. HbFe(II)NO. ESR spectra were recorded at 100K with a Bruker EMX spectrometer (X band) equipped with a high-sensitivity cylindrical cavity (ER4119HS; Bruker) as previously reported (Aldini et al., 2004). Concentration of HbFe(II)NO was determined by double integration of the signal using $CuSO_4$-EDTA as reference standard (Aldini et al., 2004; Carini et al., 2001).

Met-Hb and transferrin were measured by ESR spectroscopy using the following instrumental conditions: microwave frequency, 9.316 GHz; microwave power, 20mW; modulation frequency, 100 kHz; modulation amplitude, 5G; number of scans, 10; resolution, 1024 points; sweep time, 21 s; center field, 1400G. Stock solutions of Met-Hb were prepared by oxidizing Oxy-Hb with a slight molar excess of $K_3Fe(CN)_6$ followed by dialysis against 50 mM sodium phosphate buffer (pH 7.4). The concentration of Met-Hb solutions (always expressed per unit of heme) was determined by absorption spectroscopy (Perkin–Elmer Lambda 16) at 700, 630, 576, and 560 nm, using the Winterbourn relationship (Winterbourn, 1990). Working standard solutions were prepared by diluting the stock solution with 50 mM sodium phosphate buffer in the range 0.5–50 μM. The equation of the calibration curve (range 0.5–10 μM) was $y = 0.4772 \pm 0.012x + 0.1859 \pm 0.074$ ($r^2 = 0.9973$; SE of estimate Sy; x =0.101) and the

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calculated limits of detection and quantification by ESR were 0.4 and 1.0 μM respectively. Transferrin EPR signal was double integrated and expressed as arbitrary units.

Preparation and ESR analysis of the paramagnetic metabolites of SNP. Penta- and tetra-coordinated complexes were prepared by using $NaBH_4$ as reducing agent as previously described (Kruszyna et al., 1993). ESR analyses were carried out in the following conditions: microwave frequency, 9.316 GHz; microwave potency 20 mW; modulation frequency 100 kHz; modulation amplitude 5 G; number of scans 20; resolution 1024 points; center field 3500 G; sweep width 5000 G. The same operating conditions were used to identify the paramagnetic metabolites of SNP in blood (0.3 ml aliquots).

Data analysis. All values in the figures and text for HbFe(II)NO and Met-Hb, are expressed as means ± standard deviation (SD), those for hemodynamics as means ± standard error (SE). The statistical significance of differences between vehicle and SNP-treated animals was done by t test analysis. Statistical analysis was done using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California, USA). Significance was taken where $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Cell cultures and SNP incubation. EA.hy926 cells were cultured as previously described (Aldini et al., 2003) and cell viability determined by the calcein AM assay (Oral et al., 1998). For incubation experiments with SNP, confluent endothelial cells in 25 cm^2 flasks (~5.2 x 10^5 cells/mL), were washed twice with PBS and then incubated in PBS spiked with SNP (0.1, 0.2 and 0.3 mM in PBS, corresponding to 1, 2 and 3 nmoles added). During the experiments the flasks were maintained at 37°C under orbital shaking and protected from light exposure (by covering with aluminium foil)

Chemiluminescent detection of NO: flask-chemiluminometer connection. NO released from SNP was determined by an ozone-based chemiluminescent assay, using a Sievers Instruments Model 280 Nitric Oxide Analyzer (NOA; Sievers, Boulder, Colorado,

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USA; Sensor Medics, Milan, Italy). Because the apparatus is dedicated to liquids sampling (plasma or tissue homogenates for determination of nitrites/nitrates), it was suitably adapted in our laboratory for the NO detection directly from the incubated cells. Fig. 2 reports a schematic representation of the apparatus used. The flasks were maintained under a constant flux of helium purged through distilled water and connected to the flask through a Teflon tube, sealed with the flask screw caps. The flask outlet was connected to the reaction chamber of the chemiluminometer through a second Teflon tube. Cells were maintained at 37° C through heater coil water connected to a thermostated circulating water bath.

NO quantitation: calibration curve. Spermine NO-NOate was used as a calibrating standard, since its thermal decomposition is known to release NO at a constant rate (Maragos et al., 1991). Stock solutions of spermine NO-NOate (0.6 mM), prepared in 0.01 M NaOH, were diluted with 0.01 M NaOH to obtain working solutions at the following final concentrations: 0.05, 0.075, 0.1, 0.2 and 0.3 mM. Aliquots (10 µL) of each working solution were injected into the reaction vessel containing PBS (6 mL) maintained at 37°C. The concentration range for calibration, expressed as nmoles of injected spermine NO-NOate was 0.5 – 3 nmoles, corresponding to 0.95 – 5.7 nmoles NO. Calibration standards were analyzed in duplicate in three independent runs. The calibration curves were constructed by weighted (1/x2) least-square linear regression analysis of the peak area against analyte concentration (nmoles NO). The lower limit of quantitation (LLOQ) was determined as the lowest concentration with values for precision and accuracy within ± 20% and a signal-to-noise (S/N) ratio of the peak areas ≥ 10. The extraction efficiency of NO from the flask was determined by comparing the chemiluminometric response of spermine NO-NOate added to the flask to that of an equimolar concentration added to the conventional reaction vessel (extraction efficiency 100%). The overall absolute extraction efficiency was measured as the ratio of the two areas under the curves (AUCs) obtained, and expressed as percentage.

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Results

Identification of blood paramagnetic species. Fig. 3 shows a representative ESR spectrum (A) of a blood sample after anesthesia and before surgery (T_{00}), with 3 main signals attributed to Met-Hb (g = 5.714), transferrin (g = 4.110) (see upper panel), and to the Cu^{2+} of ceruloplasmin (g = 2.054). As observed in previous studies (Aldini et al., 2004), no signal was detected for HbFe(II)NO (see the display of the 3100-3500 G region on the right), to confirm that the endogenous levels in pigs are below the limit of ESR detection (0.25 μM). In all the animals subjected to 30 min equilibrium after surgery and before vehicle or SNP infusion (time T_0), the signal relative to HbFe(II)NO becomes detectable (Fig.3 spectrum B), as evidenced by the presence of the three lines of the HbFe(II)NO signal centered at g = 2.009 (indicated by the arrows in the 3100-3500 G region spectrum). In Fig. 3 are also reported the ESR spectra of blood samples taken 12 min (T_{HI}) and 24 min (T_{EI}) after the start of SNP infusion (spectra C, D), where the additional signal at $g \approx 2.00$ significantly increases in respect to the T_0. Attribution was achieved by subtracting the T_{00} spectrum from those recorded at T_0, T_{HI} and T_{EI}. The difference spectra (Fig. 4 panel A reports that relative to T_{00} subtracted from T_{EI}) shows two distinct signals, that are significantly increased in respect to T_0: at g = 4.110, attributed to the Fe^{3+} of transferrin (whose increase indicates iron release from SNP), and a signal centered at g = 2.009 with a hyperfine coupling constant $A_N = A_X$ = 17 G, that overlaps that reported for the T state of nitrosylated purified α-subunits of hemoglobin (Fig.4, B and C) (Yonetani et al., 1998). The increase of the HbFe(II)NO signal after SNP infusion was evident in all the treated animals, while no significant differences in the HbFe(II)NO signal intensity was observed either during or after the vehicle infusion.

Because early in the in vitro reaction of SNP with erythrocyte lysates the ESR signals of both $[Fe^{(II)}(CN)_5NO]^{3-}$ and $[Fe^{(II)}(CN)_4NO]^{2-}$ species were observed (Wilcox et al., 1990), we have

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prepared and analyzed by ESR the relative standards, in order to verify if their formation takes place also in vivo. ESR analyses indicate that no detectable signals relative to the penta- or tetra-coordinated species were detected in blood samples withdrawn during or post-SNP infusion.

Nitrosylhemoglobin levels. Fig.5 shows the time-course of HbFe(II)NO formation from vehicle and SNP-treated animals. Quantitative analysis indicates a significant increase in the blood levels of all the pigs following surgery (T_0), which rose to 1.30 ± 0.91 μM (n = 8), very likely due to endothelial activation induced by arterial and venous catheterization. HbFe(II)NO levels did not significantly change during and after vehicle infusion, and remained in a concentration range between 0.87 and 1.45 μM. After 12 min of SNP infusion, HbFe(II)NO content rose from 1.18 ± 0.26 μM to 3.07 ± 0.15 μM ($p< 0.05$), reaching a C_{max} of 4.48 ± 1.21 μM at the end of infusion (T_{EI}) (0.80 ± 0.52 μM in vehicle-treated animals; $p<0.01$). The kinetics of HbFe(II)NO at the subsequent observation times confirm an exponential decay and a half-life ($t_{1/2}$) of 14 min, as previously demonstrated in pigs following infusion of a saturated NO solution (Aldini et al., 2004). The HbFe(II)NO blood content reached approximately the pre-infusion value 30 min after the stop of the infusion (1.48 ± 0.61 μM; $p > 0.05$) and remained almost stable up to the last observation time (T_{180}).

Methemoglobina and transferrin levels. In previous studies we have demonstrated that plasma NOx (nitrite + nitrate) can not be used in pigs as a reliable marker of the oxidative metabolism of NO, because the very high basal levels (≈ 100 μM at T_0, 2-3 fold greater than those found in other animal species and in man) do not allow to accurately measure minimal variations due to exogenous NO (Aldini et al., 2004). For this reason Met-Hb, a product of the oxidative reaction between Oxy-Hb and NO (Gladwin et al., 2000), can be considered as a valid and alternative marker on NO oxidative metabolism in pigs.

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The results, reported in Fig.5, indicate that basal levels (T_0) of Met-Hb are 6.71 ± 0.33 μM, a value that remained almost stable in vehicle-treated animals during and post saline infusion. Unlike to that observed following NO administration (Aldini et al., 2004), SNP infusion did not affect Met-Hb levels: no significant differences were observed in the drug-treated group at all observation times.

Fig. 5 shows the time-course of the ESR signal relative to blood transferrin in vehichle- and SNP-treated animals. Saline infusion did not modify transferrin levels, the ESR signal at all post-infusion times being comparable to that at T_0. In SNP-treated animals a time-dependent increase of the transferrin signal was recorded, which was already significant at half-infusion (T_{HI}), and plateaued at T_{EI}. The levels significantly decreased only at T_{120}, then gradually fell back to pre-infusion levels.

Haemodynamic effects. As shown in Fig 6, SNP infusion evoked an immediate and significant ($p<0.05$) decrease in mean systemic and pulmonary blood pressures. The acute hypotensive state occurring with SNP infusion was compensated by adequate hemodynamic responses: the decreased blood pressure was matched by a significant increase in heart rate and a moderate elevation in cardiac output. Although the latter parameter could not be measured at T_{HI}, while the hypotensive peak occurred, not to interfere with the drug infusion, the relative values achieved at T_{EI} showed a trend of increase, consistent with the physiological events which intravenous sodium nitroprusside is reported to rouse. In other words, SNP acted directly on vascular smooth muscle causing vasodilation. As it was expected, during the hypotensive period systemic and pulmonary vascular resistances also decline in SNP-group, although to levels which were not statistically significant; in particular, there was a reduction in SVR of 31% and of 15% in PVR.

In vitro studies: method development and validation. The first step of the in vitro studies was to develop and validate a suitable methodology to monitor NO release from

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endothelial cells incubated with SNP. To do this, the flask containing the cell monolayer was maintained under a constant flux of helium to guarantee the extraction and transport of NO to the chemiluminometric system. Extraction efficiency from the flask was calculated using spermine NO-NOate in PBS as a standard because this polyamine/NO adduct spontaneously decomposes to NO at 37°C and pH 7.4, with a known rate constant for decomposition (half-life at 37°C= 39 min), and with a stoichiometric conversion factor of 1.9 (Maragos et al., 1991). Fig.7 shows the chemiluminometric signal (recorded in continuo) obtained by adding 1.5 nmoles spermine NO-NOate to 6 ml PBS in the 25 cm^2 flask. A rapid increase of the NO signal is obtained immediately after injection, whose intensity, fairly constant for approximately 20 min, progressively and linearly decreases with time, to reach basal levels after 220 min.

The instrumental responses obtained by injecting the spermine NO-NOate working solutions were integrated and calculated as AUCs. The calibration curves were fit over the entire calibration ranges (1.41-5.70 nmoles injected), using a $1/x^2$ weighted quadratic fit and showed good linearity with correlation coefficient (r^2) greater than 0.998. The equation for the calibration lines was $y = 160700 \pm 21240x - 107000 \pm 68010$. The AUCs values relative to each concentration level were comparable to those obtained by directly injecting the spermine NO-NOate working solutions into the conventional reaction vessel for liquids sampling (RSD ≤ 10%), to indicate an almost superimposable extraction efficiency (≈ 100%).

SNP incubation with endothelial cells. Fig. 8A reports the time-course of the chemiluminometric response obtained by incubating endothelial cells at 37°C in the absence of any added substrate (cell viability was unaffected up to 180 min of incubation). As expected, no release of NO was detected up to 180 min incubation due to the anaerobic conditions generated by a constant flux of helium that do not permit the endogenous generation of NO by NO-synthase. A similar chemiluminescent profile was seen by

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incubating SNP (2 nmoles) in PBS and in the absence of endothelial cells, thus to exclude any artifactual and spontaneous release of NO from SNP (Fig.8B). When 1 and 2 nmoles of SNP were added to the cell monolayer (Fig.8, C and D, respectively), a dose-dependent increase in the NO signal was observed already after few minutes of incubation, to indicate the ability of endothelial cells to catalyze NO release from the drug. The time-course of NO formation is similar at both dosages: maximal formation is observed after 30 min incubation, to slowly decrease and return to basal levels within 130 min (1 nmole) and 180 min (2 nmoles). Cell viability was not affected at all the SNP doses tested.

Because it is known that SNP spontaneously releases NO photochemically (Bates et al., 1991), at the end of the experiments (≈ 180 min) the reaction flasks were illuminated by a tungsten lamp in order to evaluate the completeness of the SNP biotransformation process. As shown in Fig. 8 (C, D) no further increase of the NO signal was detected following the light exposure, in respect to a SNP solution (C, inset), to indicate the ability of endothelium to induce a total conversion of SNP to NO. Quantitative analysis indicates that SNP bioconversion to NO is dose-dependent and accounts for more than 75% of the incubated doses. The remaining 25% is very likely converted to other metabolic species such as S-nitrosothiols or Fe-NOSR complexes (Wilcox et al., 1990).

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Discussion

It has been previously demonstrated that under aerobic and anaerobic conditions, SNP can undergo a one-electron exchange reaction with haemoglobin, which produces Met-Hb and cyanide. This one-electron reduction of SNP is required for NO release and its transfer to other heme(II). The reaction between SNP and Hb has been demonstrated to occur both in solution and in intact or lysate red blood cells, with formation of nitrosylated Hb, but no evidence for its in vivo occurrence have been until now provided (Wilcox et al., 1990; Kruszyna et al., 1993).

The results here reported demonstrate for the first time, in an in vivo animal model, that SNP is rapidly bioconverted in blood, with release of iron and HbFe(II)NO formation. ESR analysis of blood samples from SNP-infused pigs provided in fact evidence that transferrin and HbFe(II)NO are the only detectable paramagnetic metabolites generated in vivo. Although no quantitative data have been generated in this study, the increase in the transferrin ESR signal during SNP infusion can be considered an unequivocal index of a rapid compartmentalization of released Fe(II) into transferrin, deriving from decomposition of $[Fe(II)(CN)_4]^{2-}$. Regarding the paramagnetic metabolites of SNP, both $[Fe(II)(CN)_4]^{2-}$ and $[Fe(II)(CN)_5]^{3-}$ were not detected during or following the SNP infusion, suggesting that the reduced penta- and tetra-coordinated SNP species are transient metabolic intermediates and that the following equilibria are in vivo mainly shifted towards the reaction products.

$$[Fe^{(III)}(CN)_5NO]^{2-} + e^- \leftrightarrows [Fe^{(II)}(CN)_5NO]^{3-} \quad \textbf{(1)}$$

$$[Fe^{(II)}(CN)_5NO]^{3-} \leftrightarrows [Fe^{(II)}(CN)_4NO]^{2-} + CN^- \quad \textbf{(2)}$$

$$[Fe^{(II)}(CN)_4NO]^{2-} + HbFe^{(II)} \leftrightarrows [Fe^{(II)}(CN)_4]^{2-} + HbFe^{(II)}NO \quad \textbf{(3)}$$

Reaction **(2)** generates cyanide, whose toxicity is well documented, as well as the possible cyanide poisoning at excessive total SNP doses or infusion rates (Friederich and Butterworth,

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1995). Anyway, cyanide is rapidly cleared by enzymatic transsulfuration reactions (rhodanase and mercaptopyruvate sulfurtransferase) (Nagahara et al., 1999; Scheffler, 2001) and nonenzymatic means in erythrocytes, mainly by interaction with both ferric and ferrous heme proteins, giving highly stable complexes, values of the association equilibrium constant being higher than 10^5 M^{-1} (Milani et al., 2004). Hence, by considering the ability of most adults to detoxify by the enzymatic routes up to 50 mg total SNP (Friederich and Butterworth, 1995), and the high reactivity towards heme proteins (not determined because outside the aim of this work), we can reasonably assume that the *in vivo* rapid subtraction of cyanide would shift equilibrium **(2)** in favour of the tetra-coordinated form, which is obligatory for NO transfer. The $[Fe^{(II)}(CN)_4NO]^{2-}$ species is in turn in equilibrium with HbFe(II)NO and with $[Fe^{(II)}(CN)_4]^{2-}$ species. By considering the HbFe(II) excess, the favourable kinetics of HbFe(II)NO formation ($k_{on} = 10^7$ M^{-1} s^{-1}) (Yonetani et al., 1998), and the metabolic conversion of $[Fe^{(II)}(CN)_4]^{2-}$ with transferrin formation, we can deduce that also equilibrium **(3)** is mainly shifted toward the reaction products.

Hemodynamic parameters indicate that SNP induces a significant reduction in systemic and pulmonary arterial pressure, the vasodilating effect started from the beginning of the infusion (T_0). In particular the infusion of SNP produced a mark lowering in mean arterial blood pressure, which fell during the infusion and returned to pre-injection values upon cessation of infusion, increase in cardiac output and a decrease in peripheral resistance. The vasodilating effect induced by SNP could be mediated by HbFe(II)NO, a stable bioactive storage form of NO, able to transport the vasoactive mediator distally from the site of administration (Aldini et al., 2004). By considering a total blood volume in pig of approximately 2L, and the amount of HbFe(II)NO determined at the end of infusion (C_{max} 4.15 ± 0.69 vs. 0.80 ± 0.52 μM), the efficiency of SNP bioconversion to HbFe(II)NO accounts for ≈17% of the administered dose (≈ 40 μmoles in 24 min). Taking into account

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that 5 nM (EC50) is the NO concentration required to induce a vasoactive response in aortic ring (Brandes et al., 2000), the amount of HbFe(II)NO following the SNP injection should be high enough to potentially induce a vasoactive response. However, in previous experiments carried out in the same animal model we found that an equimolar dose of a saturated NO solution (24 min infusion in pigs) was much more efficiently converted to HbFe(II)NO (equivalent to 40% of the administered dose) but did not induce any haemodynamic response under physiological conditions (Aldini et al., 2004). Taken together, these data exclude any role for HbFe(II)NO in the vasodilating mechanism of SNP and further strength the hypothesis that NO captured by deoxygenated hemes is not an effective way to subsequently transduce NO bioactivity, at least in physiological conditions, as also confirmed by the slow off-rate ($\approx 10^{-3}$-10^{-5}) of HbFe(II)NO (Kim-Shapiro et al., 2006).

Although methemoglobinemia is a well known side effect of SNP infusion, in our experimental conditions no significant increase of Met-HB was observed during and after SNP infusion. The data are explained by considering the dose employed (1.67 μmol/Kg) which is significantly lower in respect to the dose (more than 46 μmole/Kg) typically required to generate 10% methemoglobinemia (Friederich and Butterworth, 1995). Met-Hb, beside to be the product of the redox reaction between Heme(II) and SNP, is also produced by the oxidative reaction of Oxy-Hb and NO and for this reason considered as a marker of NO bioinactivation by oxidative metabolism (Gladwin MT, et al., 2000). We previously found that an equal dose of a genuine NO solution (1.67 μmol/Kg) induced a significant increase of Met-Hb in respect to the basal content, and that the amount of NO entering the oxidative metabolism was ≈25%. Taken together the data well indicate that NO administered as SNP does not undergo a significant oxidative dependent bioinactivation. Very likely the reduced

tetra-coordinated species preferentially donates NO to deoxyhemoglobin with formation of the nitrosyl-derivative rather then to the oxygenated form, with formation of Met-Hb.

By considering that HbFe(II)NO is not involved in the vasodilating mechanism of SNP, a direct release of NO form SNP in the vascular target should be considered to explain the vasodilating mechanism. This also taking into account, on the basis of HbFe(II)NO and MetHb levels, that a significant proportion of infused SNP does not undergo a metabolic conversion in blood and it is potentially available to reach the vascular district and to be bioactivated at cellular level. This hypothesis is further sustained by considering that SNP undergoes a 30% extraction from the blood during a single passage through peripheral vascular beds in the rat in vivo (Kreine and Reske, 1982). To act as vasodilating agent, SNP might be metabolized by vascular tissues to NO, as already demonstrated in smooth muscle cells (Kowaluck et al., 1992). These considerations prompted us to investigate in vitro the ability of vascular endothelial cells to release NO from SNP. Through a novel developed (and suitably validated) experimental model, based on the sensitive chemiluminometric detection of released NO, it was possible to demonstrate that more than 70% of the incubated SNP dose is converted by endothelial cells to NO. Because of the claimed fundamental role of the sulfhydryl-containing compounds (GSH, cysteine) in inducing NO release form SNP, we can reasonably postulate that NO generation at endothelial level might follow the mechanism recently proposed by Grosso and D'Angelo (2005). An electron-transfer process can be invoked as the key step, which leads to the formation of the reduced SNP radical and the corresponding S-nitrosothiol, the ending product of NO that can be considered the real storage and transporter of NO. Under anaerobic conditions S-nitrosothiols can undergo thermal decomposition by homolytic cleavage of the S-N bond that leads to nitric oxide and sulfanyl radicals in a reversible reaction. As a consequence, the decomposition rate is strongly

decreased by the presence of endogenous and/or exogenous nitric oxide (Grossi and Montevecchi, 2002). Because in our conditions NO is continuously removed by the stream of helium, the decomposition rate is increased, favoring the escape of NO from the reaction vessel. The remaining 25% of unrecovered NO is very likely converted to other metabolic species, such as Fe-NOSR, as previously demonstrated by Rochelle et al. (Rochelle et al., 1994), or is conserved as S-nitrosothiols (not determined in this study).

In conclusion, the results of the in vivo studies demonstrate that NO released in the blood compartment and transported as HbFe(II)NO is not involved in the vasodilating mechanism. In vitro studies clearly indicate that endothelium is able to metabolize SNP, with the formation of stoichiometric amounts of NO, the agent responsible for vasodilation. Hence, we can postulate that the amount of the administered drug in our *in vivo* model that escapes the bioconservative pathway (more than 80% of the dose), reaches the endothelium: this means that the pharmacological response of SNP is mediated by a direct mechanism of NO release/metabolization of the parent compound at the cellular target. Fig. 9 summarizes the proposed metabolic scheme for SNP bioactivation and vasodilating mechanism.

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Footnotes

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Legends for Figures

Figure 1 - SNP infusion in pigs: experimental protocol.

Figure 2 - Schematic representation of the modified chemiluminometric apparatus for determination of NO released from endothelial cells incubated with SNP. Cells were maintained at 37° C through heater coil water and the NO generated by EC in equilibrium with the gas phase, is stripped by a constant flux of helium. The flask outlet is connected to the reaction chamber of the chemiluminometer through a Teflon tube. Abbreviations: EC: endothelial cells (EA.hy926); PMT: photomultiplier tube.

Figure 3 - Representative ESR spectra of blood samples drawn before (T_{00}, A and T_0, B) and during (T_{HI}, C; T_{EI}, D) SNP infusion. Spectra A-D were recorded by using a wide scan range (center field of 2400 G, sweep width of 3200 G) in order to identify all the paramagnetic blood metabolites and the following instrumental conditions: microwave power, 20mW; modulation frequency, 100 kHz; modulation amplitude, 5G; number of scans, 1; resolution, 1024 points. Met-Hb and transferrin (upper box, A'') were determined by setting the center field at 1400 G (sweep width of 1200 G) and increasing the number of scans to 20. For a better visualization, the signal relative to Met-Hb has been magnified in respect to transferrin. Spectra in the right box were recorded using a center field of 3300 and are relative to T_{00} (A') and T_0 (B'). The arrows in spectrum B' indicate the appearance of three lines centred at g = 2.009 and attributed to HbFe(II)NO.

Figure 4 – Representative difference spectra relative to T_{00} subtracted from T_{EI} and recorded with a scan range 800-4000 G (A) and 3000-3600 G (B). Spectra (C) and (D) are relative to standard samples of HbFe(II)NO and $[Fe^{(II)}(CN)_5NO]^{3-}$, respectively.

Figure 5 – Blood levels of HbFe(II)NO, MetHb and time-course of transferrin ESR signal in pigs treated with saline (○) or SNP solution (■). The data are represented as the mean ± S.D.

(n=4). t test analysis was used to compare the two groups at each time point (*, $P < 0.05$; **, $P < 0.01$;***, $P < 0.001$).

Figure 6 – Time-dependent effects of SNP (■) and vehicle (○) infusion on MAP, MPAP and HR. Values are mean percentage ± SE. t test analysis was used to compare the two groups at each time point (*, $P < 0.05$; **, $P < 0.01$).

Figure 7 - Calibration of the chemiluminometric system for quantitative analysis of NO: time-course of NO release obtained by adding 1.5 nmoles spermine NO-NOate to 6 ml PBS in the 25 cm^2 flask

Figure 8 - Time-course of NO release determined by chemiluminescence analysis in the following experimental conditions: cultured endothelial cells (A), PBS spiked with 2 nmoles SNP (B); cultured endothelial cells incubated with 1 nmole (C) and 2 nmoles (D) of SNP.

Figure 9 - Proposed metabolic scheme for SNP bioactivation and vasodilating mechanism

Figure 1

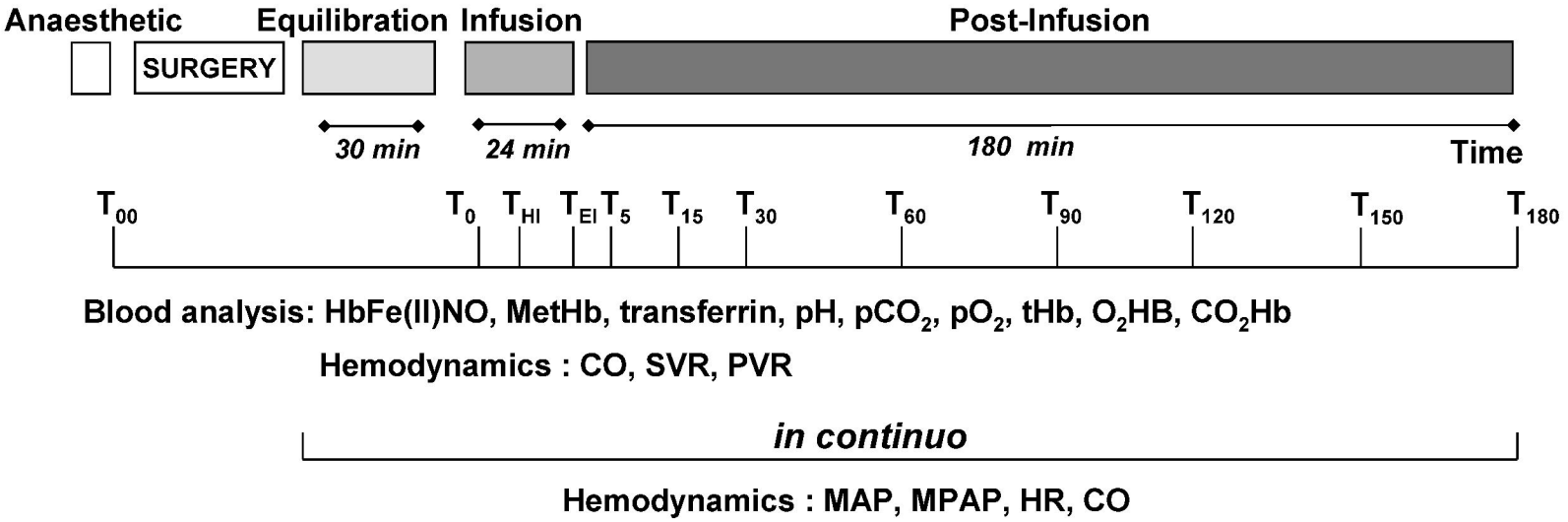
Anaesthetic
Equilibration
Infusion
Post-Infusion
SURGERY
30 min
24 min
180 min
Time
T00
T0
THI
TEI
T5
T15
T30
T60
T90
T120
T150
T180
Blood analysis: HbFe(II)NO, MetHb, transferrin, pH, pCO2, pO2, tHb, O2HB, CO2Hb
Hemodynamics : CO, SVR, PVR
in continuo
Hemodynamics : MAP, MPAP, HR, CO

Figure 2

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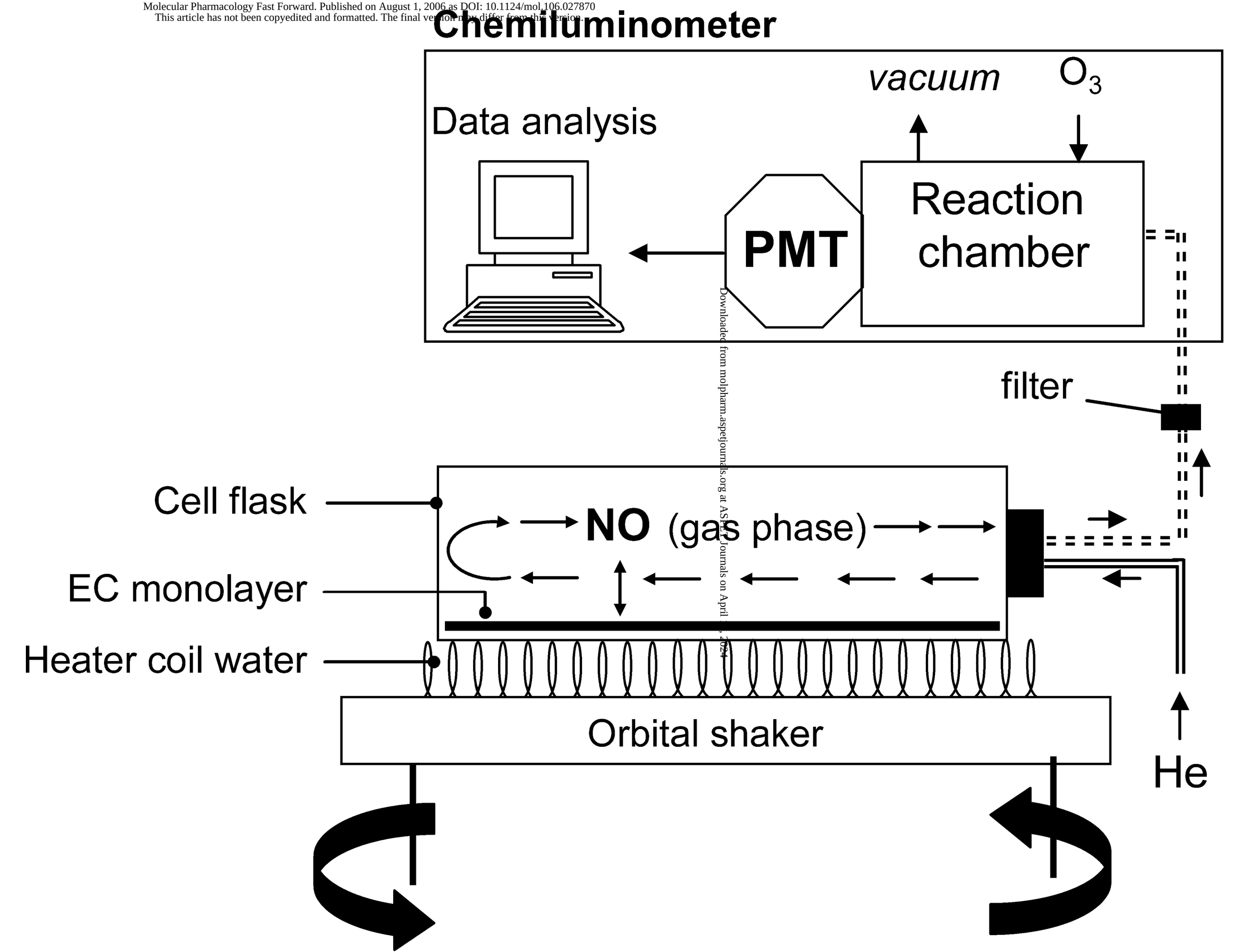
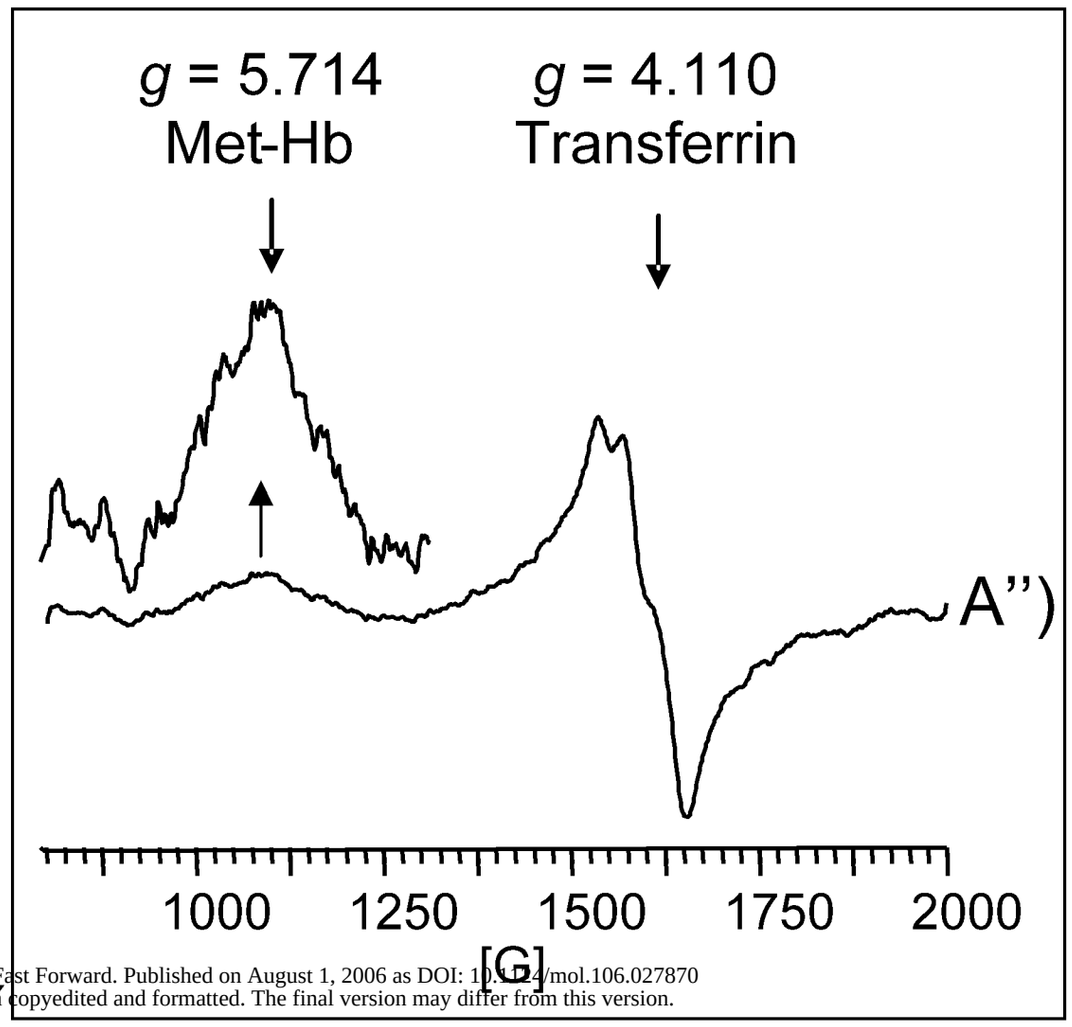
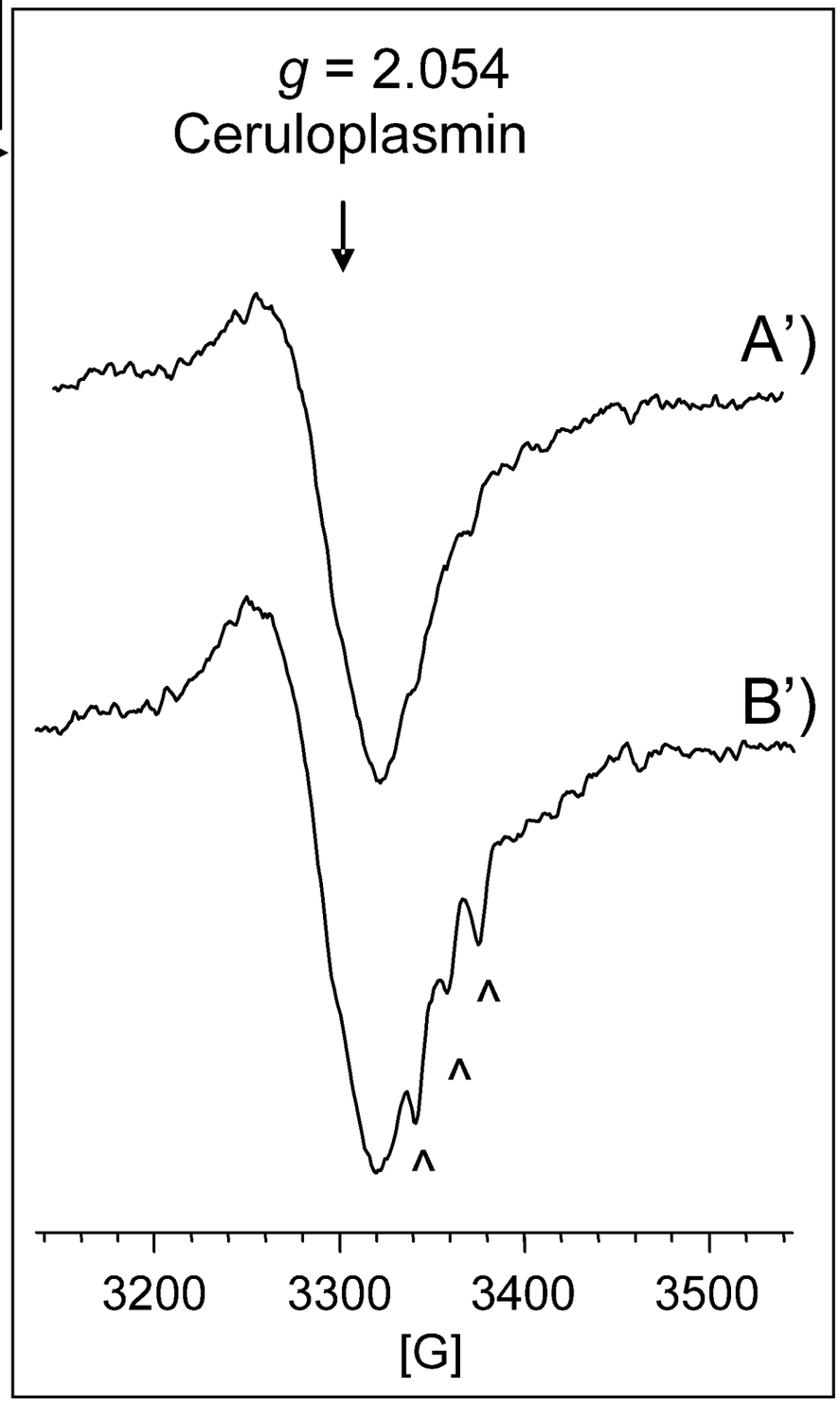


Figure 3





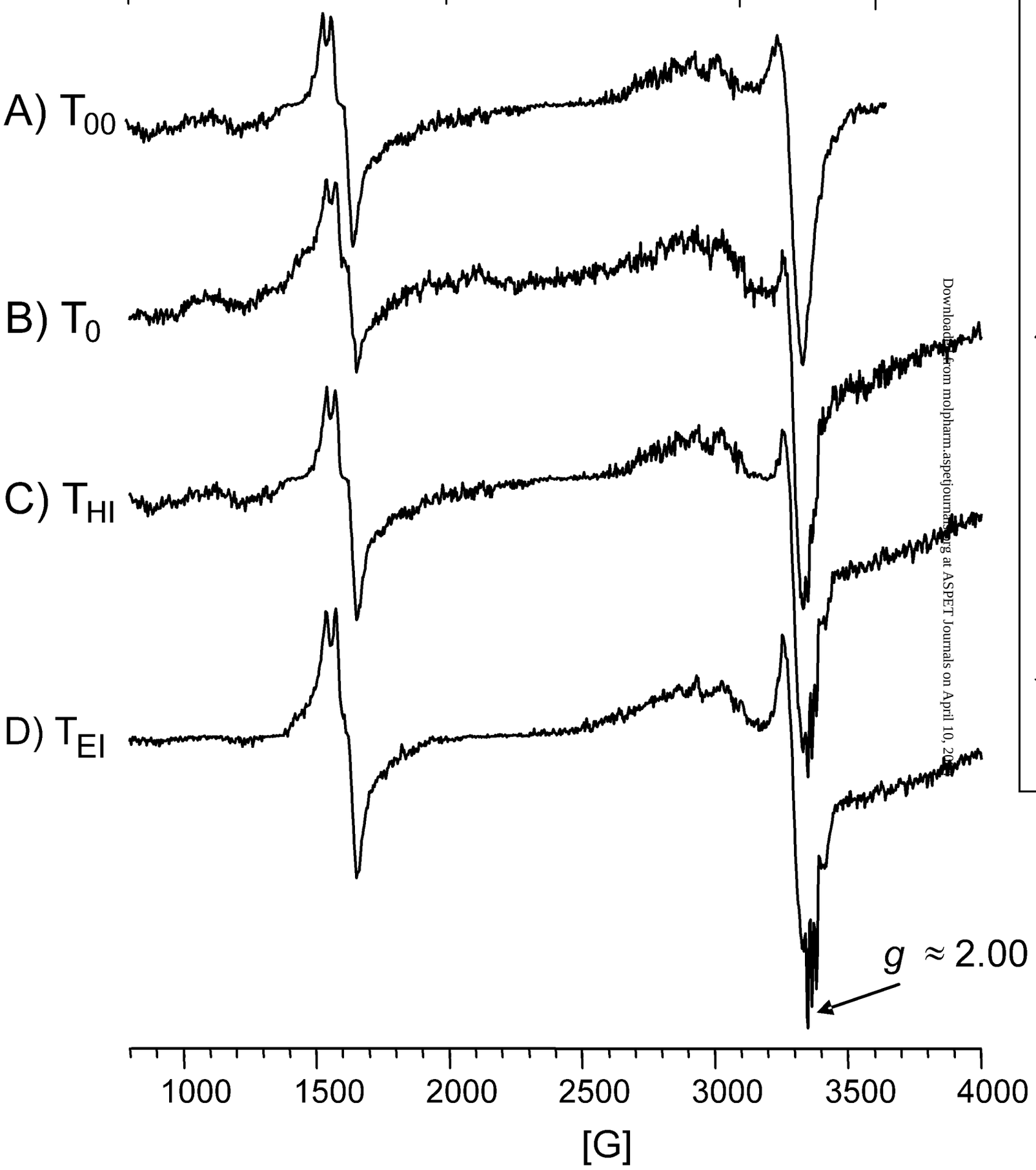
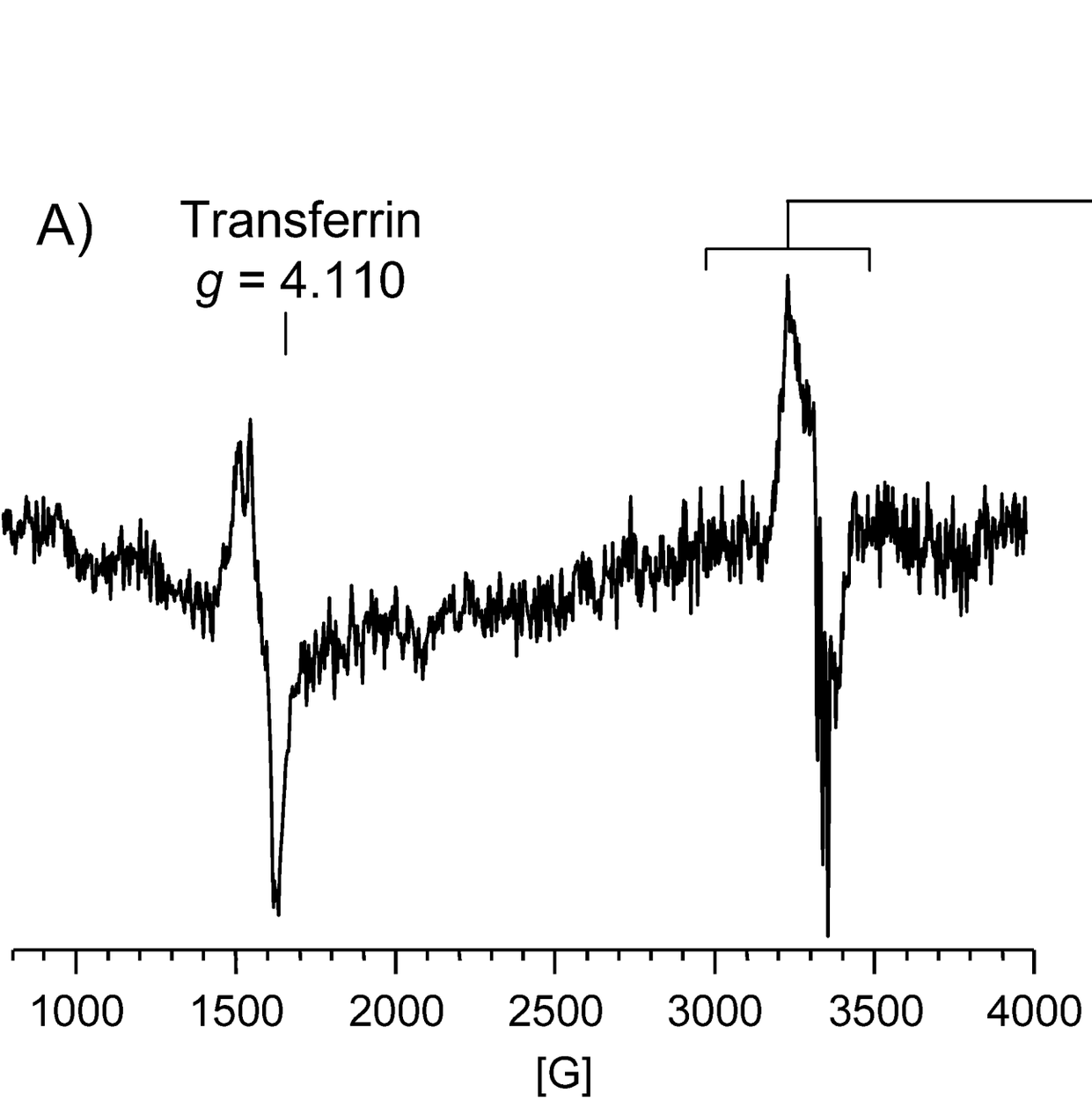


Figure 4



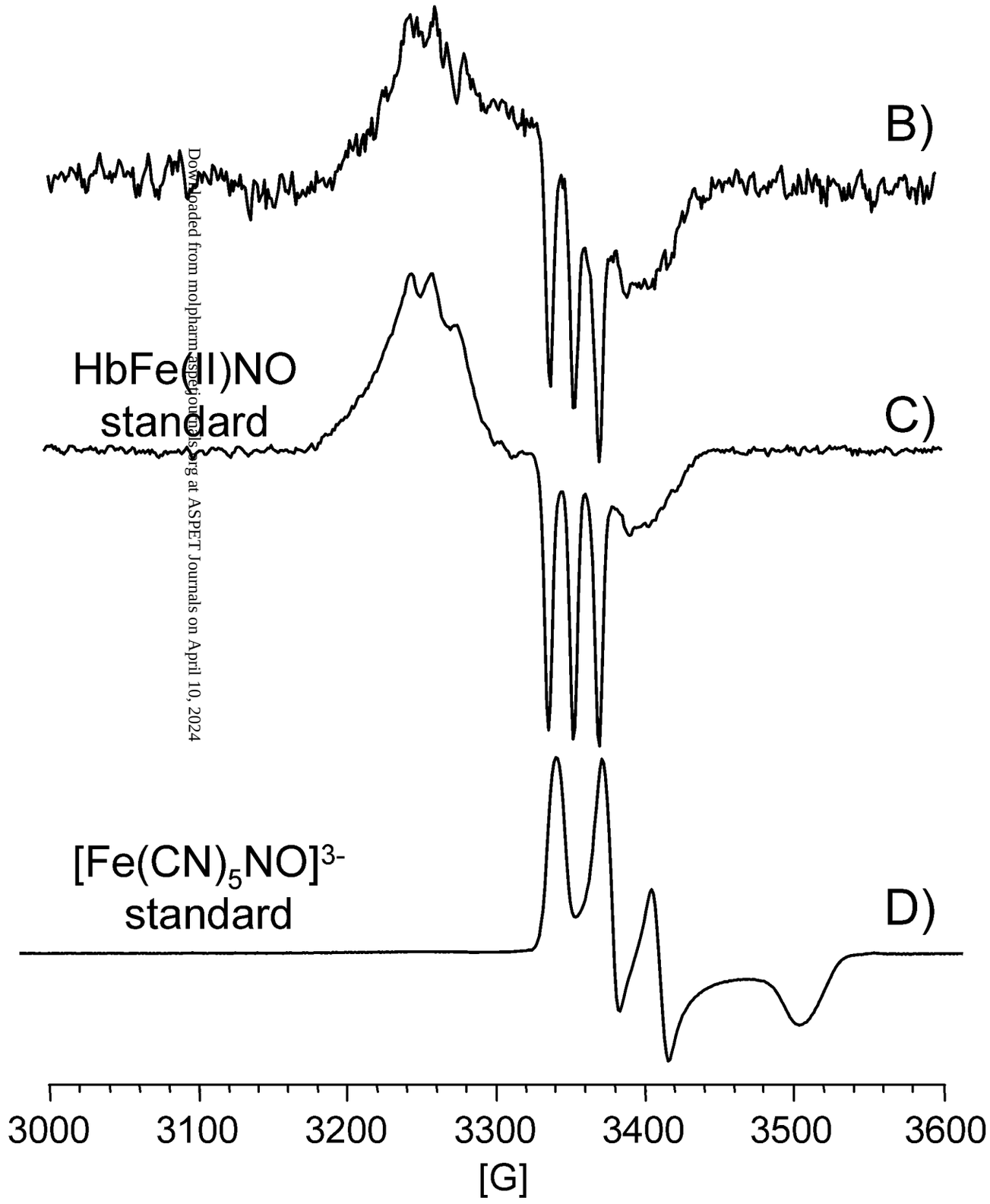
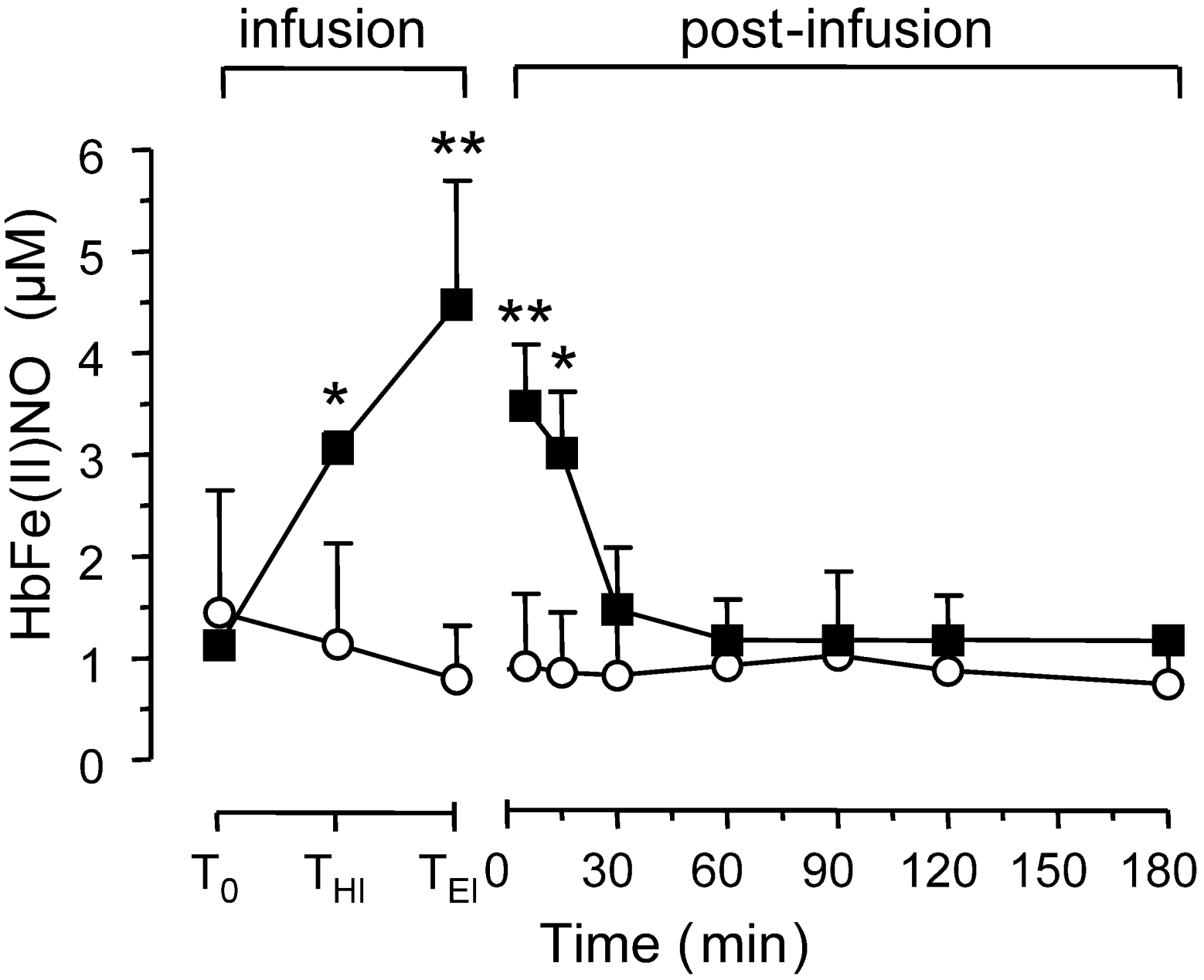
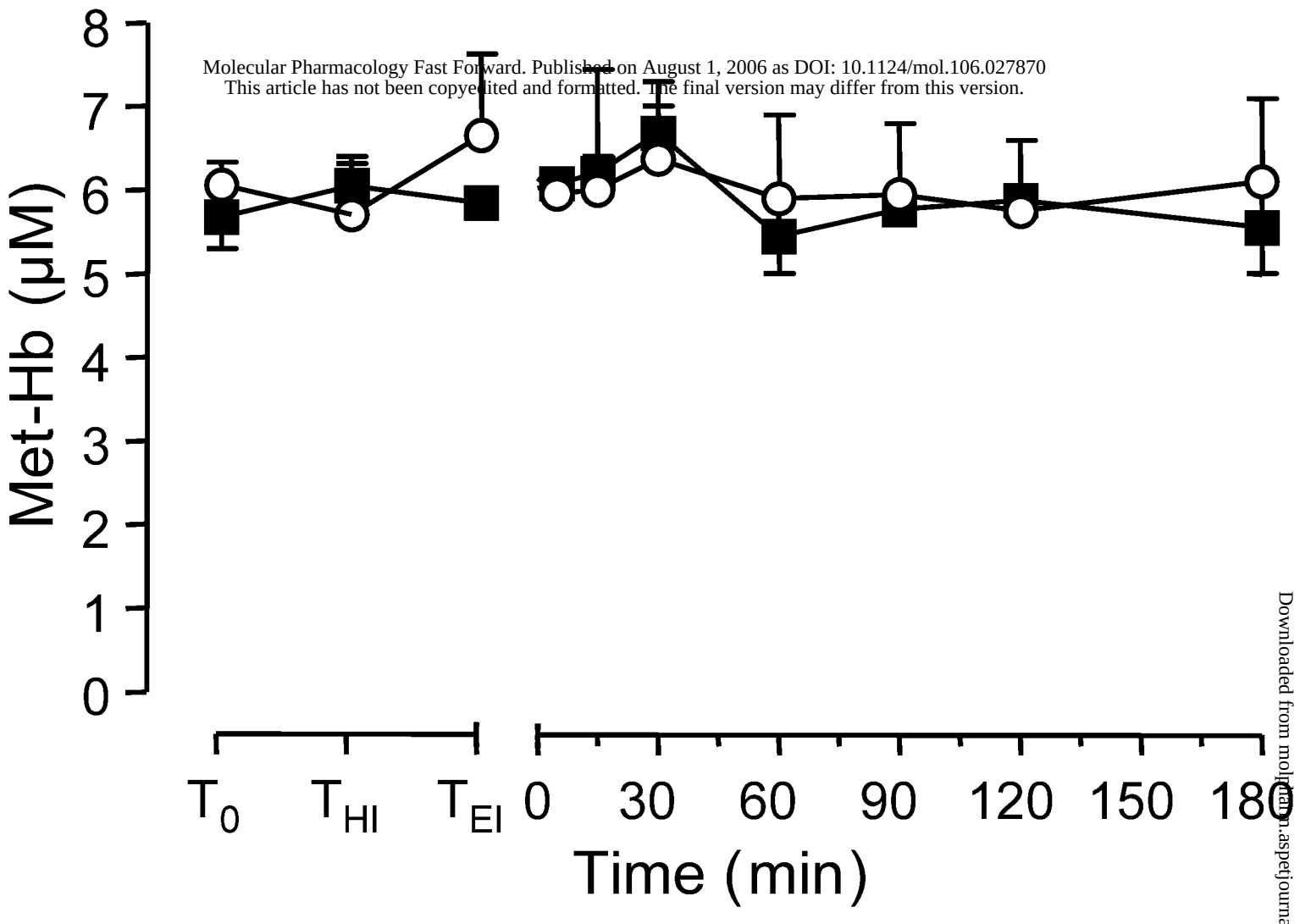


Figure 5





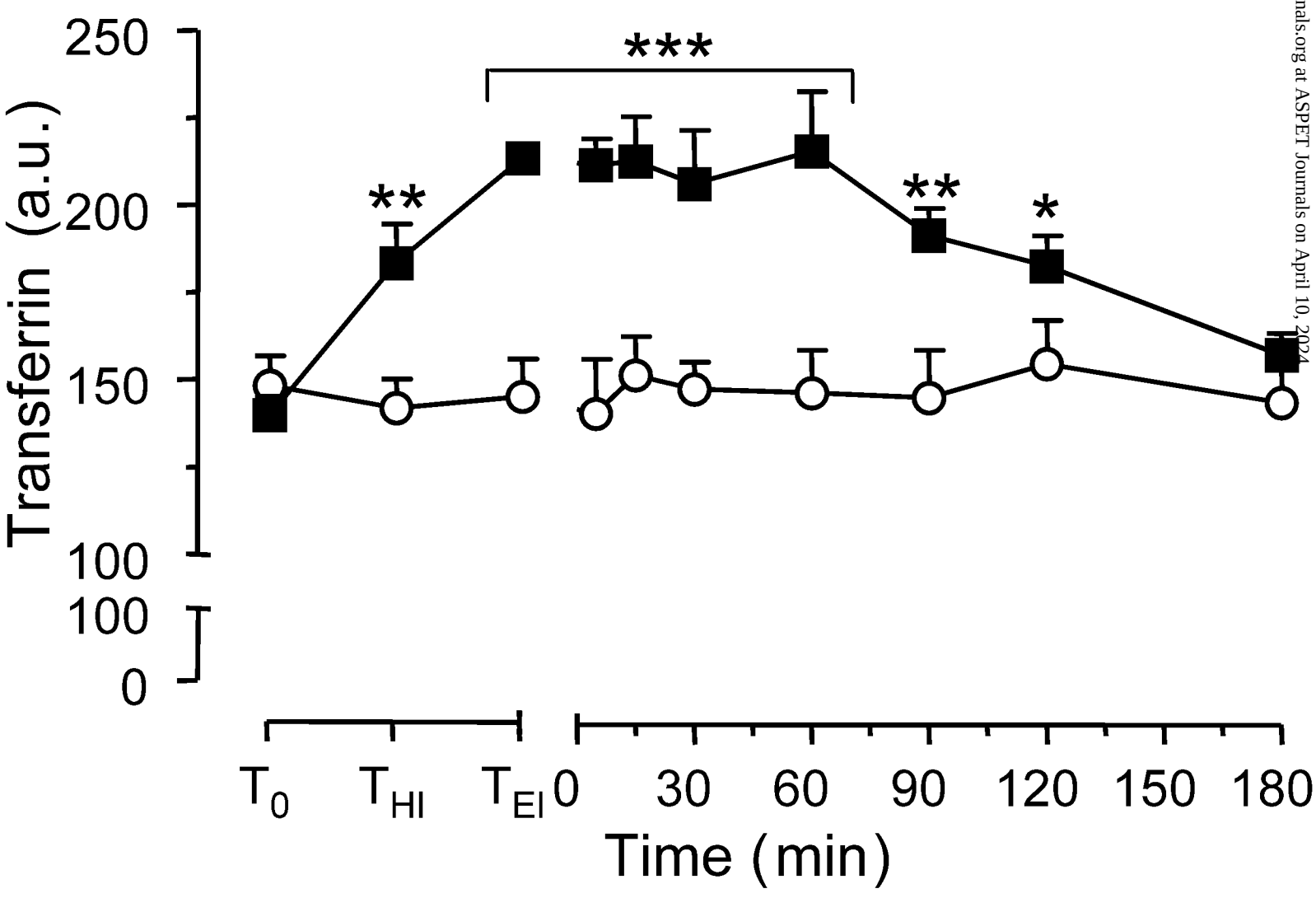


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Figure 6

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Figure 7

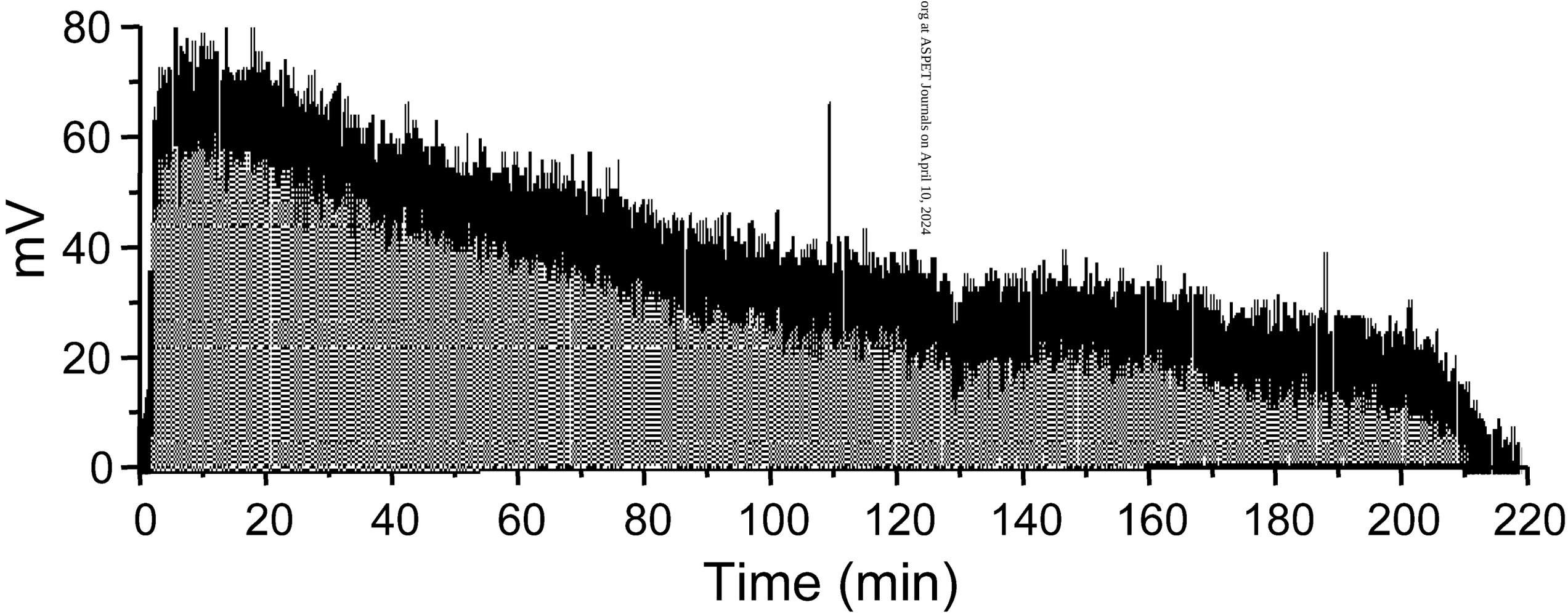

Figure 8

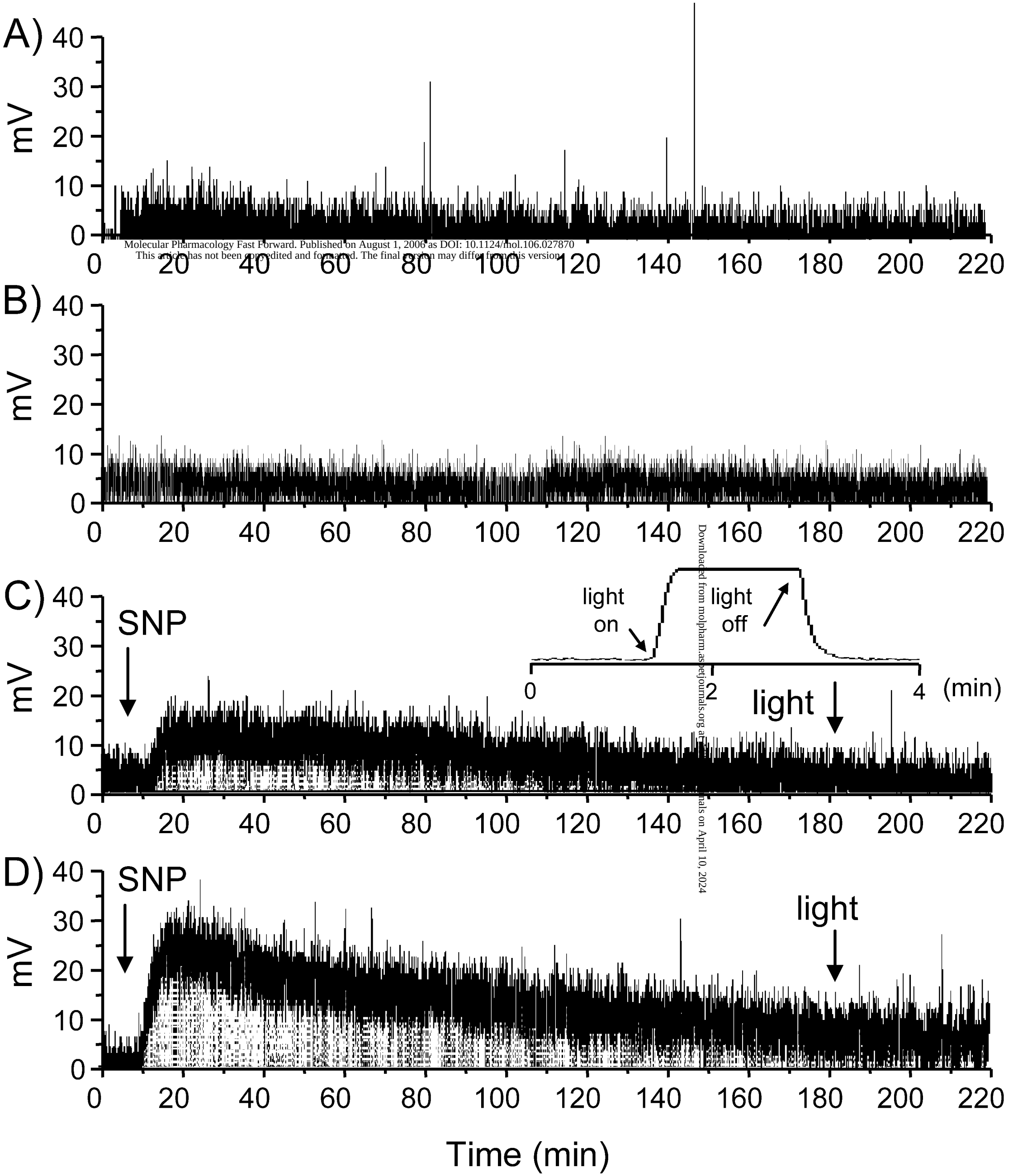

Figure 9