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Title Page

Acetoxymethoxycarbonyl Nitroxides as EPR Pro-Imaging Agents to Measure O_2 Levels in Mouse Brain: A Pharmacokinetic and Pharmacodynamic Study

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Abstract

Measurement of O_2 concentration and distribution in brain is essential to understanding the pathophysiology of stroke. Low-frequency electron paramagnetic resonance (EPR) spectroscopy with a paramagnetic probe is an attractive imaging modality that can potentially map O_2 concentration in the brain. In a previous study, we demonstrated that, after intraperitoneal administration of 3-acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl [**1**] to mice, this nitroxide crossed the blood-brain barrier into brain tissue where, after hydrolysis, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**2**] was liberated and entrapped. This pilot study suggested that 3-acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl [**1**] is a pro-imaging agent that can deliver 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**2**] to brain tissue, where O_2 levels can be estimated. In the present study, we conducted a series of pharmacokinetic and pharmacodynamic experiments designed to assess the uptake of structurally disparate nitroxides into brain tissue and retention, after hydrolysis, of the anions of the corresponding nitroxide acids. From these findings, nitroxide [**1**] and *trans*-3,4-di(acetoxymethoxycarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**5**] meet the requirement as EPR pro-imaging agents for mapping O_2 distribution in the brain following stroke.

Introduction

Real time estimates of O_2 levels in tumors and in the brain in living animals are an important criterion in the treatment of many cancers and in understanding the pathology of stroke, epilepsy, and traumatic brain injury (Tsuzuki et al., 2000; Zauner et al., 2002). However, measurement of O_2 levels in animal tissues using different imaging modalities is not a trivial task. For instance, while O_2 is paramagnetic, electron paramagnetic resonance (EPR) spectroscopy cannot directly detect this molecule at 37^{o}C, and requires the development of molecular probes that can report O_2 concentrations in different experimental models. Once such family of stable free radical probes is trityls, whose EPR spectral lines are broadened in the presence of O_2 due to the interaction of the two paramagnetic species (Elas et al., 2003). Not surprisingly, changes in the EPR spectral linewidths of trityl radicals and other stable free radicals have been used to estimate O_2 concentrations in homogenous solutions and isolated cell preparations (Elas et al., 2003; Khan et al., 2003; Shen et al., 2003; Kutala et al., 2004; Liu et al., 2004). With the development of low-frequency EPR spectroscopy with the capability to image free radicals (Halpern et al., 1989; Halpern and Bowman, 1991), reliable real-time estimates of O_2 concentration in the vascular bed of a mouse tumor has recently been achieved using a polyanionic trityl radical (Elas et al., 2003).

Owing to their charge, however, trityl radicals are not particularly useful for measurement of tissue oxygenation in the brain, because delivery of these free radicals to brain tissue at sufficiently high concentration for imaging purposes is difficult, at best. Recently, we demonstrated that after intraperitoneal administration of 3-acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**1**], this nitroxide was able to cross the blood-brain barrier. Thereafter, upon esterase hydrolysis, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**2**] was entrapped in brain tissue (Shen et al., 2006). Through EPR spectroscopy, nitroxide [**2**] was found

to accurately estimate O_2 levels in homogeneous aqueous solutions (Shen et al., 2006). In light of this successful pilot study, we now describe results from a series of *in vivo* experiments designed to assess uptake of structurally disparate nitroxides that cross the blood-brain barrier after different routes of administration. From these experiments, nitroxide [**1**] and *trans*-3,4-di(acetoxymethoxycarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**5**], but not (2,2,5,5-tetra-methylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N*,*N*-diacetic acid diacetoxymethyl ester [**3**] (Fig. 1), exhibited favorable pharmacokinetic and pharmacodynamic profiles. As nitroxide [**1**] is easier to synthesize and is more soluble in aqueous buffers than nitroxide [**5**], it appears that nitroxide [**1**] is the best of the current family of acetoxymethoxycarbonyl-containing nitroxides as an EPR pro-imaging agent for quantitating O_2 levels in mouse brain.

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Materials and Methods

Chemicals. All chemical reagents and solvents were of American Chemical Society grade or better and were used without further purification. Silica gel (230-400 mesh, EMD Chemicals, distributed by VWR International, Bridgeport, NJ) was used for column chromatography. 3-Acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**1**], 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**2**], and (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N*,*N*-diacetic acid diacetoxymethyl ester [**3**] were synthesized as describe in the literature (Rozantsev, 1970; Kao and Rosen, 2004; Rosen et al., 2005). Nitroxides [**1**] and [**3**] were dissolved in 95% ethanol as 0.5 M stock solutions. For cellular and animal experiments, the stock solution was diluted 10-fold with PBS before use. The final concentration of ethanol was 0.0075 – 0.0126 mmol/kg body weight; no adverse effects were observed at these ethanol levels. Synthesis of *trans*-3,4-di(acetoxymethoxycarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**5**] is summarized schematically in Fig. 2 and is described in detail below. Since nitroxide [**5**] was poorly soluble in 95% ethanol, a stock solution of [**5**] (0.2 M) was made in *N*,*N*-dimethylacetamide–H_2O (1:9 v/v), and then diluted 10-fold with PBS for animal experiments.

3,4-Dicyano-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**8**]. To a solution of 3-cyano-2,2,5,5-tetramethyl-1-pyrrolinyloxyl [**7**] (3.2 g, 14.4 mmol, prepared as described by Rozantzev, 1970a) in 95% ethanol (100 ml) was added 100 ml of an aqueous solution of potassium cyanide (3.05 g, 46.9 mmol) and ammonium chloride (2.6 g, 49.1 mmol). This mixture was heated at 70^{o}C for 7 h, at which point the reaction was cooled to room temperature. The solution was then saturated with NaCl and extracted with ether (5 × 100 ml). The combined ether solutions were dried over anhydrous $MgSO_4$, filtered and reduced to dryness in a rotary evaporator. The resulting mixture of compounds was purified on silica gel. A small amount of starting material was removed by elution with hexane-ether (2:1). Subsequent elution with hexane-ether (1:1) afforded *trans*-3,4-

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dicyano-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**8a**] (1.7 g, 46% yield), which was recrystallized from ether-hexane (mp = 141-142^{o}C; IR ($CHCl_3$): 2215 cm^{-1}(CN)). Increasing the polarity of the solvent to hexane-ether (1:2) eluted *cis*-3,4-dicyano-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**8b**] (0.7 g, 18% yield), which was recrystallized from ether-hexane (mp = 84^{o}C; IR ($CHCl_3$): 2220 cm^{-1} (CN)) (Mathew and Dodd, 1985).

***Trans*-3,4-di(acetoxymethoxycarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl** [**5**]. A mixture of *trans*-3,4-dicyano-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**8a**] (1 g, 5.2 mmol) and 2 M NaOH (60 ml) was warmed to 90^{o}C for 3 d, at which point the reaction was cooled and the aqueous solution extracted with ether. The remaining aqueous solution was cooled in an ice bath, acidified with 10% HCl, and extracted with ether. The organic solution was dried over anhydrous $MgSO_4$, filtered and rotary evaporated to dryness, leaving a light yellow solid. Recrystallization from acetone-benzene afforded *trans*-3,4-dicarboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**6**] (1 g, 89% yield, mp = 222–224^{o}C with decomposition) (Mathew and Dodd, 1985; Chatani, et al., 2005).

To a mixture of *trans*-3,4-dicarboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**6**] (0.6 g, 2.6 mmol) and K_2CO_3 (1.08 g, 7.8 mmol) in Me_2SO (2 ml) was added bromomethyl acetate (0.79 g, 0.51 ml, 5.2 mmol, Aldrich Chemical Co., Milwaukee, WI). The reaction was stirred at room temperature for 3 h, at which point methylene chloride (50 ml) was added. This mixture was washed with water (3 $\times$ 100 ml). The organic solution was dried over anhydrous Na_2SO_4, filtered, and reduced to dryness on a rotary evaporator. The residual Me_2SO was removed under high vacuum. The resultant yellow oil was purified by silica gel chromatography (chloroform-ether, 49:1) to afford *trans*-3,4-di(acetoxymethoxycarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**5**] (0.77 g, 85%), which was recrystallized from hexane (mp = 89–90^{o}C; IR ($CHCl_3$): 1769 cm^{-1} (broad ester peak)). Anal. ($C_{16}H_{24}NO_7$): Calculated, C = 51.34%; H = 6.46%; N = 3.74%;

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found, C = 51.35%; H = 6.41%; N = 3.75%.

Cell culture. Sprague-Dawley rats were maintained and used in compliance with the principles set forth in the "Guide for Care and Use of Laboratory Animals" and approved by the University of New Mexico Animal Care and Use Committee. Primary cultured cortex neurons were prepared from embryonic day 15 SD rats, as described previously with modification (Furuichi et al., 2005). Briefly, dissociated cell suspensions were plated at a density of 2×10^6 cells/well on poly-L-lysine-coated 6-well plates (BD Biosciences, San Diego, CA, USA) with Neurobasal/2% B27 (Gibco-BRL, Grand Island, NY) containing 0.5 mM glutamine (Sigma Chemical Company, St. Louis, MO), penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were maintained in a humidified incubator at 37˚C, in 5% CO_2-95% air. On the tenth day of culture the cells were used for experimentation.

Loading of neurons with various nitroxides. We had shown that for *in vitro* cellular studies, intracellular loading of the carboxylate form of the nitroxides increases significantly when cultured cells are incubated with the acetoxymethoxycarbonyl form of the nitroxides at room temperature (Rosen et al., 2005). Therefore, the *in vitro* cellular studies here were conducted at 21°C. Neuronal cells (2.5×10^6 cells/ml) were incubated with 50 µM of either 3-acetoxy-methoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**1**] or (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N*,*N*-diacetic acid diacetoxymethyl ester [**3**] at 21^{o}C with gentle shaking for 60 min, at which point the cells were centrifuged, and the cell pellet was washed with PBS. This washing procedure was repeated three times. The neurons were resuspended in HBSS at 2.5×10^6 cells/ml. One aliquot of the cell sample was transferred into a Teflon tube, placed into the cavity of the EPR spectrometer, and the spectrum was recorded at room temperature. This time point was considered to be t = 0 in Fig. 3. The remaining cell suspension was gently agitated at

21°C, and at 30 min and at 60 min, aliquots of the cells were removed, centrifuged, washed with PBS and resuspended in HBSS. EPR spectra of these aliquots were then acquired at room temperature. A Bruker X-band EleXsys 540 EPR spectrometer was used with the following parameters: microwave power 20 mW, center field 3340 G, modulation frequency 100 kHz, modulation amplitude 0.3 G.

Pharmacokinetics of uptake of nitroxides [1], [3] and [5] in mouse brain. We determined the preferable route of administration that will achieve the maximum retention of the nitroxide in the brain by measuring the pharmacokinetics of nitroxides [**1**], [**3**] and [**5**]. Mice (C57 strain, weighing 18-20 g) were obtained from Charles River Laboratory (Wilmington, MA). Animal housing, care and application of experimental procedures were in accordance with the institutional guidelines and approved by University of New Mexico Animal Care and Use Committee. Mice were maintained under appropriate lighting conditions for 4 days with free access to food and water before experimentation. On the day of experimentation, mice were anesthetized by inhalation of 4% isoflurane in N_2O:O_2 (70%:30%) and were maintained under anesthesia by 1% isoflurane in N_2O:O_2 (70%:30%); the mouse core temperature was maintained at 37°C by using a heating pad. In a typical experiment (as shown in Fig. 4), after a mouse was anesthetized, nitroxide [**1**] or [**3**], at the dose of 0.39 or 0.23 mmol/kg body weight, respectively, was injected intra-arterially (IA), intravenously (IV), or intraperitoneally (IP). Nitroxide administration caused no obvious changes in animal behavior or any signs of acute toxicity. For intra-arterial and intravenous injections, micro-tubing (TYGON ®, ID 0.010 in, OD 0.030 in, Saint-Gobain PPL Corp.) was cannulated before administration of nitroxide. For the arterial route, the right carotid artery was exposed surgically and the external carotid artery was ligated with a 6-0 silk suture and then the tube was inserted into the right common carotid artery and was fixed with a 6-0 silk suture. For the intravenous route, the tube was cannulated into the right

femoral vein. In experiments depicted in Fig. 6, mice received nitroxide [**1**] or [**5**] intravenously at a dose of 6 μl/g body weight of a 20 mM stock solution, prepared by diluting a 0.2 M stock solution in *N*,*N*-dimethylacetamide–H_2O (1:9 v/v) 10-fold with PBS.

In all the experimental models, the mouse was immediately transferred into the custom-made head resonator for the Bruker E540L L-band EPR system after cannulation. After the resonator was tuned, the nitroxide was administered through the micro-tubing and then EPR spectra were recorded at 1-min intervals for up to 90 min with the following EPR acquisition parameters: microwave frequency 1.12 GHz, microwave power 18 mW, center field 412 G, modulation frequency 50 kHz, modulation amplitude 1.0 G. The relative intensities of the EPR spectra of the nitroxides were measured. Typically, between nitroxide injection and acquisition of the first EPR spectrum there was a 3 min interval during which instrument tuning and optimization occurred.

Pharmacodynamics of nitroxides [1], [3] and [5] in mouse brain. After the mice (C57 strain, weighing 18–20 g) were anesthetized, either nitroxide [**1**] or [**3**] was injected intra-arterially, intravenously or intraperitoneally at a dose 0.39 or 0.23 mmol/kg body weight, respectively. EPR spectra were recorded from the mouse head using the head resonator, immediately after tuning the spectrometer and then every 2 min for 10 min. The animals were then removed from the magnet, and the thorax was surgically opened. A 23-gauge butterfly needle was inserted immediately into left ventricle, and a part of the right atrium was excised to allow the blood to drain. Normal saline (2.0–2.5 ml/min) was infused via an infusion pump through the butterfly needle until clear saline emerged from the right atrium. The animals were then returned to the spectrometer and EPR spectra were again recorded. The perfusion procedure took about 8 min on average. Pharmacodynamic experiments were also conducted to compare nitroxides [**1**] and [**5**] after intravenous administration of each compound (6 μl/g body weight of a 20 mM stock

solution). EPR spectra were recorded using the following instrumentation settings: microwave frequency 1.12 GHz, microwave power 18 mW, center field 412 G, modulation frequency 100 kHz, modulation amplitude 1.0 G.

Statistical analysis. All data are expressed as mean ± S.E. The Student's unpaired t-test was used to assess the statistical significance of differences.

Results

Intracellular retention of nitroxides [1] and [3] in primary cultures of neurons

We compared the uptake of nitroxides [**1**] and [**3**] into primary cultures of neuronal cells and the intracellular retention of the corresponding hydrolysis product, nitroxides [**2**] and [**4**] (see, Fig. 1 for the structure of the nitroxides). Neuronal cell suspensions (2.5×10^6 cells/ml) were incubated with either nitroxide [**1**] or [**3**] at 50 μM for 60 min at 21°C. After extensive washing, cells were resuspended and maintained in HBSS. Thereafter, at 0, 30 and 60 min, aliquots of these cells were centrifuged, washed and then resuspended in HBSS. EPR spectra of these cell suspensions were recorded to quantitate the nitroxide concentration that was retained by the cells. The spectral intensity of the central peak of the nitroxide triplet of each sample was measured and plotted as a function of time (Fig. 3). Several observations are worth noting from these experiments. At t = 0, neuronal cell suspensions loaded with nitroxide [**3**] had a significantly greater concentration of this nitroxide than cells loaded with nitroxide [**1**], despite the fact that nitroxide [**3**] is primarily positively charged at pH 7.4. More importantly, over the next 60 min, only ~10–20% of the initial intracellular concentration of the nitroxides was lost from the cells. These data suggest that nitroxides [**1**] and [**3**] may be excellent EPR pro-imaging agents for estimating O_2 levels in mouse brain.

Pharmacokinetics of nitroxides [1] and [3] in mouse brain via three different routes of administration

While isolated cells do not exhibit the same dynamic properties of an intact animal, based on data depicted in Fig. 3, we expected the concentrations of nitroxide [**1**] and nitroxide [**3**] in the mouse head to be similar and, perhaps, nitroxide [**3**] might achieve even higher levels in this organ. Thereafter, diffusion across the blood brain barrier followed by *in situ* hydrolysis would liberate the corresponding nitroxides [**2**] and [**4**] which, being predominantly charged at

physiologic pH, should be retained in brain tissue. After intraperitoneal administration of nitroxides [**1**] and [**3**], the peak EPR signal intensity and the $t_{1/2}$ of these nitroxides in the mouse head (i.e., brain tissue with the associated vasculature) were essentially the same (Fig. 4). In contrast, intravenous or intra-arterial administration led to a different scenario (Fig. 4B and Fig. 4C). After either intravenous or intra-arterial administration, the peak EPR signal intensity of nitroxide [**1**] was always greater than that found for nitroxide [**3**]. The disparity between nitroxides [**1**] and nitroxide [**3**] is approximately 2-fold at the maximum (Fig. 4B and Fig. 4C). With nitroxide [**1**] the $t_{1/2}$ was determined to be 35 min, 19 min and 21 min after intraperitoneal, intravenous, and intra-arterial administration, respectively (Fig. 4A, 4B and 4C). In contrast, the $t_{1/2}$ values for nitroxide [**3**] were 34 min, 13 min and 10 min, following intraperitoneal, intravenous or intra-arterial administration, respectively (Fig. 4A, 4B and 4C).

Pharmacodynamics of the nitroxides [1] and [3] in mouse brain

The pharmacokinetic studies presented in Fig. 4 cannot differentiate between nitroxides that have crossed the blood-brain barrier and become trapped in brain tissue from nitroxides that are merely passively retained in the brain vasculature. Therefore, we conducted experiments designed to measure the distribution of nitroxides in these two compartments after administration through different routes. In a typical study, a group of mice was injected intraperitoneally with nitroxide [**1**]. EPR spectra were recorded from the heads of mice before and after blood was completely displaced from the vasculature by saline perfusion. Differences in the EPR spectral peak height before and after emptying of the vasculature gave an estimate of the amount of nitroxide that was in the vascular bed as well as the amount that had been transported into and entrapped in the brain tissue. The findings of these studies are summarized in Fig. 5.

Several features of the results presented in Fig. 5 are noteworthy. Irrespective of the route of administration, the concentrations of nitroxide [**1**] and its hydrolysis product nitroxide [**2**] in

the mouse head were consistently greater than those found for nitroxides [**3**] and its hydrolysis product nitroxide [**4**] (Fig. 5A and 5B). Not surprisingly, levels of nitroxides [**1**], [**2**], [**3**] and [**4**] in the head are substantially higher after intravenous and intra-arterial as compared to intraperitoneal injection (Fig. 5A and 5B). We surmise that after intravenous and intra-arterial routes of administration, differences in nitroxide levels in the mouse head are due, perhaps, to differential binding of nitroxides to proteins in the blood and/or bioreduction (Griffeth et al., 1984), whereas after intraperitoneal injection, additional metabolism of these nitroxides and the charged nature of nitroxides [**3**] and [**4**] must be contributing factors along with bioreduction for diminished concentration of these nitroxides in the mouse head. Additionally, the peak bolus concentration attained in the brain was undoubtedly much higher for IV and IA administration than for IP injection.

After blood was completely displaced from the vasculature by perfusion with normal saline, the fraction of nitroxides [**1**] and [**3**] that crossed the blood-brain barrier and was entrapped in brain tissue (as nitroxides [**2**] and [**4**]), was virtually the same, independent of the route of administration (Fig. 5A and 5B). This finding was surprising given the highly charged nature of nitroxide [**4**]. For nitroxide [**1**], the fractional retention values in brain tissue following IP, IV or IA administration were 0.49 ± 0.12, 0.50 ± 0.11 and 0.62 ± 0.06, respectively. Corresponding values for nitroxide [**3**] were 0.61 ± 0.10, 0.56 ± 0.15 and 0.52 ± 0.06 for IP, IV or IA administration, respectively.

Pharmacokinetics of nitroxides [1] and [5] in mouse brain after intravenous administration

Because regardless of the route of administration of nitroxide [**1**] in brain tissue, its retention was substantially greater than that of nitroxide [**3**], we hypothesized that a more lipid-soluble acetoxymethoxycarbonyl analog of nitroxide [**1**] might further increase nitroxide concentration in the brain. Toward this goal, we synthesized *trans*-3,4-di(acetoxymethoxy-

carbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**5**], which can be hydrolyzed to the doubly anionic nitroxide [**6**] (Fig. 1). We determined the pharmacokinetics of nitroxides [**1**] and [**5**] after intravenous administration of each compound (Fig. 6). The pharmacokinetic curves were essentially the same, with $t_{1/2}$ values being 16 min and 13 min for nitroxides [**1**] and [**3**], respectively. These results indicated that an additional acetoxymethoxycarbonyl group at position 4 on the nitroxide ring neither increased peak concentration of the nitroxide in the mouse head nor enhanced the kinetic profile.

Pharmacodynamics of the nitroxides [1] and [5] in mouse brain

Since the pharmacokinetics of nitroxides [**1**] and [**5**] were essentially identical, we expected that there would not be a significant difference in the concentration of nitroxides [**2**] and [**6**] in brain tissue. The results shown in Fig. 7 confirm this hypothesis, even though nitroxide [**5**] is the more lipophilic of the two compounds. Although at physiologic pH nitroxide [**6**] is doubly anionic and nitroxide [**2**] is singly anionic, there was no significant difference in the fraction of each nitroxide that was entrapped in brain tissue (Fig. 7). The fractional retention values were 0.55 ± 0.08 and 0.49 ± 0.09 for nitroxides [**1**] and [**5**], respectively.

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Discussion

The long-term goal of this research is to synthesize nitroxides that, when introduced into a mouse can enter the vasculature and then diffuse across the blood brain barrier and accumulate to concentrations sufficient to quantitate O_2 levels in brain tissue by EPR imaging. In a recent publication (Shen et al., 2006), we demonstrated that after intraperitoneal administration of nitroxide [**1**], this pro-imaging agent crossed the blood-brain barrier, where it was hydrolyzed to nitroxide [**2**]. This metabolism allowed the paramagnetic compound to be entrapped in brain tissue. Based on this earlier study, we prepared two nitroxides, whose molecular differences would give us insight into structural features that might promote even higher concentrations of nitroxide in brain tissue. Because all the nitroxides have the same core structure, they are expected to be equally efficacious for O_2 sensing. By altering the functional groups attached to the core nitroxide structure, we aimed to examine how substituents affected the pharmacokinetic and pharmacodynamic profile. We first synthesized nitroxide [**3**]. Once this paramagnetic compound crossed the blood-brain barrier, hydrolysis would lead to nitroxide [**4**]. At physiologic pH, nitroxide [**4**] bears two negative charges and one positive charge, resulting in net anionic character (Fig. 1). Based on an earlier report (Keana et al., 1987), we surmised that this compound would be more resistant to bioreduction than nitroxide [**2**]. Moreover, the highly charged nature of nitroxide [**4**] would further retard its diffusion across the blood-brain barrier from brain tissue into the vasculature.

In primary cultures of neuronal cells that had been incubated with nitroxide [**1**] or [**3**], intracellular concentration of nitroxide [**4**] was considerably greater than that found for nitroxide [**2**] (Fig. 3). The loss of nitroxide [**4**] from the cells, which is through an organic anion transport mechanism (Rosen et al., 2005), paralleled our earlier studies (Shen et al., 2006). In mice, pharmacokinetics of nitroxides [**1**] and [**3**] after intraperitoneal injection were essentially the

same, with a $t_{1/2}$ of ~35 min for both compounds (Fig. 4A). In contrast, after intravenous and intra-arterial administration of nitroxide [**1**], peak concentration of this nitroxide was twice that observed when the identical experiments were conducted with nitroxide [**3**] (Fig. 4B and 4C). These results suggest that intravenous or intra-arterial injection of nitroxide [**1**] is the best route of administration for achieving the highest concentration of nitroxide [**2**] in the brain for imaging O_2. Interestingly, the results from the *in vivo* pharmacokinetics and pharmacodynamics experiments demonstrate that higher concentrations of nitroxide [**2**] as compared to nitroxide [**4**] were observed in brain tissue, which is contrary to expectations based on *in vitro* cellular studies. This difference is likely the result of the disparity between the static system, i.e., cultured neurons, and the dynamic system, i.e., the functional and complex blood-brain barrier. The findings from the present study again underscore the importance of animal experiments when investigating compounds for targeted delivery to the brain. In the present study, we inferred that the form of the nitroxide retained in brain tissue is principally the carboxylate resulting from *in situ* enzymatic hydrolysis. This inference is founded on two published studies: First, *in vitro* biochemical studies unequivocally demonstrated that carboxyl esterase rapidly hydrolyzes the acetoxymethyl ester (i.e. [**1**]), but not the methyl ester, of nitroxide [**2**] to the corresponding carboxylate (as shown in Fig. 1) (Sano, et al., 2000). Second, we had shown (Shen et al. 2006) that whereas the acetoxymethyl ester [**1**] was very effective for loading nitroxide [**2**] into brain tissue, the methyl ester was not, despite the greater lipophilicity of the methyl ester. These two studies led us to attribute the EPR signal in brain primarily to the carboxylate form of the nitroxide.

We also designed and synthesized nitroxide [**5**] based on the reasoning that inclusion of two acetoxymethoxycarbonyl groups at positions 3 and 4 would enhance the lipophilicity of the pro-imaging nitroxide. Furthermore, after hydrolysis to nitroxide [**6**], the doubly anionic nature

of this molecule would further retard diffusion from brain tissue back into the vasculature. Even though the increased hydrophobicity of nitroxide [**5**] as compared to nitroxide [**1**] dictated a ~3-fold decrease in the concentration of nitroxides used in the pharmacokinetic studies shown in Fig. 6, still we achieved brain levels of nitroxide that should allow, based on earlier studies (Shen et al., 2006), accurate estimates of O_2 levels *in vivo*. Given that nitroxide [**6**] is a dianion at physiologic pH, we were surprised that brain tissue levels of nitroxide [**6**] were not substantially higher than that found for the monoanionic nitroxide [**2**] (Fig. 7). The reason for this remains uncertain. Future studies are designed to prepare the ^{15}N-perdeuterio-nitroxide [**1**] to determine whether this acetoxymethoxycarbonyl-containing nitroxide is an even better EPR pro-imaging agent than is nitroxide [**1**] for quantitating O_2 levels in mouse brain.

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Footnotes

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Legends for Figures

Fig. 1. Structures of nitroxide pro-imaging agents and their enzymatic hydrolysis products. Nitroxides [**1**], [**3**] and [**5**] are acetoxymethyl esters, which can be hydrolyzed by esterases to the corresponding nitroxides [**2**], [**4**] and [**6**], which are carboxylates.

Fig. 2. Synthetic scheme for the preparation of nitroxides [**5**] and [**6**].

Fig. 3. Comparison of the intracellular retention of nitroxides [**1**] and [**3**] in primary cultures of neuronal cells. Rat cortical neurons (2.5×10^6 cells/ml) were incubated with 50 μM of either nitroxide [**1**] or nitroxide [**3**] for 60 min at room temperature. After 3 washes with PBS, the neurons were resuspended in HBSS at 2.5×10^6 cells/ml, and the EPR spectrum of the suspension was recorded at 0, 30, and 60 min thereafter. Each data point is the average of 3 replicate measurements.

Fig. 4. Pharmacokinetics of the uptake of nitroxides [**1**] and [**3**] in mouse head by different administration routes: intra-peritoneal (A) intravenous (B) and intra-arterial (C). Nitroxides [**1**] and [**3**] were injected into mice at a dose of 0.39 and 0.23 mmol/kg body weight, respectively (n = 4 for each nitroxide). EPR spectra were recorded from the mouse head region 3 min after injection and every minute thereafter for up to 90 min. Where not shown, error bars are smaller than the symbol.

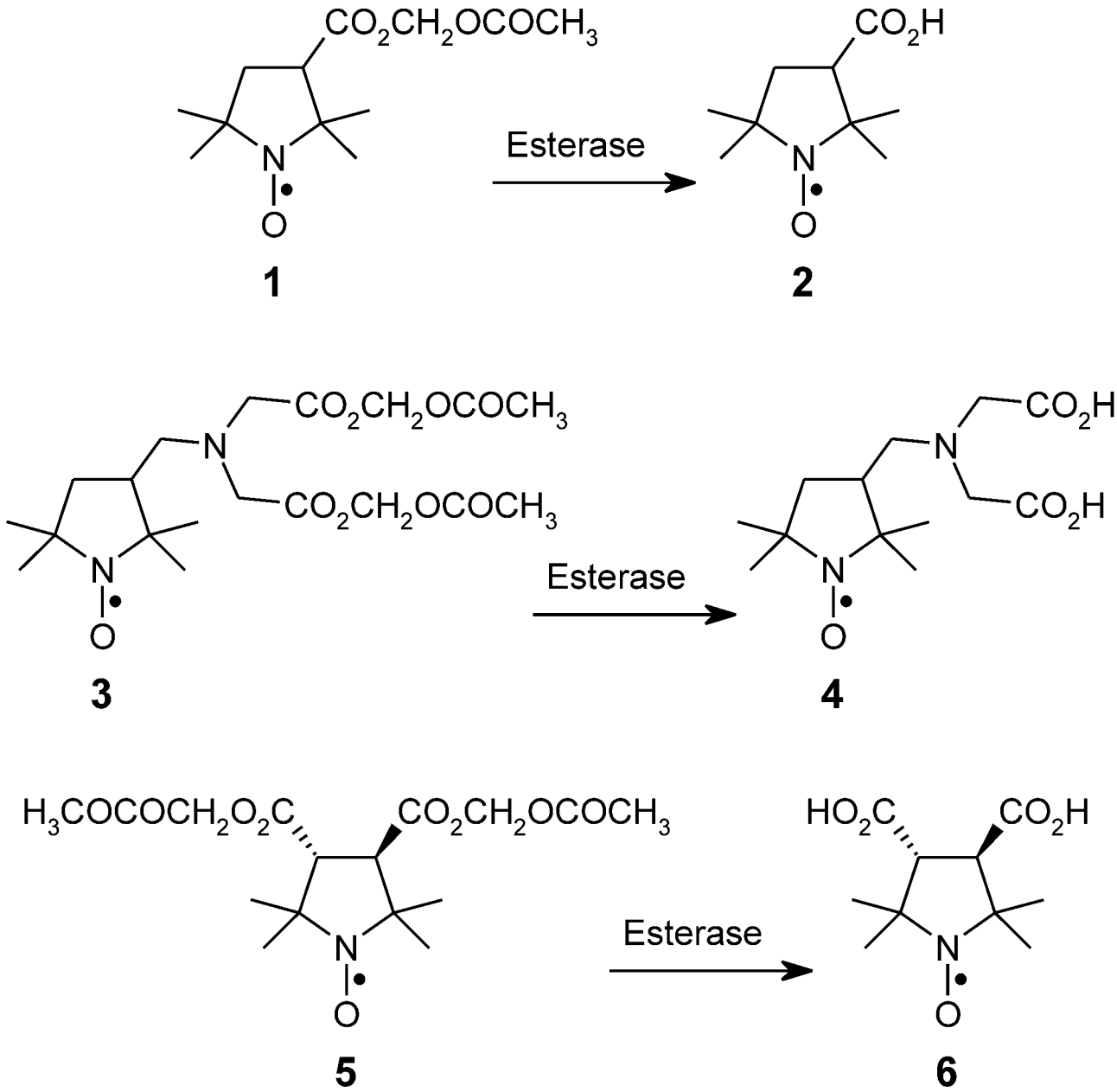
Fig. 5. Pharmacodynamics of nitroxides [**1**] and [**3**] in the mouse head. Nitroxide [**1**] (A) or nitroxide [**3**] (B) was administered at a dose of 0.39 or 0.23 mmol/kg body weight, respectively, into mice by intra-peritoneal (IP), intra-venous (IV) or intra-arterial (IA) injection. EPR spectral intensity in the mouse head was recorded with an L-band spectrometer. At 10 min after nitroxide injection, animals wee perfused with normal saline to remove blood completely from the

vasculature. Perfusion was complete in ~8 min. EPR spectral intensity was again recorded with an L-band spectrometer. The result for each route of administration is the average from 3 animals.

Fig 6. Pharmcokinetics of uptake of nitroxides [**1**] and [**5**] in mouse head after intravenous administration. Nitroxide [**1**] or [**5**] (6 μl/g body weight of 20 mM stock solution) was injected intravenously into mice (n = 4 for each nitroxide). EPR spectra were recorded from the mouse head 3 min after injection and every min thereafter for up to 60 min. For visual clarity, only plus error bars are shown on the data points for nitroxide [**1**], and only minus error bars are shown on the data points for nitroxide [**5**]. Where not shown, error bars are smaller than the symbol.

Fig. 7. Pharmacodynamics of nitroxides [**1**] and [**5**] in the mouse head. Nitroxide [**1**] or [**5**] (6 μl/g body weight of 20 mM stock solution) was injected intravenously into mice. EPR spectral intensity in each mouse head was recorded with an L-band spectrometer. At 10 min after nitroxide injection, the animals wee perfused with normal saline to remove blood completely from the vasculature. Perfusion was complete in ~8 min. EPR spectral intensity was again recorded with an L-band spectrometer. The results for each nitroxide are the average from 3 animals.

Figure 1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Esterase
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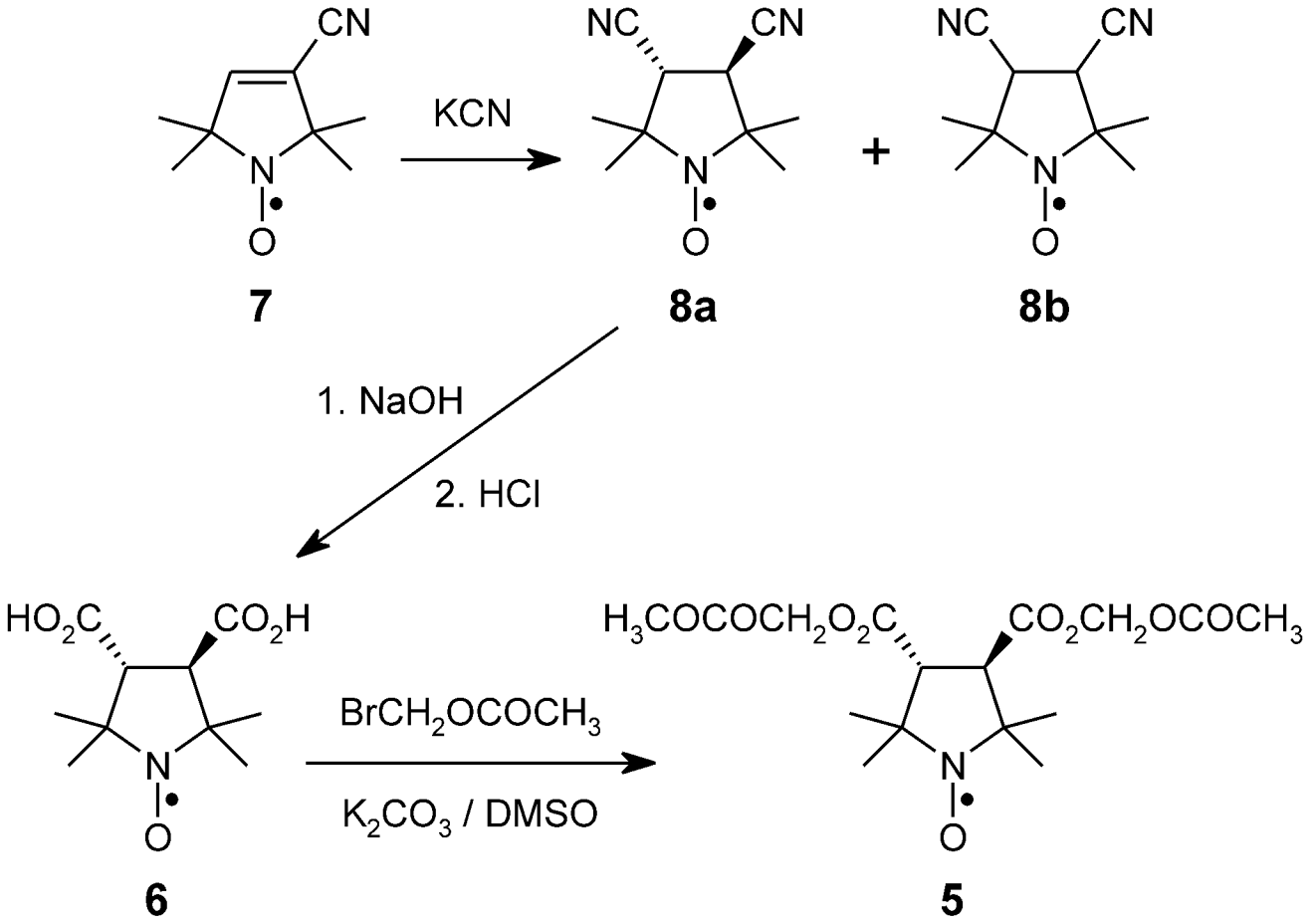
CO2H
5
6

Figure 2


CN
KCN
NC
CN
NC
CN
+
N
O
7
8a
8b
1. NaOH
2. HCl
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K2CO3 / DMSO
H3COCOCH2O2C
CO2CH2OCOCH3
6
5

Figure 3

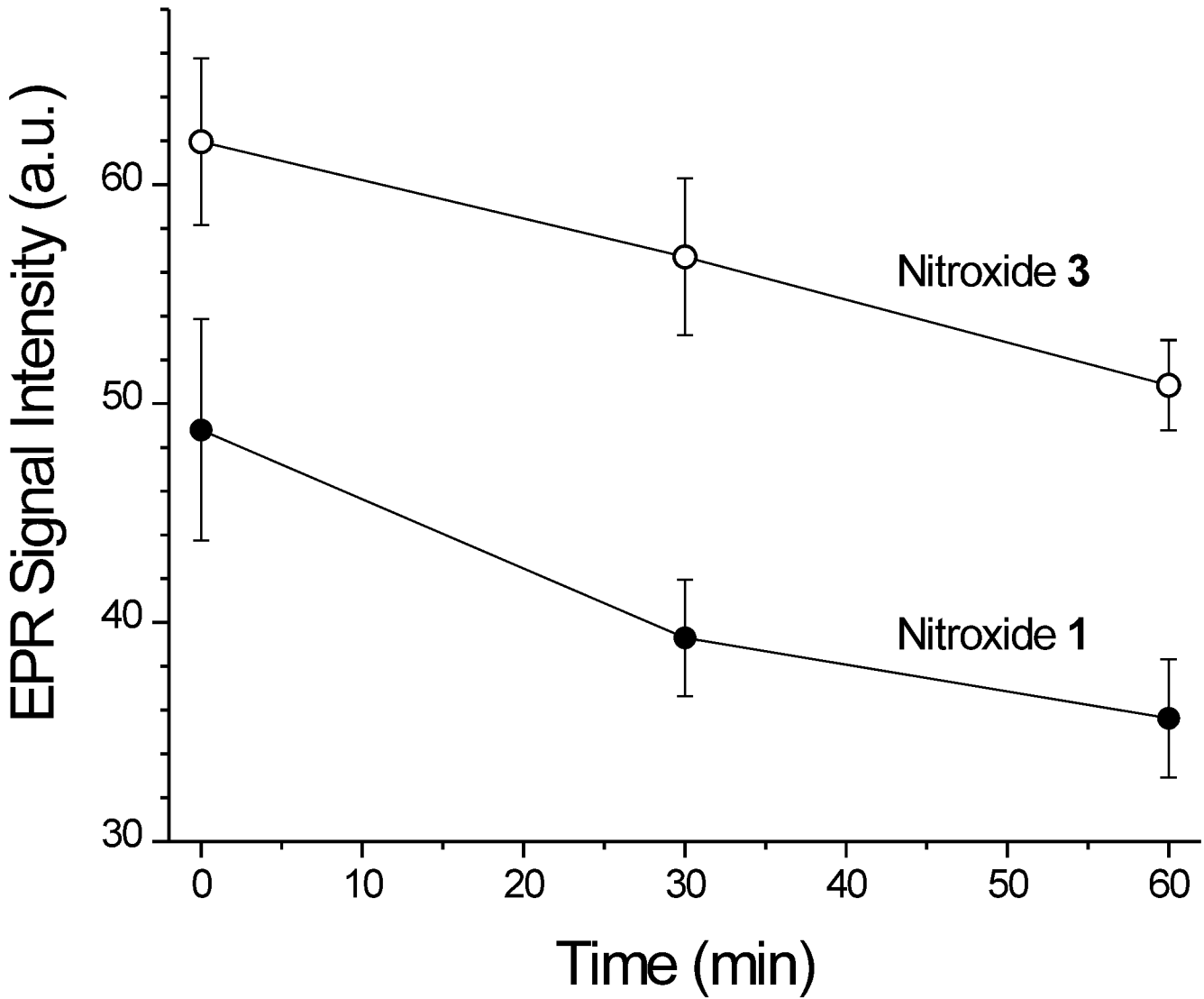
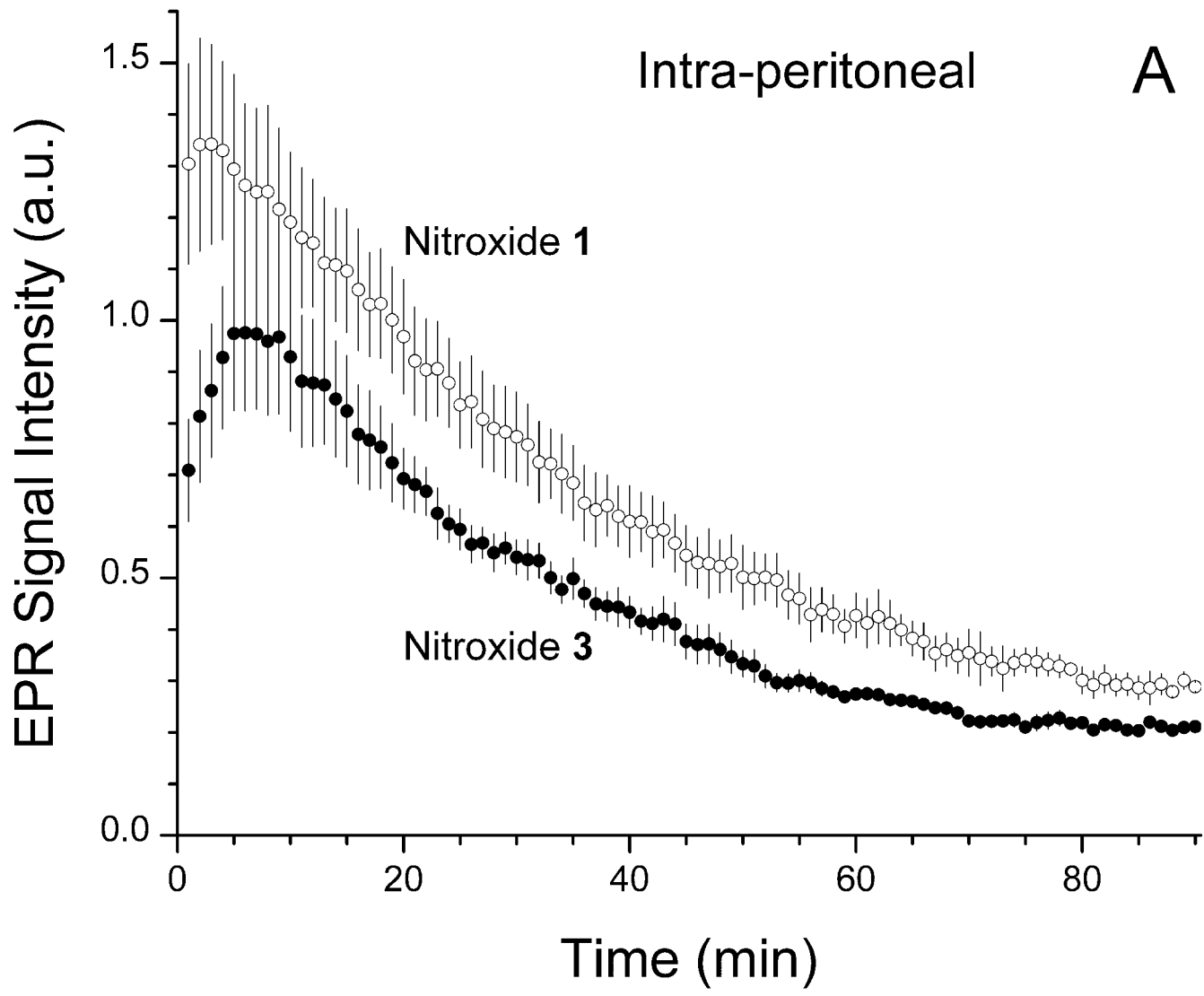
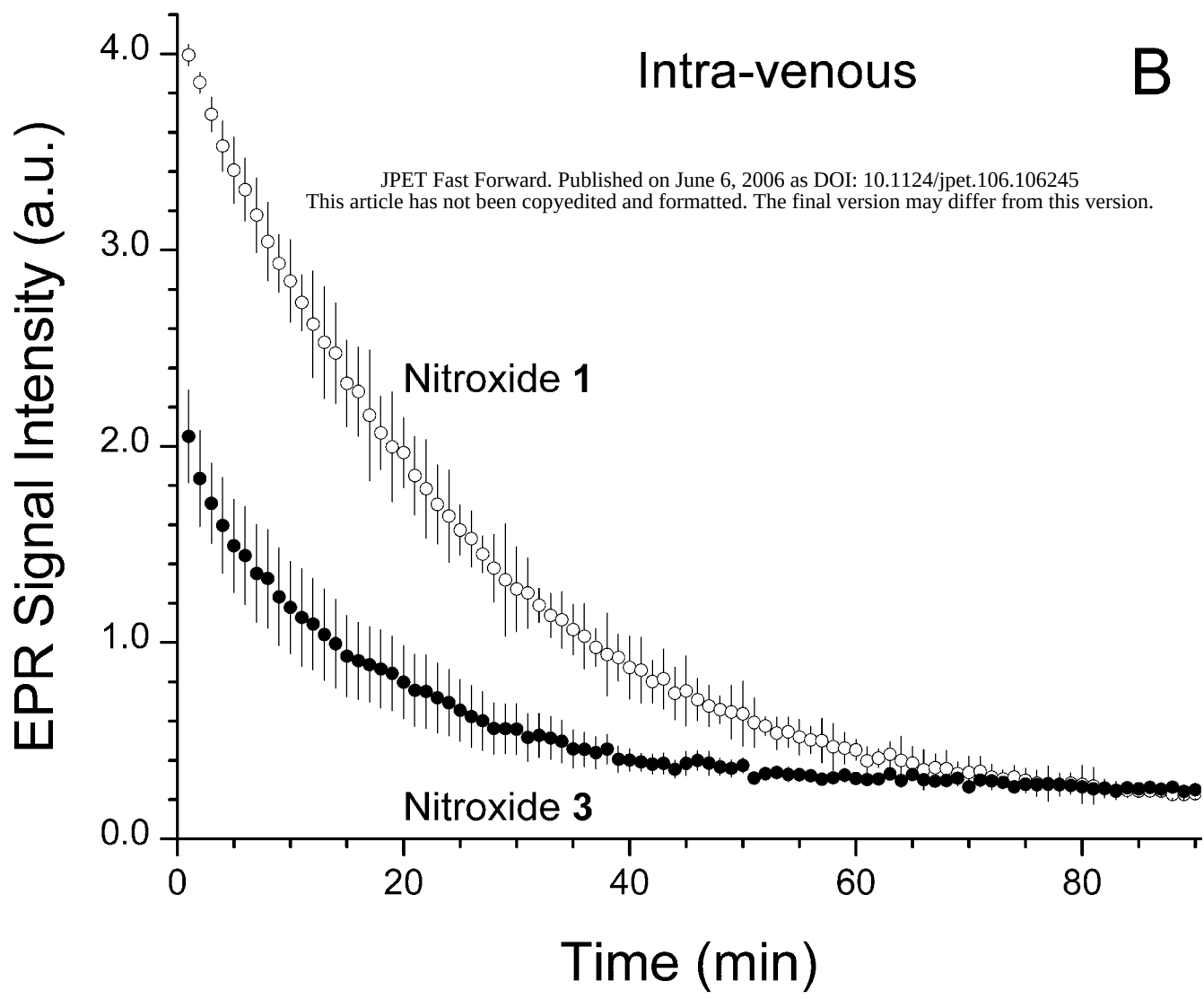

EPR Signal Intensity (a.u.)
60
50
40
30
0
10
20
30
40
50
60
Time (min)
Nitroxide 3
Nitroxide 1

Figure 4





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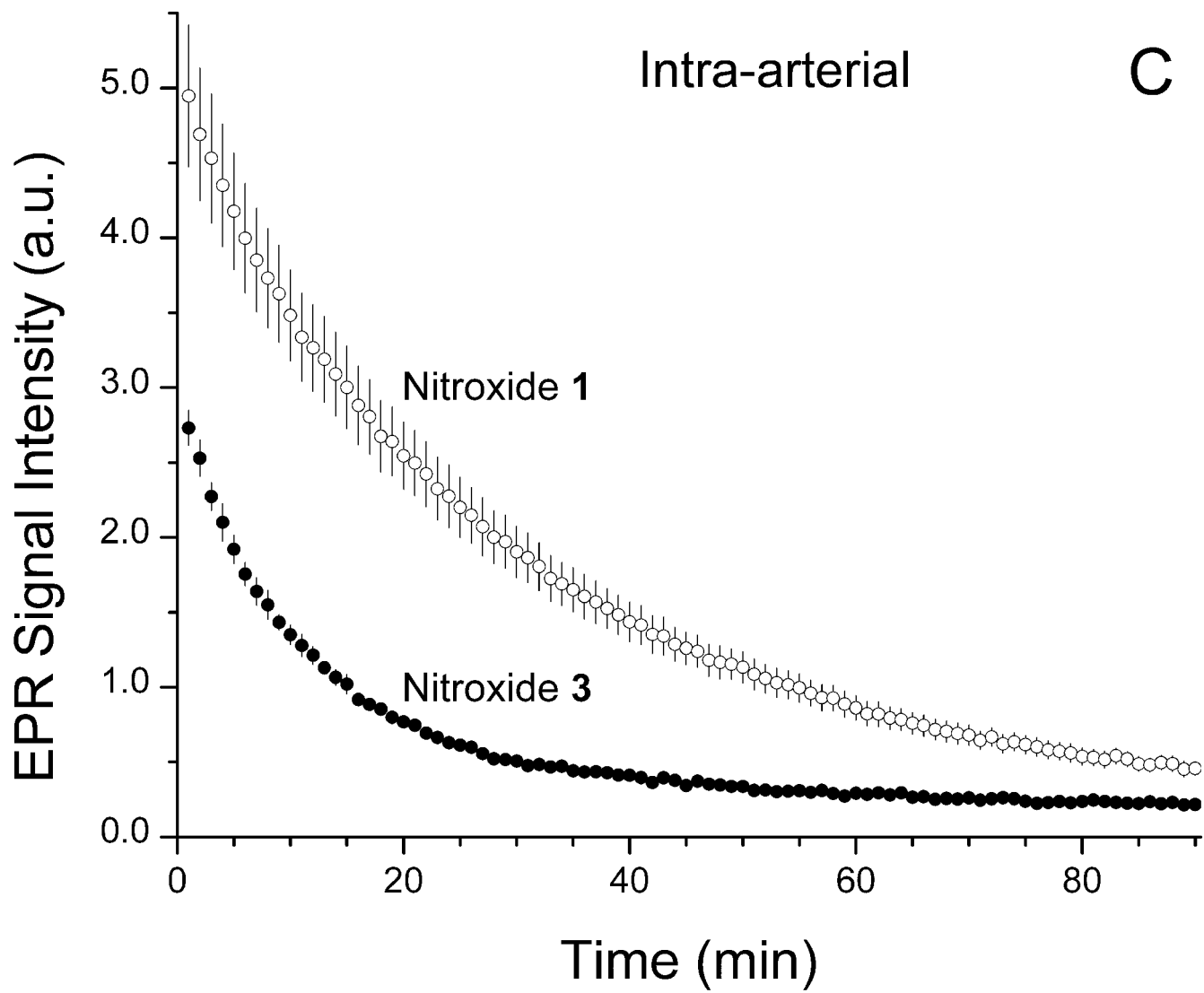
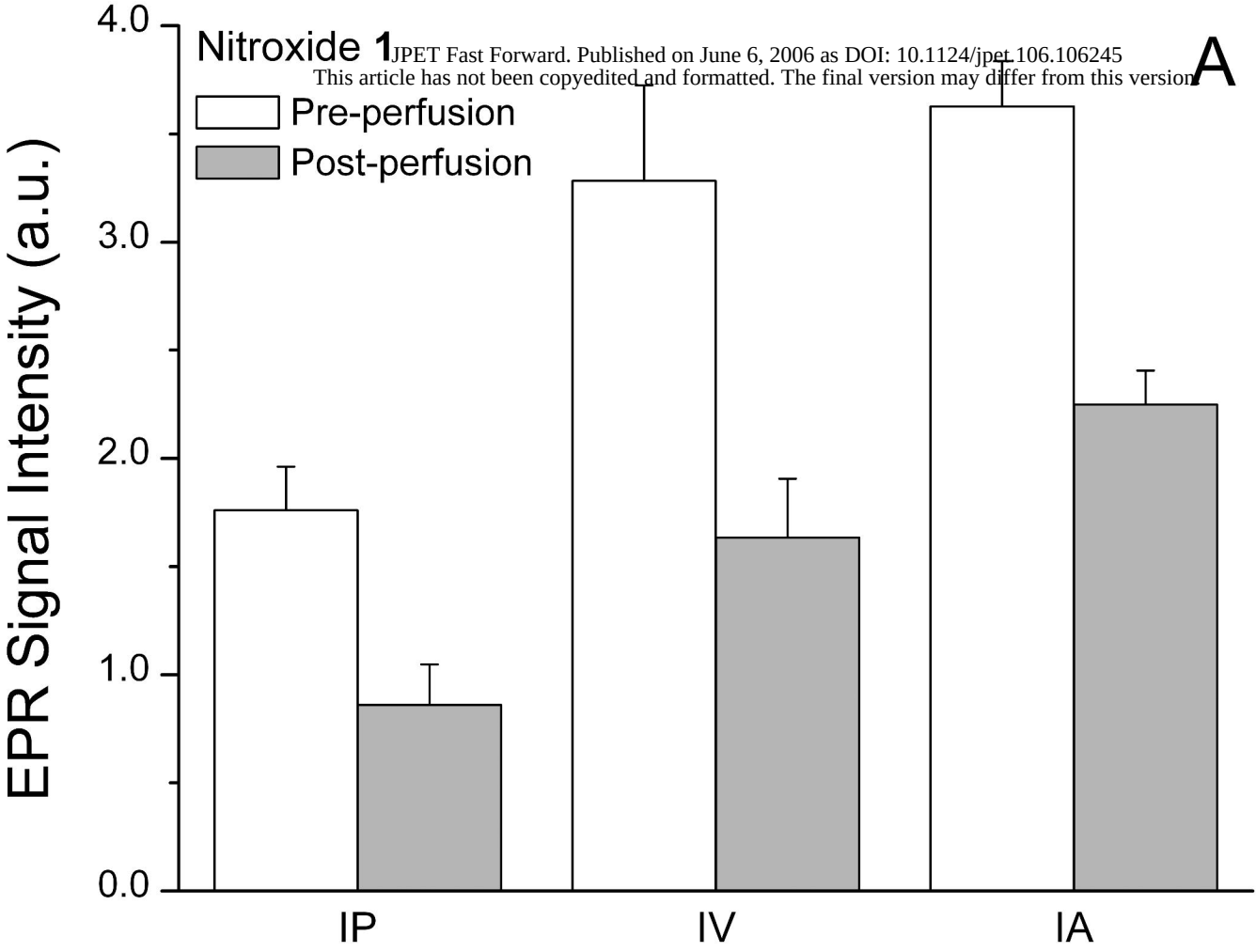


Figure 5



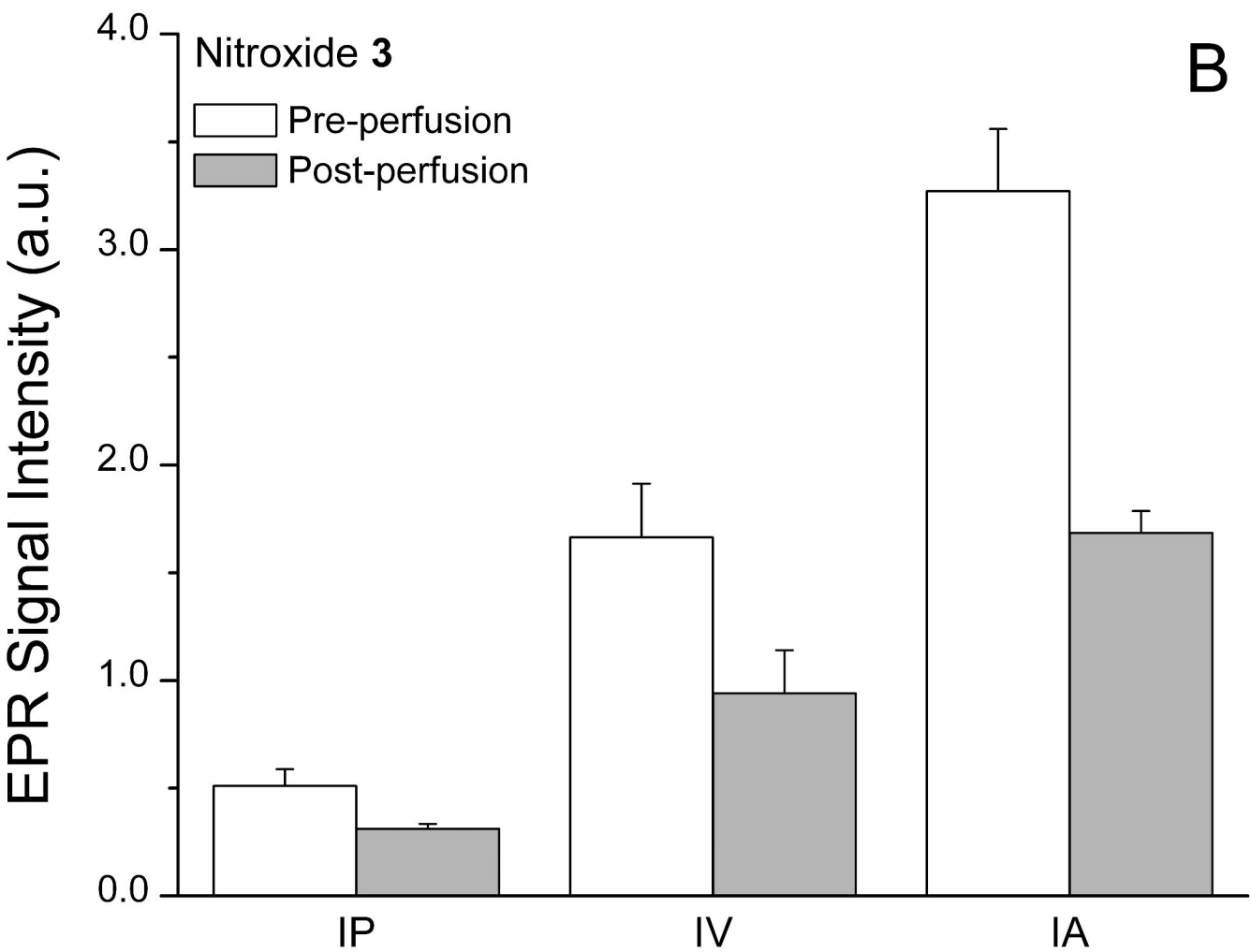


Figure 6

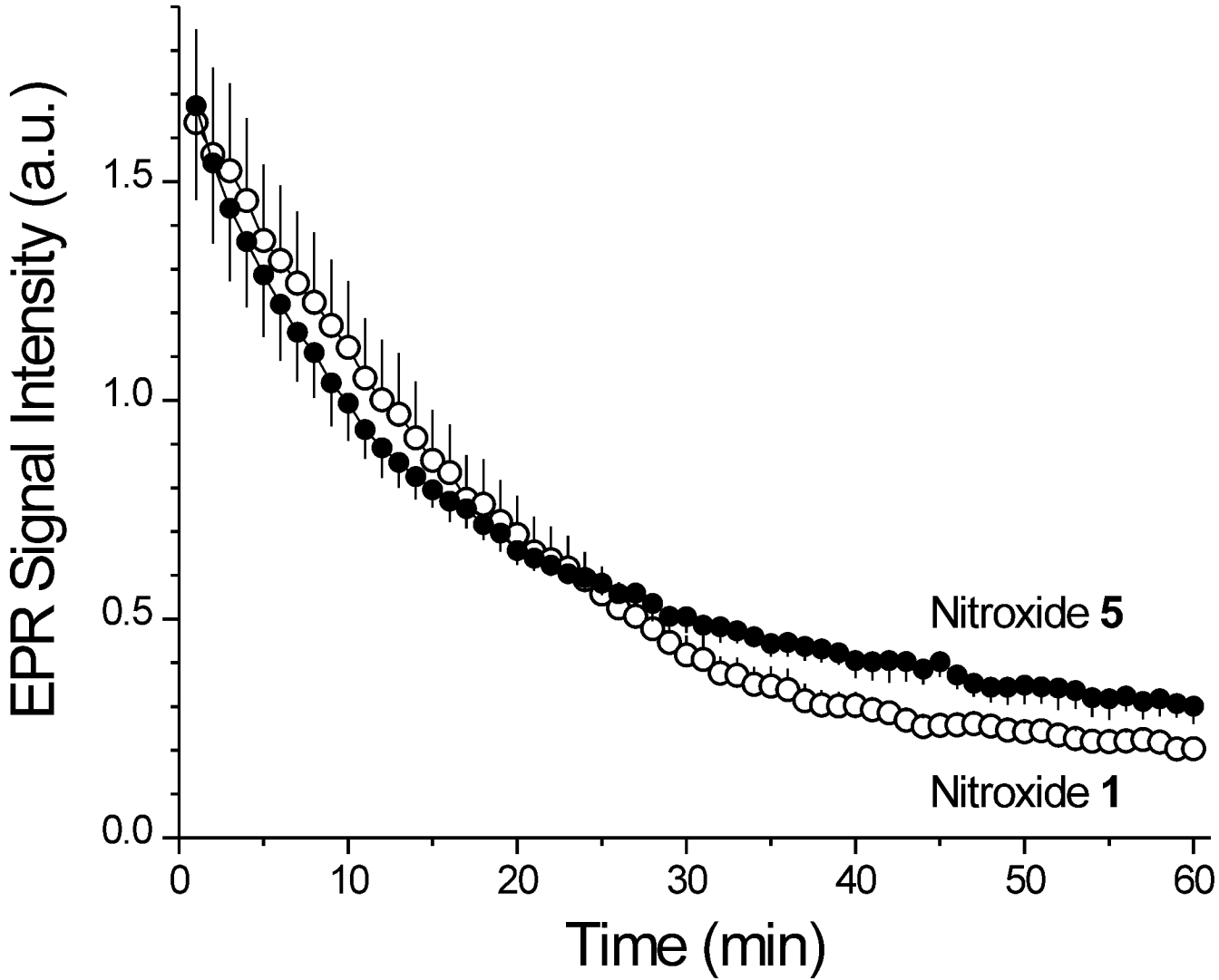
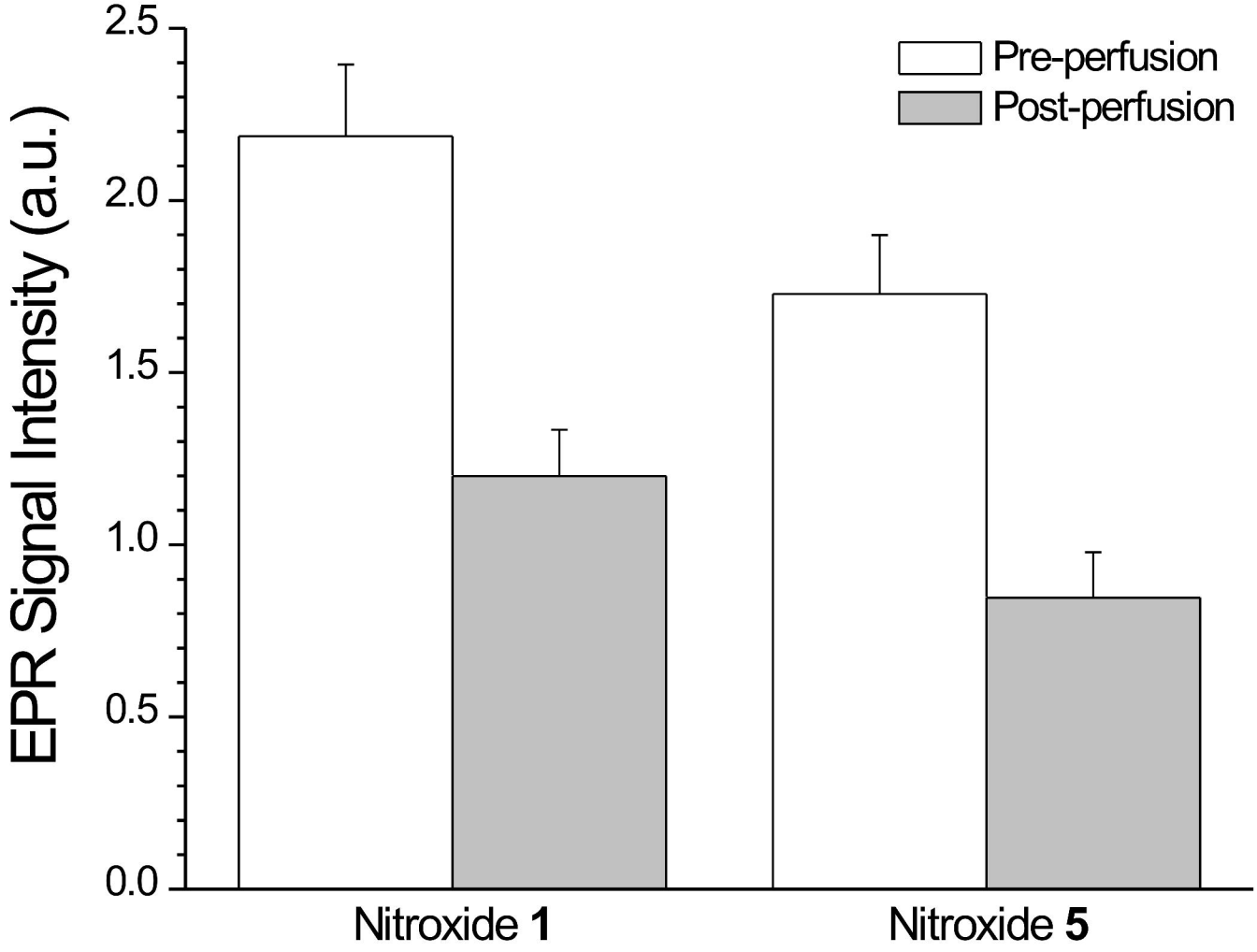

EPR Signal Intensity (a.u.)
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
Time (min)
Nitroxide 5
Nitroxide 1

Figure 7


EPR Signal Intensity (a.u.)
2.5
2.0
1.5
1.0
0.5
0.0
Pre-perfusion
Post-perfusion
Nitroxide 1
Nitroxide 5