Hemodynamic Effects of Glutathione-Liganded Binuclear Dinitrosyl Iron Complex: Evidence for Nitroxyl Generation and Modulation by Plasma Albumin

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ABSTRACT

Glutathione-liganded binuclear dinitrosyl iron complex (glut-BDNIC) has been proposed to be a donor of nitric oxide (NO). This study was undertaken to investigate the mechanisms of vasoactivity, systemic hemodynamic effects, and pharmacokinetics of glut-BDNIC. To test the hypothesis that glut-BDNICs vasodilate by releasing NO in its reduced [nitroxyl (HNO)] state, a bioassay method of isolated, preconstricted ovine mesenteric vasodilate by releasing NO in its reduced [nitroxyl (HNO)] state, a bioassay method of isolated, preconstricted ovine mesenteric was used in the presence of selective scavengers of HNO or NO free radical (NO•); the vasodilatory effects of glut-BDNIC were found to have characteristics similar to those of an HNO donor and markedly different than an NO• donor. In addition, products of the reaction of glut-BDNIC with CPTIO [2-(4-carboxyphenyl)-4,4,5-tetramethyl imidazoline-1-oxyl-3-oxide] were found to have electron paramagnetic characteristics similar to those of an HNO donor compared with an NO• donor. In contrast to S-nitroso-glutathione, which was vasodilative both in vitro and in vivo, the potency of glut-BDNIC-mediated vasodilation was markedly diminished in both rats and sheep. Wire myography showed that plasma albumin contributed to this loss of hypotensive effects, an effect abolished by modification of the cysteine-thiol residue of albumin. High doses of glut-BDNIC caused long-lasting hypotension in rats that can be at least partially attributed to its long circulating half-life of -44 minutes. This study suggests that glut-BDNIC is an HNO donor, and that its vasoactive effects are modulated by binding to the cysteine residue of plasma proteins, such as albumin.

INTRODUCTION

Nitric oxide (NO) plays an important vasodilatory role in mammals, including humans. NO synthase enzymes are the primary source of NO in the body, but its bioactivity is more complex than simple diffusion of NO from NO synthase to its site of action. Instead, evidence suggests roles for a number of NO congeners that are capable of both preserving and regulating its bioactivity. Many derivatives of NO, such as dinitrosyl iron complexes (DNICs) (Musch et al., 1991; Suryo Rahmanto et al., 2012; Vinin, 2016), S-nitrosothiols (SNOs) (Pawlowski et al., 2001), and nitrite (Cosby et al., 2003), have been proposed to stabilize, store, and transport NO bioactivity.

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Research on DNICs is limited in comparison with extensive studies on SNOs and nitrite, despite recent evidence that DNICs are the most prevalent intracellular NO adduct (Hickok et al., 2011).

Dinitrosyl iron complexes consist of an Fe(NO)2 nucleus attached to anionic ligands. These ligands are often thiols and can range in size from small molecules, such as L-cysteine and glutathione, to high molecular weight proteins with thiol groups, such as albumin (Vanin, 2009). DNICs can exist with either one or two Fe(NO)2 nuclei, termed mononuclear (MDNIC) and binuclear (BDNIC) DNICs, respectively (Supplemental Fig. 1A) (Borodulin et al., 2014).

When applied to isolated arteries, low molecular weight MDNICs and BDNICs synthesized with glutathione or L-cysteine ligands demonstrate vasodilatory potencies comparable to that of NO itself (Vanin et al., 1996, 2007; Blum-Johnston et al., 2016). Although DNIC is capable of activating isolated soluble guanylate cyclase (sGC) (Severina et al., 2003), the mechanism by which these compounds deliver NO moiety across the plasma membrane is not known. Even the redox state of the

ABBREVIATIONS: AS, Angeli’s salt; BDNIC, binuclear dinitrosyl iron complex; CPTIO, 2-(4-carboxyphenyl)-4,4,5-tetramethyl imidazole-1-oxyl-3-oxide; DNIC, dinitrosyl iron complex; DTT, 1,4-dithiothreitol; EPR, electron paramagnetic resonance; glut-BDNIC, glutathione-liganded binuclear dinitrosyl iron complex; GSH, glutathione; GSNO, S-nitroso-glutathione; HMW, high molecular weight; HNO, nitroxyl; LMW, low molecular weight; MDNIC, mononuclear dinitrosyl iron complex; NO, nitric oxide; NO+, nitrosonium; NO•, free radical nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo [4,3-a]quinoxaline-1-one; RBC, red blood cell; sGC, soluble guanylate cyclase; SNO, S-nitrosothiol; SOD, superoxide dismutase 1.
NO moiety in DNICs is uncertain. There are three different redox states of NO: nitrosonium (NO⁺), free radical NO (NO·), and nitroxy (HNO), each with similar but also distinctive biochemical properties (Stamler et al., 1992; Flores-Santana et al., 2011; Shoman and Aly, 2016). X-ray crystallography studies have indicated the NO moieties of DNICs are partially negatively charged (Tsai et al., 2015), suggesting they may release HNO. However, others have proposed that DNICs exist with a partial positive charge on the NO moiety, in which the DNICs would be more likely to release NO⁺ or even NO⁺⁺ that would be able to S-nitrosate cysteine thiols to make SNOs (Vanin, 2009; Borodulin et al., 2014), which are also potent vasodilators. Determination of the redox state of the NO species released by DNICs is a necessary step toward understanding their mechanism of bioactivity.

In addition to interests in the physiologic role of DNICs, their NO-mimetic properties have made them important therapeutic candidates (Vanin, 2009). Unlike other nitro-dilators, glutathione-ligated BDNIC (glut-BDNIC) has long-lasting hypotensive effects and is currently in clinical trials for treatment of hypertension (Chazov et al., 2012). It has been proposed that glut-BDNIC is converted into more stable plasma albumin–liganded MDNIC in the circulation (Boese et al., 1995; Timoshin et al., 2007; Suryo Rahmanto et al., 2012). Nevertheless, the kinetics and mechanism of this conversion are not clear, let alone the effects on BDNIC-mediated vasodilation.

This study was undertaken to investigate the mechanism of glut-BDNIC–mediated vasodilation in both isolated arteries and in vivo, and to characterize the relationship between the hemodynamic effects and pharmacokinetics of glut-BDNIC. Using both wire myography bioassay and chemical analysis, we tested the hypothesis that DNICs release HNO rather than NO·. Using wire myography again, we characterized the role of plasma albumin in modulating the vasodilatory effects of glut-BDNIC. Finally, we investigated the hemodynamic effects of glut-BDNIC and correlated this with its pharmacokinetics both in vitro and in vivo.

Materials and Methods

Experimental Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University, and were in accordance with guidelines of the American Physiologic Society and the National Institutes of Health.

Preparation of S-Nitroso-Glutathione, DNICs, and NO Gas. S-nitroso-glutathione (GSNO) and DNICs were prepared as reported previously (Borodulin et al., 2013b; Liu et al., 2016a). NO gas was generated by reaction of HCl and nitrite followed by purification with NaOH. Further details are provided in the Supplemental Materials.

Surgical Procedures and Bolus Injection Protocol in Sheep. Neutered male sheep 9–10 months old and weighing 37 ± 2 kg were surgically instrumented as previously reported (Liu et al., 2016a) and are described in more detail in the Supplemental Materials. After surgical instrumentation, isoflurane (1.5%–2.5%) was discontinued, and anesthesia was maintained with ketamine (1 mg/kg) and vecuronium (0.1 mg/kg; Sun Pharmaceutical, Mumbai, India) given intravenously and supplemented hourly or as needed. L-NW-nitro arginine methyl ester (45 mg/kg) and hexamethonium (1 mg/kg for initial dose; 2 mg·h⁻¹·kg⁻¹ for continuous dose) were given intravenously to block endogenous NO synthesis and sympathetic activity, respectively. After a baseline period of 30 minutes, a 1-mL bolus of NO· provided by dissolving NO gas in saline, GSNO, or glut-BDNIC was injected into the jugular vein while the mean arterial blood pressure was recorded through a catheter in the brachial artery. Average hypotensive responses during 120-second intervals were calculated and normalized to the baseline values.

Surgical Procedures and Experimental Protocol in Rats. Female rats weighing 301 ± 3 g were anesthetized and surgically prepared in a manner similar to the sheep and as described in the Supplemental Materials. After surgical instrumentation, isoflurane (2.5%) was discontinued, and anesthesia was maintained with an intraperitoneal injection of urethane (1000 mg/kg) and supplemented thereafter as required. Hexamethonium (1 mg/kg for initial dose; 2 mg·h⁻¹·kg⁻¹ for continuous dose) was given intravenously to limit neural influences. Mean arterial blood pressure and heart rate were recorded through a catheter in the carotid artery. Rats were given continuous infusions or bolus injections. In the constant infusion protocol, 25 μM glut-BDNIC or 50 μM GSNO was infused into the lower abdominal aorta at rates of 0.05, 0.1, 0.2, and 0.4 μL/min, increasing every 3 minutes, with doses providing a total of ~0.19 μmol/kg glut-BDNIC or ~0.37 μmol/kg GSNO, respectively. Since each glut-BDNIC molecule contains four NO moieties, this equates to ~0.75 μmol/kg NO moiety during the 12-minute infusion. In the bolus injection protocol, 0.29 ± 0.02 mL of 2.5 mM glut-BDNIC (2.42 μmol glut-BDNIC molecule or 9.68 μmol/kg NO moiety) was injected into the lower abdominal aorta over 5 seconds. Blood samples were then collected from the jugular vein for electron paramagnetic resonance (EPR) analyses. Average hemodynamic responses during 20-second intervals at different time points were calculated and compared with baseline values.

Wire Myography. Ovine mesenteric arterial rings (2-mm diameter and 5 mm long) were denuded of endothelium (verified with 1 μM acetylcholine) and mounted in organ bath chambers as previously described (Liu et al., 2016b). Following preconstriction with 10 μM serotonin, dose-response curves were measured for study drugs including glut-BDNIC, glut-BDNIC, PROLI-NONOate (NO· donor; Cayman Chemical, Ann Arbor, MI), GSNO, and Angelis’s salt (AS; HNO donor; Cayman Chemical). Test compounds were added to the baths 5 minutes before the contraction and maintained until the end of the experiments. These compounds included the sGC inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one; 10 μM), the NO· and HNO scavenger CPTIO (2-(4-carboxyphenyl)-4,4,5-tetramethylimidazoline-1-oxyl-3-oxide; 200 μM), superoxide dismutase 1 (SOD; 1000 U/mL), 1,4-dithiothreitol (DTT; 1 mM), bovine albumin (15 μM) (all from Sigma-Aldrich, St. Louis, MO), and heparinized plasma collected from sheep. Relaxation was normalized to the tension (100%) measured just prior to the first addition of the dose-response drug.

Spontaneous changes in the tension of control vessels that did not receive a test drug were subtracted from individual experiments before calculation of responses. Maximal absolute tensions of sheep mesenteric arterial rings in the absence and presence of different test compounds are shown in Supplemental Fig. 2.

Hydroxylamine Assay. The details are described in the Supplemental Materials.

EPR. EPR signals were recorded from paramagnetic DNICs at room temperature or 150 K using an X-Band EPR spectrometer (Bruker, Billerica, MA). Samples with CPTIO (50 μM), the NO· and HNO scavenger CPTIO (2-(4-carboxyphenyl)-4,4,5-tetramethylimidazoline-1-oxyl-3-oxide; 200 μM), superoxide dismutase 1 (SOD; 1000 U/mL), 1,4-dithiothreitol (DTT; 1 mM), bovine albumin (15 μM) (all from Sigma-Aldrich, St. Louis, MO), and heparinized plasma collected from sheep. Relaxation was normalized to the tension (100%) measured just prior to the first addition of the dose-response drug.

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Fig. 1. Role of HNO in BDNIC-mediated relaxation in isolated sheep mesenteric arteries \( (n \geq 5 \text{ for (A–F); } n = 3 \text{ for (G))} \). Glut-BDNIC–mediated relaxation in the absence and presence of the sGC inhibitor ODQ (10 \( \mu \text{M} \)) or the NO\(^*\) and HNO scavenger CPTIO (200 \( \mu \text{M} \)) (A), or the enzyme SOD (1000 U/ml, converts HNO into NO\(^*\)) or SOD (1000 U/ml) and CPTIO (200 \( \mu \text{M} \)) (B). Effects of SOD (1000 U/ml), CPTIO (200 \( \mu \text{M} \)), or SOD (1000 U/ml) and CPTIO (200 \( \mu \text{M} \)) on NO\(^*\) (from PROLI-NONOate) (C) and HNO-mediated (from AS) relaxation (D). The effects of DTT (1 mM, HNO scavenger) on AS (E) and glut-BDNIC-mediated (F) relaxation. The vessel rings were \( \sim 2 \text{ mm in diameter and 5 mm long. Information concerning the various reactions is shown in each panel. Relaxation was normalized to the tension (100%) prior to the first addition of the dose-response drug. (G) In vitro measurements to detect the production of hydroxylamine, a metabolite of HNO, from glut-BDNIC. N.D., not detected.} \)
Modification of Albumin. To test for interactions of glut-BDNIC with albumin, the protein was modified chemically. Albumin (840 μM) in phosphate-buffered saline at pH = 7.4 was incubated with the thiol modifier HgCl₂ (5 mM) and/or the histidine modifier diethylpyrocarbodine (6 mM) at room temperature for 40 minutes and then desalted using a Sephadex G-25 column with an exclusion limit of 5000 Mᵋ (GE Healthcare, Uppsala, Sweden).

Statistics. Average values are given as the mean ± S.E.M. in the text and figures. Statistical analyses were carried out with Prism, version 5.0c (GraphPad Software, La Jolla, CA), with significance accepted at P < 0.05. One-way analysis of variance with Dunnett’s test was used to test significance of changes associated with rates of intravenous infusion. Student’s t test was used where indicated. Relaxation results from wire myography experiments were quantitated and compared by fitting the data to the Gaddum/Schild model in Prism. Rates of DNIC elimination from circulating blood were quantified by fitting the concentration versus time curve to a one-phase decay function using Prism after subtraction of baseline concentrations (nil).

Results

Verification of Laboratory-Prepared DNICs. The UV-Vis spectra of glut-BDNIC and glut-MDNIC were as others have found (Borodulin et al., 2014), indicating successful synthesis (Supplemental Fig. S1). Glut-MDNIC displayed a typical EPR signal with an axial symmetric g-factor with g₁ = 2.04, g₂ = 2.014, and g₃ = 2.03. Glut-BDNIC, which itself is diamagnetic, was EPR-silent, indicating no detectable contamination with glut-MDNIC. The prepared glut-BDNIC was 99.5% ± 0.7% pure as estimated by the extinction coefficient of 7400 M⁻¹ cm⁻¹ at 360 nm (Borodulin et al., 2014).

Role of HNO in BDNIC-Mediated Relaxation. The relaxation of isolated ovine arterial rings induced by glut-BDNIC was eliminated by the sGC inhibitor ODQ (Fig. 1A; does not degrade DNICs, as described in the Supplemental Materials), indicating that the relaxation is sGC-dependent (Moro et al., 1996). CPTIO, which scavenges both NO⁺ and HNO (Goldstein et al., 2003; Samuni et al., 2010), attenuated the response to glut-BDNIC (Fig. 1A) as evidenced by a decrease in the pEC₅₀ from 6.03 ± 0.16 to 5.33 ± 0.13 (P < 0.05). To assess whether the attenuating effects of CPTIO on glut-BDNIC–mediated vasodilation were due to HNO or NO⁺ scavenging, we compared the effects of CPTIO on dose-response curves to known donors of either HNO or NO⁺ (provided by PROLI-NONOate). Similar to its attenuation of glut-BDNIC–mediated vasodilation, CPTIO resulted in a partial right shift in the dose-response curve to HNO (Fig. 1D, pEC₅₀ from 5.7 ± 0.2 to 5.1 ± 0.2). In contrast, vasodilatory responses to NO⁺ were almost completely blocked (Fig. 1C, pEC₅₀ from 6.0 ± 0.1 to an extent that the EC₅₀ could not be calculated). Notably, the effect of CPTIO on vasodilation by glut-BDNIC was similar in magnitude to its effect on the AS dose-response curve, consistent with release of HNO by glut-BDNIC.

To further test the possibility that glut-BDNIC releases HNO, the effects of CPTIO were compared in the presence and absence of SOD, which readily converts HNO into NO⁺ (Zeller et al., 2009). In the presence of SOD, the ability of CPTIO to attenuate vasodilation by glut-BDNIC was enhanced to a level comparable to its ability to attenuate vasodilation by NO⁺ (Fig. 1, A and B, P < 0.05 at log([BDNIC]) (M) = −5.30), consistent with the idea that SOD was converting HNO to NO⁺, which is more efficiently scavenged by CPTIO. SOD potentiated the right shift caused by CPTIO for both glut-BDNIC and AS, but had no effect on NO⁺-induced relaxation (Fig. 1, B–D). In further support of the involvement of HNO, the HNO scavenger DTT (Zeller et al., 2009), which significantly blocked relaxation by the HNO donor AS (Fig. 1E), also blocked glut-BDNIC–mediated relaxation (Fig. 1F). Taken together, these findings demonstrate a potent vasodilatory activity of glut-BDNIC in isolated arterial rings and suggest a role for the release of HNO.

Parallel experiments were performed using glut-MDNIC. As shown in Supplemental Fig. 3A, SOD alone did not alter glut-MDNIC–mediated relaxation, and CPTIO only partially inhibited it. However, the inhibitory effects of CPTIO were potentiated by the presence of SOD. Furthermore, glut-MDNIC–mediated vasodilation was blocked by ODQ and significantly attenuated by DTT (Supplemental Fig. 3B). These parallel findings are all consistent with glut-MDNIC acting as an HNO donor in a manner similar to glut-BDNIC.

Chemical Verification of Glut-DNICs as HNO Donor. To further test the hypothesis that glut-DNIC releases HNO, experiments were conducted based on the recently reported distinction between the reactions of HNO and NO⁺ with CPTIO (Samuni et al., 2010), which demonstrated that EPR can be used to distinguish between the products of CPTIO reacting with either NO⁺ or HNO. Consistent with previous reports (Samuni et al., 2010), we found CPTIO to have a five-peak EPR spectrum (Fig. 2A). This spectrum was rapidly converted to a seven-peak spectrum upon reaction of the CPTIO with NO⁺ provided by the NO donor PROLI-NONOate (Fig. 2B). In contrast, the five-peak CPTIO spectrum gradually disappeared over a period of 90 minutes if the CPTIO was allowed to react with HNO from excess AS (Fig. 2D). Finally, addition of an oxidizing solution of CuSO₄/H₂O₂/ferricyanide (10 μM/10 mM/1 mM) resulted in the seven-peak spectrum (Fig. 2D). These findings are consistent with previous work characterizing the EPR spectra of CPTIO products of reaction with NO⁺ or HNO, as well as oxidation of the product of the reaction of CPTIO with HNO (Samuni et al., 2010). Importantly, similar to AS, addition of glut-BDNIC to CPTIO resulted in a complete loss of the EPR spectrum, which could be restored to the seven-peak spectra by the addition of CuSO₄/H₂O₂/ferricyanide (Fig. 2E). Notably, glutathione (GSH) alone at a comparable level did not eliminate the EPR signal of CPTIO (Fig. 2C), indicating that the effect of glut-BDNIC on the CPTIO spectra was not due to GSH contamination. Taken together, these results are consistent with the idea that glut-BDNIC releases HNO, not NO⁺. In addition, the EPR signal of CPTIO was also eliminated by glut-MDNIC (data not shown), suggesting the same conclusion may be applied to glut-MDNIC.

Further experiments were performed to characterize the NO species released from glut-BDNIC. Using HEPES buffer (pH 7.4) as a reagent in a purge vessel in line with a chemiluminescence NO⁺ analyzer, the NO⁺ release from glut-BDNIC was compared with that from the NO⁺ and HNO donors. Similar to AS but different from PROLI-NONOate, glut-BDNIC released minimal amounts of NO⁺. These results were not affected by the presence of isolated mesenteric artery tissue in the purge vessel (Supplemental Fig. 4), indicating glut-BDNIC is a poor donor of NO⁺. To further investigate the
oxidative state of NO species released from glut-BDNIC, measurements of hydroxylamine, one product of HNO reduction, were obtained following incubation of glut-BDNIC in phosphate-buffered saline at pH 6.8. After 5-minute incubation at 95°C, 20 μM hydroxylamine was formed from an initial concentration of 250 μM glut-BDNIC. In contrast, levels of hydroxylamine were undetectable following incubation of NO• or GSNO (Fig. 1G). These results are again consistent with the possibility that glut-BDNIC is a donor of HNO rather than NO•.

Glut-BDNIC Loses Hypotensive Effects In Vivo. Contrary to the potent relaxation found in isolated arteries in the vessel baths (Fig. 1), glut-BDNIC did not reduce blood pressure when low doses were given by continuous infusion in rats (Fig. 3A). On the other hand, GSNO given at comparable doses was vasodilative both in vivo and in vitro (Figs. 3B and 4E). Similar results were obtained in sheep given low doses of glut-BDNIC or GSNO by bolus injection (Fig. 4A). The hypotensive responses to bolus injections of NO•, GSNO, and glut-BDNIC in sheep are shown in Fig. 4, B–D. One possible reason for the lack of response in vivo is that glut-BDNIC is converted in circulating blood into a form that does not release HNO as readily. Another possibility is that any HNO that might be released from glut-BDNIC is rapidly scavenged by reaction with other components present in the blood but not in the vessel baths. Notably, the observation that 50 μM GSNO caused vasodilation at 0.1 ml/min whereas 25 μM glut-BDNIC (which contains 100 μM NO moieties) infused at 0.4 ml/min had no effect (Fig. 3) suggests that the production of SNOs from glut-BDNIC in vivo, if it occurs at all, accounts for less than 1/8 of the NO moieties in glut-BDNIC.

Role of Plasma Albumin in the Loss of Hypotensive Effects of Glut-BDNIC. Further tests were carried out in isolated arteries to learn what component of blood might prevent glut-BDNIC–induced vasodilation. The iron chelators pyrrodoxal isonicotinoyl hydrazone and EDTA (ethylene diamine tetraacetic acid) did not affect the glut-BDNIC–mediated relaxation (Fig. 5B), indicating no role for endogenous iron chelators. Addition of heparinized plasma to the vessel bath significantly right shifted the dose-response curve of glut-BDNIC (P < 0.05; Fig. 5A), indicating that one of its components contributes to the loss of hypotensive effects of glut-BDNIC in vivo (Fig. 3A). Similar to plasma, albumin alone right shifted the dose-response curve of glut-BDNIC significantly (P < 0.05; Fig. 5C), indicating it plays a role, perhaps by either scavenging HNO or stabilizing the BDNIC and thus inhibiting its release of HNO. The first possibility was tested and ruled out by the finding that plasma did not significantly attenuate AS-mediated relaxation (Supplemental Fig. 5). To test whether the histidine or cysteine residues of albumin were involved in stabilization of glut-BDNIC, experiments were carried out with covalently modified albumin. Modification of histidine residues resulted in no change in the attenuating effects of albumin on BDNIC-mediated relaxation (Fig. 5D). Modification of the cysteine-thiols, however, resulted in a loss of the attenuating effects of albumin (P < 0.05; Fig. 5D). These results indicate that the cysteine-thiol, but not the histidine residues of albumin are involved in stabilization of glut-BDNIC.
Residue, in albumin plays an important role in attenuating the vasodilatory effects of glut-BDNICs. Whole blood contracted the isolated arterial rings significantly, and thus its effects on glut-BDNIC–mediated relaxation were not tested.

Parallel experiments were performed to evaluate the effects of plasma and albumin on vasodilation by glut-MDNIC. In contrast to the significant attenuating effects of plasma and albumin on glut-BDNIC–mediated vasodilation, neither plasma nor albumin significantly altered glut-MDNIC–induced relaxation (Supplemental Fig. 6), suggesting the functional effects of glut-BDNIC differ from those of glut-MDNIC under these conditions.

Long-Lasting Hypotensive Effects and Pharmacokinetics of BDNIC. Differing from low doses, high doses of glut-BDNIC led to long-lasting hypotensive effects in intact rats (Fig. 6A). Within 3 minutes after infusion of the EPR-silent glut-BDNIC, an MDNIC-like EPR signal was detected in blood samples, although a shallow shoulder appeared on the left side of the spectrum (Fig. 6C). A similar spectrum with a shallow shoulder could be observed after addition of glut-BDNIC to isolated blood, plasma, and albumin solutions (Supplemental Fig. 7). These findings suggest that glut-BDNIC is rapidly converted to an MDNIC-like species whether infused in vivo or added to blood, plasma, or albumin solutions in vitro. Glut-BDNIC retained some vasodilatory activity in vivo, albeit at markedly higher doses.

The intensity of the EPR signal at the first time point (3 minutes) following infusion of glut-BDNIC corresponded to 70.9 ± 2.9 μM MDNIC (Fig. 6D). This concentration is comparable to that which would be predicted based on a volume of distribution equal to the rat blood volume [4.84 μmol Fe(NO)2 nuclei infused per kg ÷ 0.068 l of blood per kg = 71.1 μM], assuming a blood volume in rats of ~0.068 l/kg (Lee and Blaufox, 1985), suggesting that all glut-BDNIC administered was converted to MDNIC-like metabolites that were retained in the blood. The time course of the DNIC

Fig. 4. Hypotension induced by bolus injections of NO*, GSNO, and glut-BDNIC in anesthetized sheep. (A) Effects of NO*, GSNO, and glut-BDNIC on mean arterial blood pressure (MAP) following a 1.0-ml bolus given in the jugular vein (n = 4). (B–D) Representative hypotensive effects of bolus injection of NO (1 ml of 2.0 mM or 54 nmol/kg) (B), GSNO (1 ml of 2 mM or 54 nmol/kg) (C), and glut-BDNIC (1 ml of 2.5 mM or 270 nmol/kg) (D). (E) Relaxation in isolated sheep mesenteric arteries in response to GSNO (n = 5). ****P < 0.0001 vs. saline (one-way analysis of variance with Dunnett’s post hoc test). ns, not significant.
disappearance from the circulation followed first-order reaction kinetics with a half-life of $\sim 44$ minutes (Fig. 6D). Thus, the long duration of the hypotensive effects may be at least partially attributed to the relatively long elimination half-life of the MDNIC-like metabolite.

**Conversion and Distribution of BDNIC in Blood In Vitro.** Given the evidence that intravenously administered glut-BDNIC is rapidly and completely converted into a MDNIC-like metabolite in vivo, we sought to characterize the conversion and distribution of glut-BDNIC in blood in vitro. Addition of EPR-silent glut-BDNIC to either lysed red blood cells (RBCs) or plasma efficiently resulted in an EPR-detectable MDNIC-like metabolite. In contrast, glut-BDNIC added to the low molecular weight (LMW) fraction of plasma remained EPR-silent (Fig. 7A). Compared with native plasma, the conversion of glut-BDNIC into EPR-detectable species was about 50% less efficient in the presence of only the high molecular weight (HMW) fraction of plasma or albumin solution (Fig. 7A). Thus, the HMW fraction of plasma and the albumin therein play important roles in converting glut-BDNIC to MDNIC-like metabolites, and this conversion may be facilitated by one or more components found in the LMW fraction of plasma.

Similar to plasma and lysed RBCs, whole blood converted glut-BDNIC into MDNIC-like metabolite (Fig. 7B). All of the EPR-detectable metabolite was recovered in the plasma compartment of whole blood, indicating that the conversion to MDNIC-like metabolite occurs in the plasma and not in the interior of RBCs. In comparison with whole blood, washed RBCs suspended in saline were less effective at converting glut-BDNIC (Fig. 7B) into an EPR-detectable species, in agreement with the conclusion that plasma and probably the albumin therein play important roles in the conversion. No MDNIC-like metabolite was detected in the RBCs of plasma-free blood, indicating that glut-BDNIC itself, without binding to plasma proteins, was also not membrane-permeable.

Following addition of glut-BDNIC to whole blood, most of the MDNIC-like metabolite was recovered in the HMW fraction of plasma, consistent with its binding to plasma proteins (Fig. 7C). In addition, after adding glut-BDNIC to blood, plasma, or dissolved albumin, the EPR spectra obtained at room temperature did not display an isotropic hyperfine structure (seen as multiple sharp peaks pointing upward at the left side of the spectrum versus down on the right side in the bottom spectrum of Supplemental Fig. 8), which is characteristic of LMW but not HMW MDNIC (Vanin, 2009). These results further support the conclusion that the glut-BDNIC in blood and plasma was converted to HMW complexes likely containing albumin.

**Discussion**

The results of the present experiments are consistent with previous work demonstrating that glut-BDNIC is a potent vasodilator via sGC activation (Vanin, 2009). However, contrary to previous propositions that DNICs are donors of NO$^-$ and NO$^+$ (Vanin, 2009; Borodulin et al., 2013a), we present both functional and chemical evidence that glut-DNIC acts by releasing HNO. In addition, our results suggest that the vasodilating potency of glut-BDNIC is markedly attenuated in the presence of plasma proteins (such as albumin), likely through an interaction with the cysteine residues.
What Is the Redox State of the NO Moieties in DNIC? The redox state of NO moieties in DNICs has been uncertain. Based on X-ray studies, the Fe(NO)2 core has been formulated as either \{Fe\(^{II}\)(NO\(^{+}\))(NO\(^{-}\))\}(9) or \{Fe\(^{III}\)(NO\(^{+}\))(NO\(^{-}\))\}(2)(9), in which the NO moieties have partial negative charges (Tsai et al., 2015; Tseng et al., 2015) and thus might be expected to be released in the form of HNO. Alternatively, others have proposed the core to exist as \{Fe\(^{I}\)(NO\(^{+}\))(NO\(^{-}\))\}(7), representing the NO moieties as positively charged (Vanin, 2009; Borodulin et al., 2013a, 2014). The latter formulation is supported by evidence that DNICs can S-nitrosate thiols to produce SNOs (Boese et al., 1995; Stojanovic et al., 2004; Bosworth et al., 2009; Vanin, 2009; Vanin and Burbaev, 2011).

The current results support the idea that DNICs are donors of HNO rather than NO\(^{+}\) or NO\(^{-}\). There are multiple reasons to put forward this conclusion. First, we found that the attenuation effects of CPTIO on glut-BDNIC–mediated vasodilation are comparable to its effects on dilation by the HNO donor AS. In addition, CPTIO inhibits vasodilation by the NO\(^{-}\) donor PROLI-NONOate more efficiently than by either glut-BDNIC or AS, suggesting that glut-BDNIC releases an NO species more like HNO than NO\(^{+}\). The addition of SOD, which converts HNO to NO\(^{-}\) (Zeller et al., 2009; Flores-Santana et al., 2011), resulted in a marked increase in the ability of CPTIO to attenuate glut-DNIC–mediated vasodilation, a phenomenon that was also observed for the HNO donor AS but not for NO\(^{-}\) itself. Likewise, vasodilation by both BDNIC and the HNO donor AS was similarly attenuated by the addition of DTT, which scavenges HNO by converting it to H\(_2\)NOH (Zeller et al., 2009). These results are all more supportive of a role for HNO than NO\(^{+}\) in BDNIC-mediated relaxation. Furthermore, based on the EPR-characterized distinction between the products of HNO and NO\(^{-}\) reactions with CPTIO (Samuni et al., 2010), we chemically confirmed glut-DNIC as an HNO donor. In addition, little NO\(^{+}\) was released from glut-BDNIC, whereas hydroxylamine, an HNO metabolite, was detected in glut-BDNIC, again suggesting that glut-BDNIC is a donor of HNO rather than NO\(^{-}\).

The potential sources and roles of endogenous HNO have been the subject of debate for several decades (Switzer et al., 2009). As the most abundant cellular NO adduct (Hickok et al., 2011), DNICs have been proposed to be the “working form” of endogenous NO activity (Vanin, 2016). Our evidence that DNICs can behave as HNO donors provides a potential mechanism of endogenous HNO formation and implies an important role for endogenous HNO.

In addition to evidence that DNICs release HNO, several experiments reported here speak against previous proposals that DNICs release NO\(^{+}\), which then might act as an S-nitrosating agent (Boese et al., 1995; Stojanovic et al., 2004; Bosworth et al., 2009; Vanin, 2009; Vanin and Burbaev, 2011). First, in contrast to the potent hypotensive effects of GSNO in...
rats and sheep, blood pressure was unaffected by administration of comparable doses of glut-BDNIC (Figs. 3 and 4). Such an observation suggests that little SNO was formed in vivo following glut-BDNIC infusion (calculated earlier as 1/8 of BDNIC infused). Further, EPR results demonstrated nearly complete recovery of both glut-BDNICs and glut-MDNICs as DNICs after their reaction with plasma, excluding the possibility they were converted to SNOs (Fig. 7C). There are several possible explanations for the discrepancy between previous reports suggesting DNICs are converted to SNOs (Boese et al., 1995; Stojanovic et al., 2004; Bosworth et al., 2009; Vanin, 2009; Vanin and Burbaev, 2011) and the lack of evidence in our current work. First, some previous experiments were performed using NO as the initiator of reactions in the presence of iron and thiols (Stojanovic et al., 2004; Bosworth et al., 2009). In these experiments, DNICs were simply an intermediate product, whereas presynthesized DNICs were used for the current experiments. Therefore, in the previous studies, the SNOs might have been a byproduct of oxidation reactions of the NO that was used for synthesizing the DNICs. Others have reported the production of SNOs from cellular DNICs under hypoxic or anoxic conditions, thus minimizing the possibility of NO oxidation reactions. However, these previous studies used HgCl₂ to selectively detect SNO concentrations, a method recently found to artifactually detect DNICs as SNOs (Keszler et al., 2017). Although recent closer examinations (Hickok et al., 2011, 2012) of this issue agree with our results that production of SNOs from DNICs is not a predominant mode of action of DNICs, if it occurs at all, we would submit that a validated method that selectively detects SNOs in the presence of DNICs is needed to address this question rigorously.

**What Are the Hypotensive Effects of BDNIC and the Underlying Mechanisms?** The hypotensive effects of glut-BDNIC have been described by previous researchers (Lakomkin et al., 2007; Chazov et al., 2012; Borodulin et al., 2013b; Timoshin et al., 2015), leading to the ongoing clinical development of glut-BDNIC for treating hypertension (Chazov et al., 2012). Our results confirm these previous reports by demonstrating a prolonged hypotensive response to high-dose bolus injection (Fig. 6). In the present experiments, the hypotension lasted even longer than comparable doses used in previous reports (Chazov et al., 2012; Borodulin et al., 2013b), perhaps due to sympathetic blockade in our animal model. Although GSNO and glut-DNICs had similar vasodilatory potencies in isolated arterial rings (Figs. 1 and 4E; Supplemental Fig. S3), their potencies differed markedly in vivo. As calculated in the Results section, GSNO was found to be at least an 8-fold more potent vasodilator than glut-BDNIC in vivo. Notably, glut-BDNIC–induced hypotension lasted for...
more than 4 hours (Fig. 6). Such persistence could be at least partly attributed to the long elimination half-life of the MDNIC-like metabolite derived from glut-BDNIC (Fig. 6D) and is likely influenced by its interaction with plasma albumin as discussed next.

**How Might Plasma Alter the Vasoactivity of Glut-BDNIC?** It has long been proposed that proteins stabilize DNICs (Vanim, 2009; Suryo Rahmanto et al., 2012), possibly attenuating their physiologic activity. Consistent with such stabilization, we found that plasma attenuated glut-BDNIC–mediated vasodilation both in vitro and in vivo. Other earlier investigators found that MDNIC could exchange its LMW thiolate ligands for either the cysteine-thiol or histidine residues in albumin to form HMW MDNICs (Boese et al., 1995; Timoshin et al., 2007). The present glut-BDNIC experiments support only the involvement of the cysteine-thiol residues, however (Fig. 5D), as the vasoactivity of glut-BDNIC in the presence of albumin was only attenuated by covalent modification of cysteine-thiol residues and not by modification of histidine residues. This finding suggests that glut-BDNIC indeed binds to albumin, possibly via its cysteine-thiol.

Notably, glut-BDNIC and glut-MDNIC added into plasma were both converted into HMW metabolites (Fig. 7C). The EPR spectrum of the HMW metabolite of glut-MDNIC is indistinguishable from that of glut-MDNIC itself. In contrast, although the glut-BDNIC changes from an EPR-silent signal to one that is similar to that of MDNICs, distinct differences in the spectra were noted (Supplemental Fig. 7). These differences may indicate that the HMW metabolites of glut-BDNIC are not physically equivalent to the HMW metabolites of glut-MDNIC. Consistent with this view, the significant attenuating effects of plasma and albumin on glut-BDNIC–mediated vasodilation were not observed for glut-MDNIC–induced relaxation (Supplemental Fig. 6). These results suggest that the HMW metabolite of glut-BDNIC is also functionally different from the HMW metabolite of glut-MDNIC. Thus, although glut-BDNIC and glut-MDNIC display similar vasodilatory and HNO-releasing characteristics in vitro, key differences exist between the physical characteristics and vasodilatory effects of their HMW products in plasma, suggesting that further characterization of the structure of the HMW glut-BDNIC metabolite is needed before concluding that it is simply an HMW MDNIC.

The reason for the decrease in vasodilatory potency of glut-BDNIC upon reacting with albumin is not well understood. One possibility is that the NO moieties in the Fe(NO)₂ core are stabilized when the DNIC is bound to albumin. The decreased vasoactivity would then be a result of a decreased rate of release of HNO from the HMW DNIC. Alternatively, the albumin-bound DNIC may not release HNO, but is instead in equilibrium with a minor fraction of LMW DNIC (Fig. 7C), which is less stable (Timoshin et al., 2015) and thus more likely to release HNO. In either case, the stabilization would prolong the hypertensive effects of DNICs and account for the relatively long biologic half-life of 44 minutes.

Because neither glut-BDNIC nor its HMW metabolites are membrane-permeable (Figs. 6D and 7B), vasodilation is not likely to involve direct interaction between DNICs and sGC. Instead, it may result from the release of HNO from the DNIC outside the cell, which can then diffuse into the smooth muscle cells and activate the sGC either directly (Miller et al., 2009) or after conversion to NO* (Zeller et al., 2009). In any event, the current experiments do not exclude the possible involvement of an undiscovered vascular smooth muscle membrane transporter or receptor-mediated dilatory signaling pathway that could be stimulated by DNICs. A further possibility is that the hypotensive effects of glut-DNIC result from the calcitonin gene-related peptide which can be induced by HNO via sGC activation (Favaloro and Kemp-Harper, 2007).

Some limitations of the present study should be acknowledged. First, although multiple lines of evidence from both functional and chemical tests support glut-BDNIC as an HNO donor, further investigation with scavengers or probes that can react selectively with HNO over NO⁺ is necessary. Although not currently commercially available, such compounds are under active development and validation (Tan et al., 2015; Zhou et al., 2017). Second, depending upon the conditions, the redox state of NO moieties in glut-BDNIC might vary. Thus, we cannot exclude the possibility that DNICs might exhibit distinct biochemical properties under a different environment.

**Conclusion**

In sum, the results of this study indicate that glut-BDNIC is an HNO donor and a poor, if at all, NO⁺ or NO⁺ (SNO) generator. Plasma proteins (such as albumin), via reactions involving its cysteine-thiol residues, modulate glut-BDNIC–mediated vasodilation in such a way that attenuates its hypotensive potency but prolongs its duration of action.

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**Authorship Contributions**

**Participated in research design:** Liu, Zhang, Schroeder, Wilson, Power, Li, Tipple, Borchardt, Blood.

**Conducted Experiments:** Liu, Zhang, Terry, Schroeder, Li, Borchardt.

**Performed data analysis:** Liu, Zhang, Terry, Schroeder, Wilson, Power, Li, Borchardt, Blood.

**Wrote or contributed to the writing of the manuscript:** Liu, Schroeder, Power, Li, Blood.

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Supplementary Materials

Hemodynamic effects of glutathione-liganded binuclear dinitrosyl iron complex:
evidence for nitroxyl generation and modulation by plasma albumin

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Supplementary Methods

Surgical procedures in sheep

Neutered male sheep nine to ten months old and weighing 37±2 kg were surgically
instrumented as previously described [1; 2]. While mechanically ventilated with 1.5 to
2.5% isoflurane (Baxter U. S., Deerfield, Illinois) in oxygen, a catheter was inserted into
a side-branch of a femoral artery and its tip then advanced to within 2mm of the main
femoral artery. Another catheter was inserted into a brachial artery to measure arterial blood pressure, heart rate, and blood gases. Other catheters were placed in a brachial vein to administer L-N⁴-nitro arginine methyl ester (L-NAME; Sigma Aldrich, St Louis, MO) and hexamethonium (Sigma Aldrich), and in a jugular vein to administer anesthetics.

After surgical instrumentation, isoflurane was discontinued and anesthesia was maintained with ketamine (1mg/kg) and vecuronium (0.1 mg/kg; Sun Pharmaceutical, Mumbai, India) given intravenously and supplemented hourly or as required.

Surgical procedures in rats

Female rats weighing 301±3 g were surgically instrumented while anesthetized with 2.5% isoflurane in oxygen. A polyvinyl catheter was inserted into a femoral artery and its tip was advanced to within 2 mm of its origin from the lower abdominal aorta. A catheter was inserted into a carotid artery to measure arterial blood pressure, heart rate, and blood gases. A catheter was placed in a jugular vein to administer hexamethonium. The other jugular vein was exposed for blood sample collection by puncture through the subclavus
After surgical instrumentation, isoflurane was discontinued and anesthesia was maintained with an intraperitoneal injection of urethane (1000 mg/kg) and thereafter supplemented as required. A bolus of hexamethonium (1 mg/kg, iv) followed by a continuous infusion (2 mg·h⁻¹·kg⁻¹, iv) was given to limit neural influences on vascular tone. Rectal temperature was monitored and body temperature was maintained with a warming pad and heat lamp. The rats were mechanically ventilated with room air, with tidal volume and ventilatory rate adjusted to maintain arterial carbon dioxide tension in the range of 35 to 45 mmHg and pH from 7.35 to 7.45.

Preparation of GSNO, DNICs, and NO gas

5 mM GSNO: 5 mM GSH in 0.5 M HCl was incubated with 5 mM sodium nitrite for 30 min on ice. The stock solution was neutralized with NaOH and diluted with 0.1 M of physiological buffered saline (PBS) at pH=7.4 containing 0.1 mM diethylene triamine pentaacetic acid (DTPA) immediately before use.
2.5 mM glut-BDNIC \([(Glut)_{2}Fe_{2}(NO)_{4}]\): 20 mM GSH, 10 mM FeSO\(_4\), and 10 mM NaNO\(_2\) were mixed and incubated for two hours at room temperature. The solution was neutralized with NaOH to pH=7.2 and incubated for another 15 h. Then the stock solution was collected after passing through a filter paper.

5 mM glut-MDNIC \([(Glut)_{2}Fe(NO)_{2}]\): To the 2.5 mM glut-BDNIC 50 mM GSH was added. The solution was alkalized with NaOH to pH=11.0 and incubated for three hours at room temperature.

Because DTPA eliminates EPR signals of glut-MDNIC, PBS or saline that does not contain DTPA was used for dilution of DNICs.

NO gas was generated by reaction of 1 M HCl and 1 M nitrite in syringes. The gas was then introduced into 1 M NaOH and shaken vigorously to eliminate the trace amount of nitrogen oxides. All solutions were deoxygenated before use. Excess amount of purified NO gas was introduced into deoxygenated saline to make a saturated solution of NO (~2 mM). NO solution was prepared freshly each time before use.
**Effects of ODQ on stability of DNICs**

Experiments were performed to test the effects of ODQ on the stability of glut-MDNIC and glut-BDNIC (n=3). Incubation of 100 μM ODQ with 50 μM glut-MDNIC at room temperature for 5 min did not decrease the EPR signal of glut-MDNIC (98±5% recovered), indicating that ODQ did not degrade MDNIC. Because glut-BDNIC is EPR-silent, it was quantified as MDNIC after conversion into MDNIC-like metabolite by incubation with plasma. Two experiments were performed: First, ODQ was incubated with glut-BDNIC for 5 min and then with plasma for another 5 min; Second, glut-BDNIC was incubated with plasma for 5 min and then with ODQ for another 5 min. Both experiments showed that the yield of MDNIC-like metabolite derived from glut-BDNIC was not affected by ODQ (97±5% recovery).

**Hydroxylamine assay**

This colorimetric assay is based on the formation of indooxine in the reaction between hydroxylamine and 8-hydroxyquinoline[3]. The lower limit of quantification is 5 μM.
Three reagent solutions were used: A: 10% (w/v) trichloroacetic acid in water; B: 1% (w/v) 8-hydroxyquinoline in ethanol; C: 1 M sodium carbonate in water. Solution A (48 µl) and the sample (240 µl) in potassium phosphate buffer (432 µl, 100 mM), pH 6.8, were placed in a 1.5 ml Eppendorf tube. Solution B (240 µl) was added with rapid mixing and the solution was allowed to stand for 3 min at room temperature. Solution C (240 µl) was added with thorough mixing for 10 to 20 s. The mixture was heated at 95°C for 5 min, followed by cooling at room temperature for 15 min. The absorbance at 705 nm was measured using a spectrophotometer. Calibration curves were generated using a dilution series of a standard hydroxylamine hydrochloride solution in water.
Supplementary Figures

Figure S1. Verification of laboratory-prepared glut-DNICs by optical and EPR methods.

A) Structures of MDNIC and BDNIC, where “L” represents ligand, glutathione in the present study. B) UV-Vis spectra of 5 mM glut-MDNIC and 2.5 mM glut-BDNIC measured by NanoDrop 2000/2000c spectrophotometer. C) EPR spectra of 50 µM glut-MDNIC and the lack of signal from 25 µM glut-BDNIC (EPR-silent) measured at 150 K.
Figure S2. Maximal tension of sheep mesenteric arterial rings in the absence and presence of different test compounds, including 2% (v/v) plasma, 15 µM albumin, 1000U/ml SOD, 200 µM CPTIO, 1000U/ml SOD+200 µM CPTIO, or 1mM DTT (n≥4; p=0.7930; one-way ANOVA).
Figure S3. Role of HNO in glut-MDNIC-mediated relaxation of isolated sheep mesenteric arteries (n≥4). glut-MDNIC-mediated relaxation in the absence and presence of 1000U/ml SOD (converts HNO into NO⁻), 200µM CPTIO (NO⁻ and HNO scavenger), or 1000U/ml SOD + 200µM CPTIO (A), the sGC inhibitor 10µM ODQ or the HNO scavenger 1mM DTT (B). Relaxation was normalized to the tension (100%) prior to first addition of the drug.
Figure S4. In vitro measurements seeking to detect NO• release from glut-BDNIC. 50 µl sample was injected into 20 ml hepes buffer (37 Celsius; pH=7.4) in a purge vessel in line with a chemiluminescence NO• analyzer. Arterial segments were added into the
purge vessel right before sample injection in B, but not in A and C. Each recording lasted for around 4min. In contrast to the robust NO· signal from 100µM NO· injection, 250 µM glut-BDNIC (1000 µM NO moieties; A and B) and 1mM Angeli’s salt (AS; HNO donor) (C) resulted in little NO· signal. A representative trace of three similar experiments is shown.
Figure S5. Effects of plasma on relaxation by the HNO donor AS \((n=4)\). Plasma does not significantly alter AS-mediated relaxation \((p=0.67\) for \(\text{pEC}_{50}\)) indicating the attenuating effects of plasma on glut-BDNIC-mediated relaxation may not result from the scavenging of HNO by plasma. Relaxation was normalized to the tension (100%) prior to the first addition of the drug.
Figure S6. Effects of plasma (2% v/v) and albumin (15µM) on glut-MDNIC-mediated relaxation (n=6). In contrast to the attenuation of glut-BDNIC-mediated vasodilation (Figure 5), plasma and albumin did not alter vasodilation by glut-MDNIC. Relaxation was normalized to the tension (100%) prior to the first addition of the drug.
Figure S7. EPR spectrum (150 K) of metabolites of 25µM glut-BDNIC and 50µM glut-MDNIC in (A) plasma, (B) blood, and (C) albumin solutions in vitro. Solid lines represent the metabolite of glut-BDNIC, while interrupted lines represent the metabolite of glut-MDNIC. Addition of EPR-silent glut-BDNIC to isolated plasma, blood, and albumin solutions creates an EPR signal that is similar in all three matrices. These spectra differ slightly from those created following addition of glut-MDNIC to these matrices in that the glut-BDNIC samples all have a shallow shoulder in the left peak (at g factor=...
~2.05). These findings suggest that glut-BDNICs are rapidly converted into MDNIC-like species whether infused in vivo (Fig 6C) or added into blood, plasma, or albumin solutions in vitro. The difference in the spectra between the metabolites of glut-BDNIC and glut-MDNIC suggests that the HMW metabolites of these compounds differ in the circulation. Information for intensity is omitted for the sake of shape comparison between the spectra.
addition to whole blood, plasma, and albumin solution, as well as that of low molecular weight glut-MDNIC standard (N^{15} labeled). The room temperature EPR spectra of glut-BDNIC metabolites in blood, plasma, and dissolved albumin are similar to each other, and none show hyperfine structure (the mirroring dents up at left vs down at right) such as that observed for LMW MDNIC (bottom tracing), indicating that the glut-BDNIC metabolites in blood and plasma are HMW complexes containing albumin.
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