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Cross-Linking of Serine Racemase Dimer by Reactive Oxygen Species and Reactive Nitrogen Species

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Abstract

Serine racemase (SR) is the only identified enzyme in mammals responsible for isomerization of L-serine to D-serine, a co-agonist at NMDA receptors in the forebrain. Our previous data reported that an apparent SR dimer resistant to SDS and β -mercaptoethanol was elevated in microglial cells after proinflammatory activation. Because the activation of microglia is typically associated with an oxidative burst, oxidative cross-linking between SR subunits was speculated. In this study, an siRNA technique was employed to confirm the identity of this SR dimer band. The oxidative species potentially responsible for the cross-linking was investigated with recombinant SR protein. The data indicate that nitric oxide, peroxynitrite, and hydroxyl radical were the likely candidates, while superoxide and hydrogen peroxide per se failed to contribute. Furthermore, the mechanism of formation of SR dimer by peroxynitrite oxidation was studied by mass spectrometry. A disulfide bond between Cys₆ and Cys₁₁₃ was identified in both SIN-1 treated SR monomer and dimer. Activity assays indicated that SIN-1 treatment decreased SR activity, confirming our previous conclusion that noncovalent dimer is the most active form of SR. These findings suggest a compensatory feedback whereby the consequences of neuroinflammation might dampen D-serine production to limit excitotoxic stimulation of NMDA receptors.

Keywords

Dimerization; Disulfide; Mass spectrometry; Nitric oxide; Oxidation; Peroxynitrite

INTRODUCTION

Identified in 1999 (Wolosker et al. 1999b), serine racemase (SR) is a fold-type II class of pyridoxal-5'-phosphate (PLP)-dependent enzyme that is responsible for most of the D-serine produced in the vertebrate CNS. Accumulating evidence indicates that D-serine physiologically regulates the activity of the NMDA receptor (NMDA-R) (Schell et al. 1997; Schell et al. 1995) via binding at the "glycine_B" site. NMDA-R is a critical glutamate receptor involved in many synaptic events associated with development, plasticity, learning, memory and excitotoxicity. Malfunction of NMDA receptors has been implicated in neurodegenerative diseases (Arundine and Tymianski 2003; Cull-Candy et al. 2001; Haberny et al. 2002; Lynch and Guttman 2002) and psychiatric disorders (Labrie et al.

2009). Highly expressed in forebrain (Xia et al. 2004), SR is localized to neurons (Kartvelishvily et al. 2006) and protoplasmic astrocytes (Schell et al. 1997), with some expression by quiescent and activated microglia (Wu et al. 2004; Williams et al. 2006).

SR has been implicated in several disease processes. The enzyme and its product D-serine are progressively increased in astrocytes and microglia in amyotrophic lateral sclerosis (ALS) and a mouse model thereof (Sasabe et al. 2007). SR expression is also elevated in the hippocampus in Alzheimer's disease and is induced by amyloid β -peptide ($A\beta$) (Wu et al. 2004); the toxicity of $A\beta$ *in vivo* is also dependent upon SR expression (Inoue et al. 2008). On the other hand, diminution of SR may contribute to the NMDA-R hypofunction that appears to underlie schizophrenia (Labrie et al. 2011).

SR has been detected in all mammalian brains examined, including human, mouse and rat. These proteins share 89% homology in their amino acid sequence, and all have a PLP-binding sequence near the N-terminus (De Miranda et al. 2000; Wolosker et al. 1999a). SR catalyzes an isomerase reaction interconverting L- and D-serine in the presence of the co-factors PLP, magnesium, and ATP; the K_M is higher when D-serine is the substrate (Wolosker et al. 1999b). SR also catalyzes an α,β -elimination reaction which produces pyruvate from either L-serine or D-serine (Foltyn et al. 2005; Strisovsky et al. 2003). This might provide a novel physiological way to down-regulate intracellular D-serine levels and participate in synaptic inactivation of D-serine.

Recent X-ray crystallography evidence confirms the dimeric structure of SR (Smith et al. 2010), which was previously determined by gel-filtration chromatography (Cook et al. 2002). But the chemistry of the dimer is still unclear. So far, three different types of SR dimer have been reported. In our previous study, the structure and activity of noncovalent dimers and disulfide-bound covalent dimers were investigated. In addition, an apparent dimer resistant to denaturation by SDS and reduction by β -mercaptoethanol (β ME) was detected in microglial cells, and this species was elevated by proinflammatory stimuli (Wu and Barger 2004; Wu et al. 2004; Wu et al. 2007). It has been well documented that in microglia, proinflammatory stimuli such as lipopolysaccharide (LPS) trigger an oxidative burst that results in high production of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals via membrane-localized NADPH oxidase; also produced are reactive nitrogen species (RNS) such as nitric oxide ($\bullet$ NO) via inducible NO synthase (iNOS); its reaction with superoxide generates peroxynitrite (Barger 2004). Under conditions of oxidative stress, ROS and RNS can lead to protein oxidation. Accumulation of oxidized protein is associated with a number of neurological diseases such as Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease (Pacher and Szabo 2008). Because inflammatory activation increases the SDS/ β ME-resistant SR dimer in microglia, we examined the capacity of diverse ROS and RNS species for crosslinks between SR subunits, the amino acids involved in crosslinks, and the impact of crosslinking on SR activity.

MATERIALS AND METHODS

Expression and purification of recombinant human SR

Full-length SR cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) of human brain mRNA and subcloned into the pTrcHisB (Invitrogen) polyhistidine expression vector as described (Wang and Barger 2011). The protein was expressed in BL21 (DE3) competent cells (Invitrogen) under induction by isopropyl-1-thio- β -D-galactopyranoside (IPTG, Fisher Scientific). Cell pellets were stored at -80°C prior to purification on a ProBondTM nickel-chelating resin column (Invitrogen) (Wang and Barger 2011). Fractions with highest concentrations of SR were pooled and extensively dialyzed

against assay buffer (50 mM phosphate buffer, pH 7.4, 58 mM NaCl) containing 0.1% Tween-20, followed by dialysis against the same buffer without Tween-20. After dialysis, proteins were kept at 4 °C for structure and activity analysis.

Enzyme assay

The activity of SR was measured by the velocity of racemization reaction of L-serine to D-serine, which was coupled to D-serine chemiluminescence assay as described (Wang and Barger 2011). Porcine kidney D-amino acid oxidase (DAO, Calzyme Laboratories) was used to clear L-serine (Sigma) of contaminating D-serine prior to use in assays. SR activity assay was performed with 2.5 µg recombinant enzyme in the presence of 50 mM phosphate buffer, pH 8.0, 52 mM NaCl, 15 µM PLP, 1 mM ATP, 0.2 mM DTT, 1 mM MgCl₂ and 10 mM pretreated L-serine. After incubation at 37 °C for 30 min, the reaction was terminated by boiling for 5 min. Control reactions were performed with an enzyme preparation that had been boiled prior to reaction, and values from these controls were subtracted from experimental values. The D-serine produced during incubation was monitored by a chemiluminescence assay: 10 µl of sample or D-serine standard was added to 50 µl of buffer containing 100 mM Tris, pH 8.8, 50 mM NaCl, 2 U/ml horseradish peroxidase (HRP, Sigma), 16 µM luminol (Sigma), and 0.8 µg/ml FAD. After a 10- to 20-minute delay, required to decrease the nonspecific luminol luminescence, 60 µl of *R. gracilis* DAO (0.033 U/ml, recombinant; courtesy of L. Pollegioni, U. Insubria, Varese, Italy) was added. A Veritas Microplate Luminometer (Turner Biosystems Inc) was used to detect the production of H₂O₂ before and after addition of DAO. The value obtained before DAO signal was subtracted from what was obtained after DAO.

Generation of reactive oxygen species and nitrogen species

Xanthine oxidase (XO, Sigma) was purified by Superdex HR200 gel filtration column (GE) equilibrated with 50 mM phosphate buffer, pH 7.4, 58 mM NaCl. Its activity was determined by measuring the rate of uric acid production at 290 nm in 50 µM hypoxanthine ($\epsilon_{\text{mM}}=12.2 \text{ mM}^{-1}\cdot\text{cm}^{-1}$) according to a protocol from Sigma. Superoxide was generated by incubation of XO with hypoxanthine or pterin at ambient oxygen. Hydroxyl radical was produced by incubation of hydrogen peroxide with 100 µM Fe²⁺/EDTA through the Fenton reaction (Halliwell and Gutteridge 1992). Peroxynitrite was generated by three methods: 1: •NO released by spermine NONOate combined with superoxide generated by XO reactions as indicated above; 2: Simultaneous production of •NO and superoxide by the autooxidation of 3-morpholiniosydnonimine hydrochloride (SIN-1, Sigma). 3: Chemically synthesized peroxynitrite prepared using a quenched-flow reaction apparatus as described by Reed et al. (Reed et al. 1974). For the last, an aqueous solution of 0.6 M sodium nitrite was rapidly mixed with an equal volume of 0.7 M hydrogen peroxide containing 0.6 M HCl and immediately quenched with the same volume of 1.5 M NaOH; all reaction solutions were kept on ice; the concentration of peroxynitrite was determined spectrally in 0.3 M NaOH ($\epsilon_{302 \text{ nm}} \approx 1670 \text{ M}^{-1}\cdot\text{cm}^{-1}$) (Hughes and Nicklin 1968) before use.

Cell culture

The HAPI microglia cell line was generously provided by Dr. James R. Conner (Pennsylvania State University) and was maintained in minimal essential medium with Earle's salts (MEM, Invitrogen) plus 10% fetal bovine serum (FBS, Invitrogen).

Nitrite assay

The relative amount of nitric oxide released from HAPI cells was determined by Griess reaction of nitrite as described previously (Bodles and Barger 2005). Nitrite concentrations

were determined from a standard curve established by using known concentrations of sodium nitrite.

Western blot analysis of SR

HAPI cell extracts with same amount of protein were resolved by 10% SDS-PAGE with 2.5% β -mercaptoethanol in the sample buffer. Protein was electrophoretically transferred at 100 V for 1.5 h onto nitrocellulose membranes, which were then blocked for 1 h at room temperature with 0.2% I-Block (Applied Biosystems). Blots were probed overnight at 4 °C with a monoclonal antibody generated against mouse SR (BD Biosciences) or a polyclonal antibody generated against human SR (Abcam), each at 1:500. Then the blots were incubated with alkaline phosphatase-conjugated secondary antibody: rabbit anti-mouse at 1:400 or goat anti-rabbit at 1:3000. Blots probed with the monoclonal SR antibody were developed with BCIP/NBT chromagen (Vector Laboratories), and blots probed with the polyclonal SR antibody were developed with Western-Light detection system (Applied Biosystems). For quantification, image files were imported into Scion Image Alpha 4.0.3.2., and the densitometry thus measured was plotted with nonlinear regressions in Prism 3.03.

RNA interference (siRNA)

SR siRNA, a double-stranded RNA (dsRNA) sequence of 21-nucleotide residues in length (GGA AUU CCU GCU UAC AUU GTT) specific to the coding region of mouse SR mRNA, as well as negative-control dsRNA, were purchased from Ambion. HAPI cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) plus 10% FBS in 6-well plates and dsRNA preparations were introduced via siPORTTM NeoFXTM (Ambion) according to the manufacturer's protocol.

GeLC-MS/MS

SDS-PAGE gel bands were excised and subjected to in-gel trypsin digestion as follows. Protein-containing gel slices were destained in 50% methanol (Fisher), 100 mM ammonium bicarbonate (Sigma-Aldrich). Gel slices were then dehydrated in acetonitrile, followed by addition of 100 ng porcine trypsin (Promega) in 100 mM ammonium bicarbonate (Sigma-Aldrich) and incubation at 37 °C for 12–16 hours. Peptide products were then acidified in 0.1% formic acid (Fluka). Tryptic peptides were separated by reverse-phase HPLC on a 10-cm C18 column using a NanoLC 2D system (Eksigent) and ionized by electrospray upon elution, followed by MS/MS analysis using an LTQ-Orbitrap mass spectrometer (Thermo). Peptides were identified from MS/MS spectra by database searching using the Mascot search engine (Matrix Science).

Statistical analysis

Data are expressed as mean $\pm$ S.D. Data were analyzed using one-way ANOVA followed by Bonferroni *post hoc* comparisons. A *p*-value of ≤ 0.05 was considered statistically significant.

RESULTS

Confirmation of SDS/ β ME-resistant SR dimer elevated by LPS

Treatment of microglial cells with bacterial endotoxin LPS elevates an apparent SR dimer which is resistant to denaturing and reducing agents (Wu and Barger 2004). To confirm the identity of this band as an SR dimer, siRNA was employed to identify SR proteins on the gel (Figure 1A). LPS treatment of HAPI cells increased an SR-immunoreactive band on reducing gels, and SR siRNA down-regulated this band in a dose-dependent manner, indicating that the protein elevated by LPS does indeed contain SR. Our previous data also

demonstrated that LPS induces SR expression by activating transcription factor activator protein-1 (AP-1) (Wu and Barger 2004). To determine whether the elevation of SDS/ β ME-resistant SR dimer may also involve post-transcriptional effects, both SR dimer and monomer bands were analyzed by densitometry. This quantification indicated that LPS treatment increased the ratio of the SR dimer to total SR protein (dimer plus monomer, Figure 1B), suggesting that the effects of LPS may include post-translational modification of SR.

It is well known that LPS can activate NADPH oxidase to produce ROS such as superoxide, hydrogen peroxide, and hydroxyl radical. LPS also elevates expression of iNOS, which catalyzes $\bullet$ NO formation from arginine. Rapid reaction of equimolar $\bullet$ NO and superoxide forms peroxynitrite. ROS, $\bullet$ NO, and peroxynitrite are capable of oxidizing protein to form cross-linked products (Stadtman 2001). Thus, the role of ROS, $\bullet$ NO, and peroxynitrite in formation of SDS/ β ME-resistant SR dimer were investigated here.

The effect of $\bullet$ NO on recombinant SDS/ β ME-resistant SR dimer

Homeostasis of redox status *in vivo* is complicated by multiple ROS and RNS species and endogenous antioxidants. To simplify the potential reactions and examine the effects of the each major ROS and RNS species on cross-linking formation in SR, experiments were performed with recombinant human SR protein *in vitro*. Previously, Mustafa *et al.* (2007) reported that SR can be S-nitrosylated. To study the effect of $\bullet$ NO on cross-linking formation, recombinant SR was incubated with the $\bullet$ NO donor spermine NONOate, which thermally decomposes and releases $\bullet$ NO with a half-life of 39 min at pH 7.4 and 37 °C. SDS-PAGE under reducing conditions indicated that spermine NONOate increased the SDS/ β ME-resistant SR dimer in a concentration-dependent manner (Figure 2A, B). In addition, when the concentration of spermine NONOate was over 200 μ M, high molecular-weight aggregates (above 170 kDa) were formed while SR monomer decreased (Figure 2A), indicating that $\bullet$ NO fostered the formation of both intra- and interdimer cross-linking of SR.

The effect of superoxide on recombinant SDS/ β ME-resistant SR dimer

The continuous generation of $O_2^{\cdot-}$ was established by XO/hypoxanthine and XO/pterin systems. Proteolytic activity was detected in commercially obtained XO preparation (data not shown); therefore, gel-filtration chromatography was employed to remove this presumptive contaminant, and a protease-inhibitor mixture (0.2 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin) was added in all XO reactions. After incubation of recombinant SR with XO with or without hypoxanthine or pterin, reducing SDS-PAGE showed that XO alone created more SDS/ β ME-resistant dimer compared to no treatment (Figure 3A, Lane 3). To determine the effects of $O_2^{\cdot-}$, the production of a SDS/ β ME-resistant dimer band in the presence of XO/hypoxanthine or XO/pterin was normalized to that produced by XO alone. After a 2-h incubation, SDS/ β ME-resistant SR dimers did not increase further with the addition of hypoxanthine or pterin to XO (Figure 3A, B; Lanes 5, 6). As another test of $O_2^{\cdot-}$, potassium superoxide was added as a bolus into recombinant SR and immediately followed by vortex mixing for 5 min at room temperature. Still, no increase of stable SR dimer was detected (data not shown). Together, these data suggest that $O_2^{\cdot-}$ is not sufficient to cause cross-linking of SR.

The effects of peroxynitrite on recombinant SDS/ β ME-resistant SR dimer

The peroxynitrite anion ($ONOO^-$) can be spontaneously formed by the reaction of $\bullet$ NO and $O_2^{\cdot-}$. To mimic the situation in HAPI cells treated by LPS, a continuous flux of $\bullet$ NO/ $O_2^{\cdot-}$ was generated by adding a $\bullet$ NO donor (spermine NONOate) to the XO/hypoxanthine and XO/pterin systems. Reducing SDS-PAGE indicated that SDS/ β ME-resistant SR dimer and higher molecular-weight aggregates (above 170 kDa) were elevated in the $\bullet$ NO/XO/pterin

system (compared to XO alone or •NO plus XO) but not in the •NO/XO/hypoxanthine system (Figure 3A and B). A possible reason for this difference between pterin and hypoxanthine is that oxidation of the latter by XO results in accumulation of uric acid, a well-known ONOO⁻ scavenger (Balavoine and Geletii 1999). These results suggest that ONOO⁻ is more effective than •NO or O₂^{•-} in cross-linking SR.

To confirm the role of ONOO⁻ in SR cross-linking, two other sources were tested. First, chemically synthesized sodium peroxynitrite (NaONOO), produced from acidified nitrite and H₂O₂, was incubated with recombinant SR. Reducing SDS-PAGE showed that NaONOO at 100 μM and 150 μM significantly increased the SDS/βME-resistant SR dimer (Figure 4A and B). At higher concentrations (200–300 μM), NaONOO produced some high molecular-weight SR aggregates (greater than 170 kDa) and decreased levels of monomer and dimer (Figure 4A and B). This may indicate that NaONOO stimulated cross-linking within SR dimers at low concentrations, whereas it favored cross-linking between SR dimers at high concentrations. Another interesting phenomenon is that NaONOO treatment altered the gel mobility of SR monomer. This may result from the formation of intramolecular cross links or from nitration and/or nitrosylation of amino acid side chains.

As a second test of the role of reactive nitrogen species, ONOO⁻ was generated by the simultaneous production of •NO and O₂^{•-} by the auto-oxidation of SIN-1. These two radicals combine to form ONOO⁻ (Matalon et al. 2009). Compared to above experiments using NaONOO, which has a half-life ≤ 1 s at pH 7.4, SIN-1 can provide prolonged exposure to lower concentrations of ONOO⁻. This better mimics the profile of production in activated microglial cells (Matalon et al. 2009). Similar to NaONOO treatment, incubation of recombinant SR with increasing concentrations of SIN-1 resulted in elevated SDS/βME-resistant SR dimer in a dose-dependent manner (Figure 5A and B). In addition, SIN-1 treatment caused formation of high molecular-weight aggregates of SR protein.

The effects of H₂O₂ and hydroxyl radical on recombinant SDS/βME-resistant SR dimer

In vivo, O₂^{•-} is rapidly converted to H₂O₂ by superoxide dismutase (SOD), which in turn is reduced to water by catalase or glutathione peroxidase. Further, reduced transition metals can catalyze the partial reduction of hydrogen peroxide to hydroxyl radical (OH•), which is one of the strongest oxidants in nature. Thus, the effects of H₂O₂ and OH• on formation of the SDS/βME-resistant SR dimer were tested. Recombinant SR was incubated with increasing concentrations of H₂O₂ and resolved with SDS-PAGE. No elevation of SDS/βME-resistant SR dimer was detected with H₂O₂ treatment (data not shown).

Hydroxyl radical was produced by Fenton reaction: $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\bullet + \text{OH}^-$. Recombinant SR was incubated with increasing concentrations of H₂O₂ and 100 μM Fe²⁺/EDTA, and the products were resolved with reducing SDS-PAGE. The results showed that hydroxyl radical elevated SDS/βME-resistant SR dimer in a concentration-dependent manner (Figure 6A and B). It also generated some high molecular-weight aggregates. Unlike •NO and ONOO⁻, high concentrations of OH• did not decrease SR monomer and dimer, which may indicate that the oxidation mechanism of OH• is different from that under other conditions.

Identification of modified amino acids in SIN-1 treated recombinant SR by mass spectrometry

The above evidence indicates that hydroxyl radical and peroxynitrite are stronger oxidants than •NO with regard to SR cross-linking. Although hydroxyl radical increased SR dimer at a lower concentration than peroxynitrite, hydroxyl reactivity is less specific and can include the formation of covalent bonds within the peptide backbone (Stadtman 2001; Alvarez and

Radi 2003). Peroxynitrite oxidation of protein is implicated in many human diseases, including most neurodegenerative disorders (Pacher and Szabo 2008). To identify the specific amino acids involved in cross-linking of SDS/ β ME-resistant dimer, recombinant SR was exposed to SIN-1 treatment followed by SDS-PAGE separation. The SR monomer and dimer bands were fragmented by in-gel trypsin digestion. Tryptic peptides were separated by reverse-phase HPLC followed by MS/MS analysis (Figure 7). Using this approach, a disulfide bond was identified between Cys₆ and Cys₁₁₃ in both SR monomer and dimer. Cys₆ and Cys₁₁₃ appeared to be involved in disulfide bonding unequally. In monomer, unmodified Cys₆ peptide was not detected, while a significant amount of unmodified Cys₁₁₃ was detected. In dimer, the unmodified Cys₁₁₃ peptide is totally absent. This evidence implies that Cys₁₁₃ is involved in a disulfide link more frequently in the dimer than in the monomer.

Although dityrosine cross-linking, oxidation of methionine, and nitrosylation of cysteine residues are routinely observed by mass spectrometry from recovered peptides of peroxynitrite-treated protein samples (Ducrocq et al. 1999), no other modifications besides the Cys₆-Cys₁₁₃ disulfide bond were detected after trypsin digestion of SIN-1 treated recombinant SR.

Inhibition of recombinant SR activity by SIN-1 treatment

To determine how SR activity is affected by intra- and/or inter-molecular cross-linking, the recombinant enzyme was treated with SIN-1. After dialysis to remove the SIN-1, activity assays were performed. SIN-1 treatment greatly decreased SR activity at 25 μ M (Figure 8). Peak elevation of SDS/ β ME-resistant SR dimer was apparent at SIN-1 concentrations above 100 μ M (Figure 5). This discrepancy between effects on activity and physical detection of cross-linking may indicate that intramolecular changes occurred, perhaps at the enzyme's active site, prior to intermolecular cross-linking. In our previous study, noncovalent SR dimer was found to be more active than dimer connected by disulfide bonds. In the above experiments, the SR sample without SIN-1 treatment was equivalent to the disulfide-linked dimer (achieved by omitting DTT from the dialysis buffer after affinity chromatography).

No change of SDS/ β ME-resistant SR dimer was detected in LPS-treated HAPI cells under manipulation of iNOS and NADPH oxidase

The effects of reactive oxygen and nitrogen species on purified SR suggests that these molecules may contribute to the effects on SR exerted by inflammatory activation of microglial cells. To test the requirements for $\bullet$ NO and $\bullet$ NO-derived reactants, HAPI cells were treated with 1400W, an inhibitor of iNOS, and apocynin, an inhibitor of NADPH oxidase, with or without LPS application. Griess assays indicated that LPS stimulated $\bullet$ NO production, and 1400W completely blocked this elevation (Figure 9A). Apocynin also attenuated the LPS-evoked $\bullet$ NO production by ~30% (Figure 9A). Surprisingly, inhibition of iNOS or NADPH oxidase did not produce any substantial reduction of SDS/ β ME-resistant SR dimers triggered by LPS treatment (Figure 9B). Likewise, no increase of cross-linked SR dimer was observed after HAPI cells were treated with $\bullet$ NO donors such as S-nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP) (data not shown). Nor were H₂O₂ alone, H₂O₂ combined with Fe²⁺/EDTA, or SIN-1 effective in intact cells (data not shown).

DISCUSSION

We previously found that inflammatory stimulation of microglia elevates the levels of a stable SR dimer (Wu and Barger 2004; Wu et al. 2004; Wu et al. 2007). Activation of microglia typically involves a programmatic superoxide production from NADPH oxidase

and $\cdot\text{NO}$ production from iNOS, and this dramatically enhances oxidative conditions in the cells. It is possible that these conditions result in oxidative cross-linking of SR subunits. The oxidative species responsible for this cross-linking of SR protein were explored here. Superoxide and H_2O_2 had no measurable influence, but evidence was gathered in support of $\cdot\text{NO}$, peroxynitrite, and hydroxyl radical elevating the SDS/ βME -resistant SR dimer *in vitro*. In cell culture, inhibition of iNOS or NADPH oxidase did not impact the level of SDS/ βME -resistant SR dimer in LPS-treated cells.

Protein-protein cross-linking that is resistant to reducing reagents can be formed by multiple mechanisms of protein oxidation. Chemical conjugations can occur between the backbone of two polypeptide chains and/or the side chain of amino acids such as lysine, tyrosine, histidine, and tryptophan (Stadtman 2001; Stadtman 2006; Zhang et al. 2003). In this study, it was determined that $\cdot\text{NO}$, peroxynitrite, and hydroxyl radical can oxidize recombinant SR to form cross-linking between dimer subunits. The protein band pattern on reducing SDS-PAGE indicated that the mechanism of oxidation by hydroxyl radicals is probably different from those of peroxynitrite and $\cdot\text{NO}$. Hydroxyl radical can extract a hydrogen atom from the α -carbon of amino acids to generate two carbon-centered protein radicals which react with one another to form $-\text{C}-\text{C}-$ protein cross-linked products (Stadtman 2001). Peroxynitrite can oxidize tyrosine residues leading to $-\text{Tyr}-\text{Tyr}-$ cross-linked derivatives (Stadtman 2001). No cross-linking of proteins has been detected to date for $\cdot\text{NO}$ *per se*, but its peroxynitrite product is implicated. It is now well recognized that peroxynitrite potently oxidizes and damages proteins, DNA, and lipids; these modifications play an important role in diverse pathophysiological conditions such as inflammation, neurodegenerative diseases, and cardiovascular disorders (Pacher and Szabo 2008). Since dityrosine is the most likely cross-link occurring in proteins oxidized by peroxynitrite, it is possible that it contributed to the SDS/ βME -stable SR dimer after SIN-1 treatment. Mass spectrometry did not identify dityrosine cross-links from recovered peptides, but some of the peptides formed by this process may not have been optimally processed for detection. For SR dimer analysis, under the conditions used here, only 54% of the amino acids and 55% of the tyrosine residues were recovered in mass spectra. Any dityrosine present may have been within peptides which were undetected; indeed, the combination of fragments joined by a dityrosine may have been above the ~ 1600 m/z limitation of the LC-MS system utilized. Surprisingly, the only modified amino acids detected by mass spectrometry were Cys₆ and Cys₁₁₃, which were involved in disulfide bonds in both SR monomer and dimer. The MS/MS analysis cannot distinguish whether this link is intramolecular or intermolecular. In monomer, unmodified Cys₆ peptide was not detected, while a significant amount of unmodified Cys₁₁₃ was detected. This evidence implies that only a small fraction of Cys₆ is involved in intramolecular disulfide bonds with Cys₁₁₃ and that most Cys₆ residues were involved in disulfide linkages with other cysteine residues or underwent other modifications such as nitrosylation. In dimer, the unmodified Cys₁₁₃ peptide was absolutely absent, which may indicate that Cys₁₁₃ is involved in a disulfide link or other modifications more frequently in the dimer than in the monomer. But, it is still unlikely that disulfide bonds were responsible for the SR dimers observed under conditions of heating and strong reducing agents. It seems more likely that the stable dimer observed in activated microglia results from some chemical conjugation of amino acids other than cystine disulfides. It is possible that microglial activation initially results in an elevation of SR expression (via the AP-1 transcription factor, as described (Wu and Barger 2004)), a substantial fraction of which would be active, noncovalent dimer. The subsequent oxidative conditions may then cross-link these dimers via as yet undefined chemistry.

Although our previous study indicated that an elevation of the stable SR dimer was accompanied by an increased D-serine production in HAPI cell medium under inflammatory activation (Wu and Barger 2004; Wu et al. 2004; Wu et al. 2007), the data reported here

indicated that SIN-1 treatment decreased recombinant SR activity, arguing against any connection between formation of the stable dimer and elevated D-serine production in activated microglia. The possible explanation for this discrepancy is that microglial activation may entail influences on multiple events which govern D-serine levels in culture medium including D-serine release, SR activity regulation by protein-protein interaction and phosphorylation, SR turnover rate, expression of DAO, etc. Oxidative inactivation of SR is only a single component of the scenario and may occur subsequent to an initial rise in SR activity. An interesting observation is that Cys₁₁₃ was reported to be S-nitrosylated by ·NO, which led to a dramatic inhibition of enzyme activity (Mustafa et al. 2007). Structural analysis reveals that the ATP-binding site is in close proximity to Cys₁₁₃, and nitrosylation of Cys₁₁₃ interferes with ATP binding to SR (Mustafa et al. 2007). If the structure of the free thiol group of Cys₁₁₃ is critical for ATP binding to SR, the disulfide bond between Cys₆ and Cys₁₁₃ identified in this study is likely to distort the enzyme's quaternary structure and thereby have the same effect on ATP binding as nitrosylation of Cys₁₁₃, namely, a decrease in enzyme activity.

The fact that SR can be oxidatively inactivated by ·NO and peroxynitrite suggests a model for D-serine signaling in the brain under conditions of neuroinflammation (Figure 10). Proinflammatory stimulation such as LPS induces release of glutamate from microglia via the xc- exchange system (Barger and Basile 2001; Barger et al. 2007), potentially contributing to excitotoxicity. Excessive calcium influx through NMDA receptors activates calmodulin and neuronal NOS (nNOS) in postsynaptic neurons, leading to robust production of ·NO. Thus generated, ·NO diffuses to adjacent astrocytes or neurons to oxidize SR, which inhibits enzyme activity. In addition, ·NO reacts with superoxide leaked from mitochondria (Turrens 2003) to form peroxynitrite, which strongly inhibits SR locally. Thus, as a compensatory event, the activity of NMDA receptors might be regulated by lowering D-serine production in adjacent neurons and glia through inactivation of SR. This model provides a potential negative feedback loop to regulate NMDA receptor neurotransmission in neuroinflammatory situations.

Although ·NO, peroxynitrite, and hydroxyl radical were capable of stimulating cross-linking between SR subunits *in vitro*, the *in vivo* situation is more complicated. Accumulation of oxidized proteins in a specific cell type is dependent upon multiple factors such as the rate of generation of various oxidant species, the ratio of each species to the entire oxidant pool, the reactivity of proteins to each oxidant species, the amount and efficiency of antioxidant defenses, and the rate of repair and elimination of oxidized macromolecules (Stadtman 2001). Thus, it is difficult to predict which species are responsible for the formation of the SDS/βME-resistant SR dimer in activated microglial cells. In addition to ·NO production from iNOS, proinflammatory stimuli activate NADPH oxidase to elevate the production of ROS, including hydroxyl radicals. In cell culture, we found that an iNOS inhibitor was unable to completely attenuate SR dimer formation. However, the specific inhibition of ·NO production leaves ROS production largely intact, and it possible that abnormal levels of peroxynitrite can be formed from reaction of a basal level of ·NO with the elevated superoxide. Moreover, our work with purified enzyme indicates that hydroxyl radicals produce more SR crosslinking than does ·NO. When applied alone, nitric oxide and peroxynitrite donors, hydrogen peroxide, and the Fenton reaction were each insufficient for cross-linking SR in HAPI cells. It is possible that this SR dimer requires multiple oxidants, and the contribution of each possible candidate must be determined by further investigation.

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Figure 1. RNA-interference confirmation of SDS/βME-resistant SR dimer in HAPI cells
HAPI cells were transiently transfected with negative-control RNA (“cRNA”; 20 nM) or SR siRNA (20 nM, 50 nM) in DMEM plus 10% FBS as described under “Materials and Methods”. After 24 h of transfection with dsRNA, HAPI cells were washed with PBS, supplemented with DMEM plus 1% FBS and treated with 30 ng/ml LPS for another 48 h. **A:** Western blot detection of SR protein. HAPI protein was extracted and resolved by 10% SDS-PAGE with 2.5% β-mercaptoethanol in the sample loading buffer. SR protein was detected by western blot with anti-SR antibody. **B:** Densitometric analysis of SR bands. The Y-axis represents the ratio of SR dimer to total SR protein (dimer plus monomer). Data are expressed as fold-change relative to the no-treatment values; mean ± S.D. (** $p < 0.05$).

Figure 2. Effects of •NO in generating recombinant SDS/βME-resistant SR dimer
 Recombinant SR (8.3 μM) was incubated with the indicated concentrations of spermine NONOate (sNONOate) in 50 mM phosphate buffer, pH 7.4, 58 mM NaCl with 100 μM diethylenetriaminepentaacetic acid (DTPA) for 2 h at 37°C. **A:** The mixture was resolved by 10% SDS-PAGE with 2.5% β-mercaptoethanol in the sample loading buffer, and the gels were stained with Coomassie blue. **B:** Densitometric analysis of SR dimer, expressed as an absolute value on an arbitrary densitometric scale; mean ± S.D. ($p=0.0133$). The spermine NONOate doses at 200 μM and 800 μM have statistical significance.

Figure 3. Role of superoxide and peroxynitrite in generating recombinant SDS/βME-resistant SR dimer

Recombinant SR (8.3 μM) was incubated with the 100 μM spermine NONOate (NO), 10 mU/ml XO, 300 μM pterin, and 300 μM hypoxanthine as indicated in 50 mM phosphate buffer, pH 7.4, 58 mM NaCl with 100 μM DTPA for 2 h at 37°C. Protease inhibitors mixture (0.2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) was also added in all XO samples. Since the generation of superoxide consumes oxygen, all the reactions were performed in the vials with lid open. **A:** The mixture was resolved by 10% SDS-PAGE with 2.5% β-mercaptoethanol in the sample loading buffer. The gels were stained with Coomassie blue. Lane 1, no treatment; Lane 2, NO; Lane 3, XO; Lane 4, XO+NO; Lane 5, XO+pterin; Lane 6, XO+hypoxanthine; Lane 7, XO+pterin+NO; Lane 8, XO+ hypoxanthine +NO. **B:** Densitometric analysis of SR dimer. Data are expressed as fold-change relative to those obtained with XO alone; mean ± S.D. (* $p < 0.014$; ** $p < 0.001$).

Figure 4. Effects of synthetic peroxynitrite in generating recombinant SDS/βME-resistant SR dimer

A bolus addition of chemically synthesized peroxynitrite (NaONOO) at indicated concentrations into reaction mixture containing 8.3 μM recombinant SR in 50 mM phosphate buffer, pH 7.4, 58 mM NaCl with 100 μM DTPA was immediately followed by vortex mixing and incubation for 10 min at 37°C. **A:** The mixture was resolved by 10% SDS-PAGE with 2.5% β-mercaptoethanol in the sample loading buffer. The gels were stained with Coomassie blue. **B:** Densitometric analysis of SR dimer, expressed as an absolute value on an arbitrary densitometric scale; mean ± S.D. ($p=0.0014$). The NaONOO doses at 100 μM and 150 μM have statistical significance.

Figure 5. Role of SIN-1 in generating recombinant SDS/βME-resistant SR dimer
 Recombinant SR (8.3 μM) was incubated with the indicated concentrations of SIN-1 in 50 mM phosphate buffer, pH 7.4, 58 mM NaCl with 100 μM DTPA for 2 h at 37°C. **A:** The mixture was resolved by 10% SDS-PAGE with 2.5% β-mercaptoethanol in the sample loading buffer. The gels were stained with Coomassie blue. **B:** Densitometric analysis of SR dimer, expressed as an absolute value on an arbitrary densitometric scale; mean ± S.D. ($p=0.0107$). The SIN-1 doses at 100 μM, 200 μM, 400 μM and 800 μM have statistical significance.

Figure 6. Role of hydroxyl radical in generating recombinant SDS/βME-resistant SR dimer
 Recombinant SR (8.3 μM) was incubated with the indicated concentrations of H₂O₂ in 50 mM phosphate buffer, pH 7.4, 58 mM NaCl with 100 μM Fe²⁺/EDTA for 2 h at 37°C. **A:** The mixture was resolved by 10% SDS-PAGE with 2.5% β-mercaptoethanol in the sample loading buffer. The gels were stained with Coomassie blue. **B:** Densitometric analysis of SR dimer, expressed as an absolute value on an arbitrary densitometric scale; mean ± S.D. ($p < 0.0001$). The H₂O₂ doses at all indicated concentrations have statistical significance.

Figure 7. Mass spectrometric analysis of SIN-1 modified recombinant SR

Recombinant SR (15 μ M) was incubated with 100 μ M SIN-1 in 50 mM phosphate buffer, pH 7.4, 58 mM NaCl with 100 μ M DTPA for 2 h at 37 $^{\circ}$ C. The mixture was resolved by 10% SDS-PAGE with 2.5% β -mercaptoethanol in the sample loading buffer, and the gel was stained with GelCode blue. SR dimer and monomer bands on SDS-PAGE gel were excised and analyzed by mass spectrum as described under "Materials and Methods". **A:** MS/MS of

2⁺ peptide at 861.67 m/z; **B**: MS/MS of 3⁺ peptide at 574.91 m/z; **C**: Peptide identified with modified disulfide linking.

Figure 8. Activity assay of SIN-1 treated recombinant SR

Recombinant SR (8.3 μM) was incubated with the indicated concentrations of SIN-1 in 50 mM phosphate buffer, pH 7.4, 58 mM NaCl with 100 μM DTPA for 2 hours at 37 °C. The mixture was dialyzed against the same buffer as above for 1 h, followed by pH 8.0 dialysis buffer (50 mM phosphate buffer, 52 mM NaCl, 0.2 mM DTT, 15 μM PLP) for another 2 h at 4 °C. SR activity was then assessed as described under “Materials and Methods.”

Figure 9. Effects of •NO in formation of SDS/βME-resistant SR dimer in HAPI cells
HAPI cells were treated with 30 ng/ml LPS, 100 μM 1400W, and 100 μM apocynin as indicated for 24 h. **A:** Measurement of •NO production in conditioned HAPI medium by nitrite assay; mean ± S.D. (** $p < 0.01$); **B:** Western blot detection of SR dimer. Total cellular protein was extracted and resolved by 10% SDS-PAGE with 2.5% β-mercaptoethanol in sample loading buffer. SR protein was detected by western blot with polyclonal anti-SR antibody.

Figure 10. Schematic model of a potential self-regulating feedback on SR

Proinflammatory stimuli such as A β and LPS activate NADPH oxidase (“NOX”) in perineuronal microglia, elevating production of superoxide and release of glutamate; such stimuli also elevate the expression of iNOS and SR. The latter’s product D-Ser may cooperate with glutamate to activate extrasynaptic NMDA-Rs, which appear to be particularly neurotoxic. The resulting calcium load activates calmodulin-dependent neuronal NOS, which may contribute to the NO produced by microglial iNOS; the NO reacts with superoxide to produce peroxynitrite. Inactivation of SR by peroxynitrite may attenuate these extrasynaptic events, leaving intact the physiological activation of synaptic NMDA-Rs by more appropriate sources of agonist, including D-Ser released from astrocytes and, perhaps, presynaptic terminals.