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Copper Disrupts S-nitrosothiol Signaling in Activated BV2 Microglia

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Abstract

Microglia, the primary resident immune cells of the central nervous system (CNS), respond rapidly to pathogens and injury by secreting immune mediators including nitric oxide (NO). The reaction of NO with the anti-oxidant glutathione forms S-nitrosoglutathione (GSNO), the major pool of biologic NO in the body. GSNO is degraded by GSNO reductase (GSNOR). Recently, we have shown that copper (Cu(I)) inhibits the release of NO in lipopolysaccharide (LPS)-stimulated BV2 microglia and induces BV2 microglia to acquire a mixed a profile with both pro- and anti-inflammatory characteristics. Since GSNOR is the critical enzyme in GSNO metabolism, we sought to determine whether Cu(I) affects GSNOR activity and S-nitrosothiol (SNO) accumulation in activated BV2 microglia. Our results show that GSNOR protein expression is reduced by Cu(I) treatment in LPS-stimulated BV2 microglia. Our results also show a decrease in S-nitrosothiol content despite a reduced GSNOR expression. This effect is most likely due to Cu(I) reacting with the central thiol of the S-NO bond resulting in the degradation of SNO. A dose of 1 μ M Cu(I) did not affect SNO protein accumulation in LPS-stimulated BV2 microglia, however, a dose of 100 μ M Cu(I) inhibited SNO protein in accordance with inhibition of S-nitrosothiols. These data provide direct evidence that Cu(I) disrupts S-nitrosothiol homeostasis and NO metabolism, and, thus, provide new insights into the mechanisms involved in microglia-mediated-CNS disorders.

Keywords

Microglia; Inflammation; Copper; Nitric Oxide; GSNO; GSNOR

1. Introduction

As resident effector cells of the central nervous system (CNS), microglia actively monitor the surrounding microenvironment (Nimmerjahn et al., 2005) and are the first to respond to inflammation, infection or trauma by undergoing a series of morphologic, phenotypic and

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functional changes (Gertig and Hanisch, 2014). Activation is marked by increased production of immune-related proteins (Gehrmann et al., 1995; Ransohoff and Perry, 2009), ability to migrate (Ullrich et al., 2001) and to enter the cell cycle and to proliferate (Kim and de Vellis, 2005). In addition to playing an important role in innate immunity, microglia are also involved in adaptive immunity by becoming antigen-presenting cells (Shrikant et al., 1996).

Once activated, microglia express high levels of inducible nitric oxide (NO) synthase (iNOS, NOS2) and release mediators that include pro-inflammatory cytokines, prostaglandins and NO (Ransohoff and Perry, 2009). NO easily crosses cell membranes and diffuses readily in tissue and fluids (Coleman, 2001). While chemically reactive because of its unpaired electron, for a radical, NO is relatively stable and has a physiologically-relevant half-life (Stamler, 1994). Production of NO is dependent on arginine, NADPH and O₂ with NO and citrulline as its products. iNOS is one of three distinct enzymes that belong to the NOS family (Butler and Nicholson, 2003). Although very little iNOS is present in the absence of stimulation, once synthesized it releases a high flux of NO that does not require an increase in intracellular calcium because the Ca²⁺/calmodulin complex is bound with high affinity to iNOS even at basal calcium levels (Butler and Nicholson, 2003). Once released NO can produce cellular signaling through a wide variety of mechanisms including nitrosylation of metal and thiol moieties and nitration of lipids and proteins (Foster et al., 2003). The protective or damaging effects of NO release depend on its flux rate, local environmental conditions, and cell type (Gow and Ischiropoulos, 2001). Many of the biological effects of NO are mediated by the S-nitrosylation of low-mass and protein thiols to form S-nitrosothiols (SNO) (Foster et al., 2009). S-nitrosoglutathione (GSNO), an endogenous S-nitrosothiol, is considered to be the primary source of bioactive NO in the body and to be critical to NO signaling (Hogg, 2002). GSNO reductase (GSNOR) regulates GSNO concentration by degrading it through consumption of NADH. ADH Class III, a glutathione-dependent formaldehyde dehydrogenase, is the only GSNOR present in the brain and, as such, it plays a critical role in modulating NO chemical reactivity (Westerlund et al., 2005). GSNOR is differentially distributed in the brain where it is expressed at high levels in the hippocampus and cerebellum and more modestly in the deeper layers of the cortex (Galter et al., 2003). Degradation of GSNO results in the regulation of protein S-nitrosylation-directed signaling. Many neurodegenerative diseases, including Alzheimer's, Parkinson's and amyotrophic lateral sclerosis, are characterized by activated microglia and dysregulation of NO metabolisms (Graeber et al., 2011). The high content of polyunsaturated lipids and a high flux of reactive oxygen species produced during neurochemical reactions contribute to CNS vulnerability to oxidative stress (Ozcelik and Uzun, 2009).

Previous studies have shown that Cu(I) neurotoxicity includes induction of ROS with involvement of the NF- κ B pathway and apoptosis (Butterworth, 2010) and that, in its Cu(I) state, Cu(I) alters microglia phenotype and inhibits nitric oxide release (Rossi-George et al., 2012).

Cu(I) has a great affinity for thiols (SH groups) and is an effective catalyst for the formation and degradation of SNOs (Burg et al., 2000). Thus, high levels of Cu(I) can be toxic for the CNS. Cu(I) is an essential transition metal whose ability to accept and donate electrons

dissolved in de-ionized water. Eight h later 0, 100, or 1000 ng/mL lipopolysaccharide (LPS, from *Escherichia coli* 0128:B12; Sigma, St. Louis, MO) was added to the Cu(I)- or Cu (II)-containing medium. Twenty micromolar of 1400W Dihydrochloride (Tocris Bioscience, Ellisville, MO) was added immediately before LPS challenge.

2.3 Nitrite measurement

The nitrite release to the medium was used as a measure of NO production. Nitrite determination was performed using a potassium iodide and acetic acid mixture at room temperature and analyzed with the Ionics/Sievers Nitric Oxide Analyser 280 (NOA 280). The concentration of nitrite was calculated from a standard curve generated using NaNO₂ (Sigma Chemical Company, St. Louis, MO).

2.4 H₂O₂ Release

The rate of hydrogen peroxide formation by 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit, Life Technologies, Grand Island, NY) was measured in BV2 cells according to the manufacturer's instructions. The rate of hydrogen peroxide production was measured spectrophotometrically at excitation 560 nm and emission 587 nm.

2.5 Biotin Switch Assay and Immunoblot assay of S-nitrosylated proteins

Detection of SNO-proteins was performed via an adaptation of the biotin switch method. BV2 cell lysate (30 µg total protein) was incubated in HEN buffer (25 mM Hepes, pH 7.7/0.1 mM EDTA/0.01 mM neocuproine), and 20 µM *N*-ethylmaleimide (NEM) at 37 °C for 30 min to block free thiols. Excess NEM was removed by protein precipitation using double volume of cold acetone. After protein resuspension in HENS buffer (HEN buffer containing 1% SDS) SNO bonds were decomposed by adding 20 mM sodium ascorbate and 0.1 mM CuCl. The newly formed thiols were then linked with NEM-Biotin (Pierce, Rockford, IL). Immunoblot analysis of SNO-protein was performed with Bio-Dot SF apparatus following the manufacturer's instructions (Bio-Rad, Hercules, CA). After biotin switch assay, 5 µg total protein was applied onto a nitrocellulose (NC) membrane. The membrane was then blocked with PBS containing 5% nonfat dry milk for 1 h at room temperature, and incubated with horseradish peroxidase (HRP)-conjugated monoclonal anti-biotin antibody (1:10,000; Sigma) at room temperature for 1 h. After repeated washes the NC membrane was visualized by using SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL); the results were recorded on a film (Eastman Kodak, Rochester, NY). Signals were visualized on a Chemidoc MP System (Biorad, Hercules, CA) and optical density of dots quantified by Quantity One 4.6.5 software after subtracting background density.

2.6 GSNOR activity assay

BV2 cells were lysed in enzyme assay buffer (50 mM Tris-HCl (pH 7.4), 0.5mM EDTA and 1mM PMSF). Protein concentration was determined by Bradford assay (Biorad, Hercules, CA). Twenty g of protein was diluted 10-fold into enzyme assay buffer with 300 µM NADH. In order to remove Cu(I) and chemicals which may affect enzyme activity assay, proteins in

whole cell lysate were purified by passing them through a G25 Sephadex column (Biorad, Hercules, CA). GSNO-dependent NADH consumption was determined spectrophotometrically by measuring change in absorbance at 340 nm for 60 min at 25°C in the presence or absence of 1mM GS NO. Enzyme activity was calculated by slope generated from kinetic of NADH consumption rate (slope) and normalized by the total input protein (Units/mg). Slope (A/min) = Absorptivity (M/cm)*[NADH]/min*length (cm). GSNOR activity was expressed as $\mu\text{mol NADH degraded min}^{-1} \text{ mg}^{-1} \text{ protein (U/mg protein)}$.

2.7 Immunoblotting

BV2 cells were lysed in ice-cold buffer (Hepes: 20 mM, NaCl 150 mM, glycerol 10%, Triton-X 100 1%, EGTA 1 mM, MgCl_2 1.5 mM, , pH 7.4, containing protease inhibitors PMSF 1 mM, Na Pyrophosphate 10 mM, NaF 50 mM, Na Orthovanadate 2 mM, Lactacystin 1 μM , AEBSF 1 mM, EDTA 0.5 mM, Bestatin 65 μM , E-64 0.7 μM , Leupeptin 0.5 μM and Aprotinin 0.15 μM). An equal amount of protein for each sample (as determined by Bradford assay) was separated by 10% SDS-polyacrylamide gel electrophoresis (NuPage; Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with anti-GSNOR antibody (1:1000; Proteintech, Chicago, IL) and subsequently incubated in goat anti-rabbit IgG HRP-conjugated antibody (1:5000; Invitrogen, Carlsbad, CA). ECL reagents (GE Healthcare, Piscataway, NJ) were used as detection system according to the manufacturer's instructions. The membrane was subsequently immersed in stripping buffer (Thermo Scientific, Rockford, IL) and, after extensive rinsing, blocked, incubated with polyclonal rabbit anti-mouse iNOS antibody (1:1000; Santa Cruz Biotechnologies, Santa Cruz, CA), followed by incubation with goat anti-rabbit IgG HRP-conjugated antibody (1:5000; Invitrogen, Carlsbad, CA). Protein loading control was achieved by reprobing the membrane with anti-GAPDH monoclonal antibody (1:5000; Millipore, Bellerica, MA), followed by secondary antibody (1:2000; Invitrogen, Carlsbad, CA). Protein was visualized with ECL. The results were recorded on a film (Eastman Kodak, Rochester, NY) after each ECL development. Signals were visualized on a Chemidoc MP System; optical density of bands was quantified by Quantity One 4.6.5 software (Biorad) and normalized to GAPDH, after subtracting background density.

2.8 Statistical Analysis

Each experiment was repeated a minimum of 3 times and each time determination was made in duplicate. Data are expressed as means $\pm$ SE and analyzed for significance using T-tests or ANOVAs followed by post hoc tests when appropriate. A p value of <0.05 was considered significant.

3. Results

3.1 LPS-induced release of H_2O_2 of activated BV2 microglia is abrogated by Cu(I)

Superoxide and H_2O_2 production in microglia are indicators of activated microglia by a variety of stimuli. Culture medium of BV2 cells incubated with LPS (0, 100 or 1000 ng/mL) was collected at 0, 6, 9, and 24 h post stimulation. Both 100 and 1000 ng/mL LPS resulted in significant H_2O_2 release starting at 6 h (Figure 1, Panel A) until 24 h later. To assess the effects of Cu(I) on H_2O_2 release, a single dose of either 1 or 100 μM Cu(I) was used as a

pre-treatment 8 h prior to exposure to 100 or 1000 ng/mL LPS. Aliquots from the medium were taken 24 h after Cu(I) challenge. H₂O₂ production at 24 h is shown in Figure 1, Panel B. While 100 μ M Cu(I) mitigated the effects of both doses of LPS in a statistically significant manner, 1 μ M Cu(I) did not.

3.2 Cu(I) affects nitrite release and SNO formation in BV2 microglia

Activated microglia express iNOS and release NO; the reaction of NO with GSH results in GSNO. Having shown that 100 μ M Cu(I) inhibits H₂O₂ release from BV2 microglia, we sought to determine the effects of Cu(I) on NO metabolism in activated microglia. In order to validate our system and corroborate our previous findings, we measured nitrite release in LPS-challenged BV2 microglia. Figure 2 shows that both 1 and 100 μ M Cu(I) significantly reduced nitrite release when BV2 cells were stimulated with both doses of LPS. Cu(II) however had no significant effect when cell were stimulated with the lower (100 ng/mL) dose of LPS, but a statistically-significant reduction in nitrite release was observed with the higher dose of LPS (1000 ng/mL). The reduced release of nitrite into the medium was not due to cell death as measured by FACS analysis (data not shown). Figure 3 shows that although measurable and irrespective of concentration or redox status, Cu alone did not result in an increase of SNO formation when compared to control (i.e., No Cu). While non-statistically significant, LPS induced a more pronounced accumulation of SNO-proteins with the higher concentration (1000 ng/mL) than the lower concentration (100 ng/mL). Figure 3 also shows that SNO content in microglia stimulated with either doses of LPS was not affected by pretreatment with 1 μ M Cu(I), while 100 μ M Cu(I) reduced SNO for both 100 and 1000 ng/mL LPS. Pre-treatment with Cu(II) did not result in a statistically significant change, irrespective of LPS dose.

3.3 Cu(I) inhibits GSNOR activity in activated BV2 microglia

In the body, GSNOR degrades GSNO by NADH consumption. Having shown that a high dose of Cu(I) significantly reduces SNO content in LPS-treated BV2 microglia, we speculated that Cu(I) would affect GSNOR activity. GSNOR activity in BV2 microglia was measured by using consumption of NADH in the presence of GSNO. Figure 4 shows that while 1 μ M Cu(I) failed to inhibit GSNOR activity, a high concentration of 100 μ M significantly reduced it when BV2 cells were stimulated with 100 ng/mL LPS, but not with 1000 ng/mL LPS. GSNOR protein was measured to verify that neither a low concentration of Cu(I) nor LPS reduces GSNOR protein expression. Figure 5 shows that while 1 μ M Cu(I) alone had no effect on iNOS expression, 100 μ M reduced it. Figure 5 also shows that while 1 μ M Cu(I) reduced GSNOR protein expression in cells stimulated with 1000 ng/mL LPS, 100 μ M Cu(I) inhibited GSNOR expression irrespective of LPS stimulation. In addition, figure 5 also shows that Cu(II) has no effect on GSNOR protein expression.

3.4 Cu(I) modulates SNO-proteins in activated BV2 microglia

Intracellularly, small S-nitrosothiol compounds modify proteins to form SNO-proteins. SNO-proteins have physiologic and pathophysiologic functions. Having shown that Cu(I) inhibits GSNOR activity without affecting iNOS expression, we examined SNO-proteins in LPS-stimulated BV2 microglia. SNO proteins were detected by biotin-switch method. While a high concentration of Cu(I) had no effect, a low concentration of 1 μ M Cu(I) increased

SNO-protein expression. In addition, the increase observed in the LPS condition was drastically reduced by a high concentration (100 μ M) but not by a low concentration (1 μ M) of Cu(I). As expected, the iNOS inhibitor 1400W partially negated LPS-induced SNO-protein expression (Figure 6).

4. Discussion

Neuroinflammation plays an important role in the pathogenesis of many neurodegenerative diseases. Activated microglia have been implicated both in the initiation and progression of neurodegeneration by acquiring different phenotypes that range from induction of neuronal injury and death through the release of cytotoxic products to promotion of injury resolution through the release of anti-inflammatory products. The landmark of inflammation is a massive induction of iNOS with the subsequent release of NO by activated microglia. S-nitrosylation of protein targets has been associated with neurodegenerative disorders including Alzheimer's and Parkinson's (Brown, 2010). Cu(I) dysfunction has also been observed in neurodegeneration (Squitti et al., 2013). The 'metal-based neurodegeneration hypothesis' is based on the observation that the generation of oxidative stress results from the production of reactive oxygen (ROS) and nitrogen species (RNS) by redox-active metal ions including Cu(I). Proteins are a major target of ROS and RNS. This study sought to extend our previous results on the effects of Cu(I) on microglia. Our results confirmed that exposure to a supra-physiologic dose of Cu(I) significantly reduces the production of nitrite in LPS-stimulated BV2 microglia, a result consistent with our previous finding showing that Cu(I) alters microglia phenotype through release of NO. Interestingly, a study by Colasanti and colleagues (Colasanti et al., 2000) found that exposure of LPS-stimulated C6 glioma cells to CuSO₄ for one hour, and measured 48 h later, had no effect on nitrite release. The different outcome could be due to the dose of LPS being one order of magnitude higher than in the present study and administered in combination with interferon (IFN) γ , the different species of Cu(I) used, or the length of exposure to Cu(I). In light of our finding that treatment with 1 μ M Cu(I) inhibits nitrite release elicited by both 100 and 1000 ng/mL LPS, we attribute the lack of effect reported by the Colasanti and colleagues to a dose of Cu(I) that is insufficient to inhibit NO formation brought about by 10,000 ng/mL LPS plus IFN γ . The addition of IFN- γ to LPS is an important difference as it was shown that while stimulation with LPS alone does not induce cell death, the combination of LPS (100 ng/mL) and IFN γ does, and it involves two distinct pathways: one mediated by NO and the other by Caspase-11 (Mayo and Stein, 2007). Our results showing that 100 μ M Cu(II) reduces nitrite release in microglia stimulated with a high dose of LPS (1000 ng/mL) is, most likely, due to Cu(II) having been reduced by copper-reducing molecules within the cell, including ascorbate, reduced glutathione and other endogenous thiols (Aliaga et al., 2016).

As a measure of ROS formation we measured the extent by which Cu(I) affected the production of hydrogen peroxide and, at the same time, to ensure that there would not be shortage of NADH substrate. Our results show that H₂O₂ production is increased over time by LPS stimulation but it is not when microglia are not activated. As expected, the higher dose of LPS (although non-cytotoxic) elicits a slightly higher (albeit nonsignificant) release of H₂O₂, an increase that is most likely due to increased cellular respiration. In addition, results pertaining to the effects of Cu(I) on H₂O₂ production mirror those of nitrite release in

that, while a high dose of 100 μM Cu(I) drastically reduces H_2O_2 production, a low dose of 1 μM Cu(I) does not. We next sought to determine the extent to which Cu(I) exposure would affect SNO formation in activated microglia. SNOs are generated by the covalent, but reversible, addition of an NO moiety to reactive sulfhydryl residues of peptides and proteins. The fine equilibrium between NO production and SNO reduction is catalyzed by de-nitrosylating enzymes including the NADH-dependent GSNOR (Foster et al., 2009) which, ultimately, reduces nitrosative stress-induced toxicity (Liu et al., 2001) by metabolizing GSNO to glutathione disulfide and a glutathione N-hydroxysulfenamide intermediate, before being reduced to ammonia. Therefore, GSNOR regulates cellular concentrations of GSNO thus resulting in the regulation of protein S-nitrosylation-directed signaling. The S-nitrosylation of proteins of LPS-activated microglia by treatment with a high dose of 100 μM Cu(I) could be attributable to the direct cleavage of S-NO bonds. Although it is not clear to us why the 100 μM Cu(I) treatment would abrogate expression of GSNOR protein irrespective of LPS stimulation while control microglia express measurable GSNOR activity and protein, we would exclude that complete abrogation of GSNOR protein be attributed to either an inhibited cellular respiration or to cell death, as indicated by the intact GAPDH expression in those conditions. Furthermore, although GSNOR expression is reduced by the high dose of Cu(I), the reduction in GSNOR activity is only observed in microglia stimulated with 100 ng/mL LPS but not by 1000 ng/mL LPS nor by 100 μM Cu(I) alone, both of which, although not reaching significance, follow the same decreasing trend as for the 100 ng/mL LPS. We speculate that expression of GSNOR protein ceases to be detectable by immunoblotting while NADH consumption is still measurable and quantifying mRNA levels of GSNOR over time would provide some insight as to this discrepancy. Except for the lower expression of iNOS levels in the 100 μM Cu(I) alone, Cu(I) did not affect expression in LPS-stimulated microglia, suggesting that NO is being released at an equivalent rate, at least, within the cell. Our results also show that Cu(II) has no effect on either GSNOR activity or protein, confirming that the reduction in GSNOR is dependent on the redox state of Cu. One-thousand ng/mL LPS elicited a statistically significant drop in GSNOR activity without affecting either GSNOR protein expression or iNOS expression suggesting that Cu(I) affects GSNOR directly. As expected, 1000 ng/mL LPS resulted in increased SNO protein expression, which was inhibited by 1400W and by 100 μM Cu(I) but not by 1 μM Cu(I). In contrast to the lack of effect on the S-nitrosylation of proteins by a high dose of Cu(I), a low dose of 1 μM Cu(I) elicited a robust expression which might be due to a low and sustained production of intracellular NO (as suggested by the weak iNOS expression in figure 5) which, in turn, would only partially perturb GSNOR. Alternatively, the addition of Cu(I) could greatly enhance S-nitrosothiols (RSNO) reduction and biotin labeling (Wang et al., 2008), thereby affecting the S-nitrosothiols decomposition by Cu(I) ions; this one electron reduction of the RSNO group would result in free thiol, NO and Cu(II), which, subsequently, would be reduced to Cu(I).

In this study, we demonstrated that while Cu(I) does not affect LPS induced iNOS expression it inhibits NO release from microglia. Cu(I)-induced GSNOR inhibition and GSNO depletion is more in line with alternative (non-inflammatory) polarization. These results extend our previous findings that Cu(I) elicits a phenotype with characteristics of both pro-inflammatory and alternative profiles (Rossi-George et al., 2012).

Because GSNO represents the main pool of biologic NO in the body, its homeostasis is critical for regulating pathogenesis in the CNS. Although our *in vitro* observations cannot be directly extrapolated to *in vivo*, Cu(I) inhibition of GSNOR and GSNO in activated microglia may have pathophysiological relevance. Recent studies suggest that GSNO administration may attenuate the severity of EAE, an animal model of multiple sclerosis (Prasad et al., 2007). High levels of Cu(I) have been found in CNS pathologies in both animal models and in humans. Thus, the disruption of GSNOR-GSNO dyad may have important implications in neuroimmunological disorders and neuroinfectious diseases. Inhibition of GSNOR by Cu(I) may represent one mechanism which leads to shifting microglia signaling in the CNS. GSNO depletion and changes in the profile of SNO-proteins may be one of the mechanisms responsible for the pathogenesis of the inflammatory process related to the disorders in the CNS. Further understanding of transient metal contributions to the regulation of RNS in the CNS may lead to the development of new strategies for treatment of neurological disorders characterized by inflammation.

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- Copper inhibits GSNOR activity in activated BV2 microglia
- Cu(I) reduces nitrite release and SNO proteins in LPS-treated BV2 microglia
- SNO-formation is inhibited by 100 μ M Cu(I) in activated BV2 microglia

Panel B

Fig. 1. Effects of Cu(I) on H₂O₂ release in LPS-stimulated BV2 microglia

A. BV2 microglia were incubated with 0, 100 or 1000 ng/mL LPS and sampled for H₂O₂ release at 0, 6, 9 and 24 h later. H₂O₂ release was detected by 10-acetyl-3,7-dehydroxyphenoxazine and measured spectrophotometrically. Data represent mean $\pm$ SEM of 3 to 5 experiments and were analyzed by ANOVA followed by Holm-Sidak post hoc method with overall significance level set at $p < 0.05$: * LPS 0 vs. LPS 1000 at 6 h, $p = 0.036$; # LPS 0 vs. LPS 1000 at 9 h, $p = 0.006$; @ LPS 0 vs. LPS 1000 at 24 h, $p < 0.001$; ‡ LPS 0 vs. LPS 100 at 6 h, $p < 0.001$; § LPS 0 vs. LPS 100 at 9 h, $p = 0.001$; + LPS 0 vs. LPS 100 at 24 h, $p < 0.001$. **B.** BV2 microglia were incubated with 0, 1 or 100 μM Cu(I) in medium 8 h before stimulation with 0, 100 or 1000 ng/mL LPS and sampled 16 h later. H₂O₂ release was detected by 10-acetyl-3,7-dehydroxyphenoxazine and measured spectrophotometrically. These results represent the mean $\pm$ SEM of duplicate cultures of 5 independent experiments and were analyzed by T-tests: * No Cu(I) + LPS 100 vs. 100 μM Cu(I) + LPS 100, $p = 0.0017$; # No Cu(I) + LPS 1000 vs. 100 μM Cu(I) + LPS 1000, $p = 0.0010$.

Fig. 2. Effects of Cu(I) on nitrite release in LPS-stimulated BV2 microglia

One or 100 µM Cu(I) was added to the medium 8 h before LPS stimulation (0, 100 or 1000 ng/mL). Sixteen h after LPS stimulation, nitrite concentration was measured in the medium by using a potassium iodide and acetic acid mixture and analyzed by NOA 280. Nitrite reduction is most drastically reduced by 100 µM Cu(I) irrespective of LPS dose than by 1 µM Cu(I). Symbols denote statistical significance ($p < 0.05$): [@] 1 µM Cu(I) + LPS 100 ng/mL vs. LPS 100 ng/mL; ⁺ 1 µM Cu(I) + LPS 1000 ng/mL vs. LPS 1000 ng/mL; [#] 100 µM Cu(I) + LPS 100 ng/mL vs. LPS 100 ng/mL; ^{*} 100 µM Cu(I) + LPS 1000 ng/mL vs. LPS 1000 ng/mL; [‡] 100 µM Cu(II) + LPS 1000 ng/mL vs. LPS 1000 ng/mL. These results represent the mean $\pm$ SEM of duplicates of 5 independent experiments.

Fig. 3. Effects of Cu(I) on SNO levels of LPS-stimulated BV2 microglia

BV2 microglia were exposed to 1 or 100 μM Cu(I) or to 100 μM nonredox-active Cu(II) added to the medium 8 h prior to stimulation with 0, 100 or 1000 ng/mL LPS. Sixteen h after LPS stimulation, SNO levels in the cell lysate were determined by a Cu(I)-cysteine reduction system. A high dose of 100 μM Cu(I) reduced SNO accumulation in LPS-stimulated BV2 (# No Cu + LPS 100 ng/mL vs. 100 μM Cu(I) + LPS 100 ng/mL, $p = 0.02$; * No Cu + LPS 1000 ng/mL vs. 100 μM Cu(I) + LPS 1000 ng/mL, $p = 0.04$). Data represent mean $\pm$ SEM of duplicate cell cultures from 3 independent experiments.

Fig. 4. Cu(I) inhibits GSNOR activity in LPS-stimulated BV2 microglia

BV2 microglia were exposed to 1 or 100 μM Cu(I) 8 h prior to LPS stimulation (100 or 1000 ng/mL). Cell lysates were filtered to remove excess Cu(I) and proteins and GSNOR activity was determined by NADH consumption in the presence of 1mM GSNO. One thousand ng/mL LPS alone reduced GSNOR activity (# No Cu(I), No LPS vs. LPS 1000 ng/mL, $p = 0.002$). One-hundred μM Cu(I) reduced GSNOR activity in BV2 microglia stimulated with LPS 100 ng/mL. * LPS 100 ng/mL vs. 100 μM Cu(I) + LPS 100 ng/mL ($p = 0.0129$). Data represent mean $\pm$ SEM of duplicate cell cultures from 3 independent experiments.

Panel A

Panel D

Fig. 5. GSNOR expression, but not iNOS, is abrogated by 100 μM Cu (I) in LPS-treated cells
A. BV2 microglia were exposed to 1 or 100 μM Cu(I) 8 h prior to LPS stimulation (0, 100 or 1000 ng/mL). Whole cell lysates were collected 16 h after LPS stimulation. An equal amount of protein measured for GSNOR and iNOS with GAPDH as internal control by western blot. The figure shows representative results of duplicate cell cultures of 3 independent experiments. **B.** Densitometry quantification of iNOS normalized to GAPDH as total protein loading. Values (mean $\pm$ SEM) represent the number of pixels of the iNOS band divided by its corresponding GAPDH band; One-hundred μM Cu(I) reduces iNOS protein expression (* Control vs. 100 μM Cu(I), $p = 0.03$). **C.** Densitometry quantification of GSNOR normalized to GAPDH as total protein loading. Values (mean $\pm$ SEM) represent the number of pixels of the GSNOR band divided by its corresponding GAPDH band. One-

hundred μM Cu(I) abrogates GSNOR expression irrespective of LPS stimulation (* No LPS vs. 100 μM Cu(I), $p < 0.001$. # LPS 100 ng/mL vs. 100 μM Cu(I) + LPS 100 ng/mL, $p < 0.001$; § LPS 1000 ng/mL vs. 100 μM Cu(I) + LPS 1000 ng/mL). One μM Cu(I) reduces GSNOR expression in cells stimulated with 1000 ng/mL LPS (+ LPS 1000 ng/mL vs. 1 μM Cu(I) + LPS 1000 ng/mL, $p < 0.001$). **D.** BV2 microglia were exposed to 100 μM Cu(II) 8 h prior to stimulation with 1000 ng/mL LPS. Whole cell lysates were collected 16 h after LPS stimulation. An equal amount of protein measured for GSNOR and with GAPDH as internal control by western blot. The figure shows representative results of duplicate cell cultures of three independent experiments. **E.** Densitometry quantification of GSNOR normalized to GAPDH as total protein loading. Values (mean $\pm$ SEM) represent the number of pixels of the GSNOR band divided by its corresponding GAPDH band.

Fig. 6. Cu(I) reduces S-nitrosylated protein formation in LPS-stimulated BV2 microglia
 BV2 microglia were exposed to 1 or 100 μ M Cu(I) 8 h prior to LPS stimulation (1000 ng/mL); the iNOS inhibitor 1400W was added to the medium concurrently with 1000 ng/mL of LPS. SNO-proteins were prepared by biotin-switch assay and visualized by dot blot. LPS induced SNO-protein formation in BV2 microglia (@ Control vs. LPS, $p = 0.020$), which was reduced by 1400W (§ LPS vs. LPS + 1400W, $p = 0.03$). A high dose of 100 μ M Cu(I) reduced SNO-protein accumulation in LPS-stimulated microglia (* LPS vs. 100 μ M Cu(I) + LPS, $p = 0.05$), whereas a low dose of 1 μ M Cu(I) increased SNO protein (# Control vs. 1 μ M Cu(I), $p = 0.003$). Values of densitometry are expressed as number of pixels in a dot. Data represent mean $\pm$ SEM of duplicate cell cultures from 3 independent experiments.