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TNF alpha-mediated NO production enhances MnSOD nitration and mitochondrial dysfunction in primary neurons: An insight into the role of glial cells

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Abstract

Tumor necrosis factor-alpha (TNF- α), a ubiquitous pro-inflammatory cytokine, is an important mediator in the immune-neuroendocrine system that affects the central nervous system (CNS). The present study demonstrates that treatment with TNF- α activates microglia to increase TNF- α production in primary cultures of glial cells isolated from wild-type (WT) mice and mice deficient in the inducible form of nitric oxide synthase (iNOSKO). However, mitochondrial dysfunction in WT neurons occurs at lower concentrations of TNF- α when neurons are directly treated with TNF- α or co-cultured with TNF- α -treated microglia than iNOSKO neurons similarly treated. Immunofluorescent staining of primary neurons co-cultured with TNF- α -treated microglia reveals that the antioxidant enzyme in mitochondria, manganese superoxide dismutase (MnSOD), is co-localized with nitrotyrosine in WT but not in iNOSKO primary neuronal cells. Importantly, the percent of surviving neurons is significantly reduced in WT neurons compared to iNOSKO neurons under identical treatment conditions. Together, the results suggest that TNF- α activates microglia to produce high levels of TNF- α and that production of NO in neurons is an important factor affecting MnSOD nitration and subsequent mitochondrial dysfunction.

Keywords

tumor necrosis factor-alpha (TNF- α); mitochondrial dysfunction; MnSOD nitration; iNOSKO; nitric oxide (NO); CNS toxicity

Introduction

Tumor necrosis factor-alpha (TNF- α) is an important cytokine participating in inflammation, cell survival and cell death processes (Gomaz-Chiarri et al., 1994). TNF- α

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affects differentiation of macrophages, the inflammatory cells, and primes neutrophils to increase secretory responses and generate reactive oxygen- and nitrogen-derived species (ROS and RNS). TNF- α also triggers cell death by apoptosis and necrosis. TNF- α can increase nitric oxide (NO) production through induction of the inducible form of nitric oxide synthase (iNOS) (Poljakovic et al., 2003).

Inflammatory processes play a critical role in the pathogenesis of human diseases including neurological disorders. The hallmark of neuroinflammation is the activation of resident microglial cells and recruitment of peripheral immune cells, as well as production of pro-inflammatory mediators and free radicals (Dickson et al., 1993; Chao et al., 1995; Espey et al., 1997). While an uncontrolled or chronic inflammatory response is an essential defense against pathogens, it may cause irreversible tissue damage. Inflammatory parameters are chronically elevated in neurodegenerative disorders and are implicated in the etiology of Alzheimer's disease, Parkinson's disease, and ischemia (Lovell et al., 1998; Liao et al., 2001; Gao et al., 2002). Activated microglial cells produce a wide array of cytotoxic factors, including TNF- α , interleukin-1 beta (IL-1 β), eicosanoids, nitric oxide (NO), and ROS. Although a few of these factors are thought to contribute to tissue repair, a majority are believed to work via mechanisms not yet fully understood to induce neurodegeneration (Streit, 2000; Minghetti and Levi, 1998; Hirsch et al., 20003; Liu et al., 2002). However, prolonged survival of the G93A SOD1 mouse, a model of ROS/RNS mediated neurodegenerative disease, treated with the arachidonic acid 5-lipoxygenase (5LOX) inhibitor nordihydroguaiaretic acid (NDGA), a potent inhibitor of microglial activation and TNF- α release, suggests that selective inhibition of microglial cell activity in vivo can be a valuable avenue of therapy (West et al., 2004).

NO and its toxic metabolite ONOO $^-$ have been implicated in the pathogenesis of many neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS). Free and protein-bound nitrotyrosine, a stable oxidation product left by ONOO $^-$, is found in sporadic and familial forms of ALS (Abe et al., 1995, 1997; Beal et al., 1997; Ferrante et al., 1997a; Toghi et al., 1999) and in transgenic mice expressing human mutations of copper-zinc superoxide dismutase (SOD-1) (Bruijn et al., 1997a; Ferrante et al., 1997b). Culture studies have shown extensive nitrotyrosine accumulation in motor neurons undergoing apoptotic death induced by intracellular incorporation of Zn-deficient SOD or by trophic factor deprivation (Estévez et al., 1998, 1999). These results suggest a role for ONOO $^-$ formation in degenerating motor neurons.

The most studied nitration of proteins is the conversion of tyrosine to 3-nitrotyrosine (Beckman et al., 1996). Nitrotyrosine adducted protein in vivo is widely used as a bioassay indicative of ONOO $^-$ generation. Nitration of tyrosine residues leads to enzyme inactivation (Beckman et al., 1996; MacMillan-Crow et al., 1996; Ischiropoulos et al., 1992). ONOO $^-$ is a potent inhibitor of mitochondrial respiration chain enzymes, including Fe-S containing enzymes (Hausladen and Fridovich, 1994; Castro et al., 1994). Exogenous NO can directly diffuse to mitochondria and reversibly inhibit cytochrome oxidase, leading to increased O $_2^{\cdot-}$ in mitochondria (Brown and Cooper, 1994; Brown, 1997). NO rapidly reacts with O $_2^{\cdot-}$ to form ONOO $^-$, a potent inhibitor of mitochondrial respiration enzymes, and enhances nitration of proteins, including MnSOD (Cassina and Radi, 1996; MacMillan-Crow et al., 1996). MnSOD nitration amplifies the increase of O $_2^{\cdot-}$ and ONOO $^-$ production leading to increased oxidative stress in mitochondria (MacMillan-Crow et al., 1996). Similarly, impaired activity of complex IV, cytochrome oxidase, has been demonstrated in AD (Davis et al., 1996).

We previously reported that adriamycin (ADR), a potent anti-cancer drug, produces TNF- α and activates iNOS expression leading to MnSOD nitration in the central nervous system

(Tangpong et al., 2007). For this report, we used primary neurons and glial cells to demonstrate that TNF- α -mediated NO production enhances MnSOD nitration and mitochondrial dysfunction and cell death in neurons treated with TNF- α -activated microglial cells.

Materials and methods

Primary neuronal and microglial cell culture

Primary neurons—Neurons were cultured from cortices of postnatal day 1 mouse brains (P1, B6C3, iNOSKO). iNOS knockout mice in the C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). The iNOS^{-/-} mice were bred into the B6C3 background and the colony was maintained at the University of Kentucky. Briefly, meninges-free cortices were isolated, trypsinized, and plated onto poly-D-lysine (0.05 mg/mL)-coated tissue culture, 6-well plates (250 cells/mm²), for 7 days in vitro before use. Neurons were grown in Neurobasal media with Glutamate and B27 supplements with a change of medium every 3 days (Invitrogen, Rockville, MD) to provide neuronal cultures consistently >95% pure and able to survive for at least 1 month in vitro. Culture purity was determined by cell counting after staining with the astrocytic marker anti-glial fibrillary acidic protein (GFAP) and the neuronal marker anti-microtubule-associated protein 2 (MAP2). Primary neurons were used at day 7 after plating without further passage.

Primary microglia—Microglia were derived from postnatal day 2 (P2, B6C3) mouse brains. Briefly, meninges-free cortices from P2 mice were isolated and trypsinized. Cells were plated onto 150 cm² tissue culture flask in DMEM with Glutamax (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS) and were fed every third day. After day 14, the confluent monolayer of cultured cells was shaken vigorously (30 min, 120 rpm, on a rotary shaker) to remove microglia. Microglial marker OX-42 was immunostained.

Activated microglia—Microglial cells were plated onto transwells (400 cells/mm²), activated with TNF- α (Sigma, St Louis, MO) for 30 min, and then transferred to co-culture with WT (B6C3) and iNOSKO primary neuronal cells for 3, 6 and 24 h.

Quantitation of secreted TNF- α

Although we and others have found that circulating levels of TNF- α are increased after a single injection of ADR, the levels observed are generally in the picomolar range and it is unclear how these levels of TNF- α could cause significant neuronal injury. As a first step toward addressing this question, we determined whether neurons were exposed to a higher level of TNF- α generated in the presence of glial cells. Condition media were taken from TNF- α -activated microglial cells co-cultured with neurons for 3 and 6 h, unactivated microglial cells co-cultured with neurons for 6 h, neurons treated with 50 pg/mL TNF- α for 6 h, and neurons treated with pre-immune serum (data not shown). Concentrations of TNF- α were determined by using mouse TNF- α colorimetric sandwich Enzyme-linked Immunosorbent assay (ELISA) following the manufacturer's instructions (mouse TNF- α /TNFSF1A immunoassay, R&D Systems, Minneapolis, MN). The TNF- α concentration in the sample was calculated from the recombinant mouse TNF- α standard curve, with a minimum detected limit typically less than 5.1 pg/mL.

Determination of NO production

NO production was estimated by measuring nitrite/nitrate in the cultured media. Neuronal cells alone, neurons co-cultured with unactivated microglia for 6 h, and neurons co-cultured with 50 pg/mL activated microglia for 3, 6 and 24 h were used in most experiments.

Samples were stored at -80°C until assayed. Nitrate was converted to nitrite with nitrate reductase (Calbiochem, Los Angeles, CA). Reduced samples were incubated with an equal volume of griess reagent (Molecular Probes, Eugene, Oregon) and the absorbance was measured at 548 nm. The total nitrite concentration was determined by comparison to the standard curve.

Cytotoxicity assay (MTT assay)

Because significant increases in nitrite/nitrate levels were only observed in WT neurons co-cultured with TNF- α -treated glial cells, we further determined cell viability and associated mitochondrial function of primary neurons using MTT assays (Gerlier and Thomasset, 1986). Briefly, neurons were seeded in a 24-well plate and incubated for 7 days. WT and iNOSKO neurons were treated with 25, 50, 100, 500 pg/mL TNF- α and control pre-immune serum for 24 h. The 50 pg/mL TNF- α activated microglia were co-cultured with neurons for 24 h. Media containing 0.5 mg/mL MTT solution were added to each well and plates were incubated at 37°C for 4 h. The medium was decanted and the reaction was stopped by the addition of 1,000 μl detergent reagent (20% SDS + 50% Dimethyl formamide in deionized water). Plates were incubated at 37°C for 1 h and the solubilized formazan solution was transferred to a 96-well plate. The absorbance was read at 595 nm.

Immunocytochemistry

Neurons and activated 50 pg/mL TNF- α microglia in both WT and iNOSKO cells were co-cultured for 3 and 6 h. The reaction was stopped by moving transwells off and fixing the neurons in 4% paraformaldehyde at 37°C for 30 min. Neurons were stained with the important mitochondrial enzyme, MnSOD antibody (Upstate, Lake Placid, NY), and nitrotyrosine (Cayman Chemical, MI), the ONOO-marker, in order to study the co-localization of MnSOD and nitrotyrosine in mitochondria. The neurons were kept overnight in a humidified box at 4°C . The neurons were washed 3 times with PBS and then incubated for 1 h with donkey antibody, conjugated with secondary antibodies coated with fluorescent dyes. The Hoechst 33342 dye was used to stain nuclear cells for determination of cell death. Excess secondary antibodies were removed by washing three times in PBS and once with deionized water. Neurons were mounted with mounting medium (Vectashield, H-100, Vector Laboratories, Burlingame, CA). Photomicrographs were obtained using a Leica confocal fluorescence microscope (Leica Microsystems Inc., Bannockburn, IL).

Statistical analysis

Statistical comparisons were made using one-way ANOVA followed by Newman-Keuls multiple comparisons test. All results are given as mean + SEM of at least three separate experiments. A value of $p < 0.05$ was considered statistically significant.

Results

Microglia enhance TNF- α levels

TNF- α levels in the conditioning media were higher by 30 and 15 fold in microglia treated with 50 pg/mL TNF- α for 3 and 6 h than in the control neurons co-cultured with unactivated microglia or neurons treated with TNF- α alone (Fig. 1). However, there was no difference in the TNF- α levels between WT and iNOSKO cells. The increased TNF- α levels were higher at 3 h than those similarly treated at 6 h after neurons and microglial cells were co-cultured.

TNF- α enhances NO production

Using the levels of nitrite/nitrate in the media from co-cultured neurons/glia, we estimated the levels of NO in neurons co-cultured with activated microglial cells treated with 50 pg/

mL TNF- α for 3, 6 and 24 h in WT and iNOSKO cells. In wild-type cells, the levels of NO were higher in neurons co-cultured with activated glial cells than in control neurons and neurons co-cultured with unactivated microglia (Fig. 2). Consistent with the lack of enhanced NO production in iNOSKO mice, there was no change in the levels of nitrite/nitrate from neuron/glial cells isolated from iNOSKO mice similarly treated.

Differential toxicity of TNF- α in wild-type and iNOS-deficient neurons

The results show that neurons directly treated with 25, 50, 100, 500, 1000 pg/mL TNF- α exhibit a dose dependent decline in MTT reduction reaching a statistical significance at 500 and 1,000 pg/mL (Fig. 3A). Wild-type neurons co-cultured with TNF- α -primed microglia have a significant reduction of cell viability at 50, 100 and 500 pg/mL TNF- α compared to control neurons and neurons co-cultured with unactivated microglia (Fig. 3B). In contrast, iNOSKO neurons co-cultured with activated microglia only show a decline in MTT reduction at the highest level of TNF- α used (Fig. 3C).

Co-localization of MnSOD with nitrotyrosine

Because it is well established that MnSOD is highly susceptible to nitrative inactivation, and we found that NO level is increased in TNF- α -treated neuronal cells, we identified MnSOD and protein containing nitrotyrosine in primary neurons using confocal microscopy. The results shown in Figure 4A demonstrate that MnSOD is co-localized with nitrotyrosine, a ONOO⁻-marker, in WT neurons co-cultured with activated microglial cells. The presence of the color yellow signifies co-localization between MnSOD; the color green, fluorescent dye and nitrotyrosine; and the color red suggests that MnSOD proteins were nitrated in WT cells at 3 and 6 h but not nitrated in iNOSKO cells (Fig 4B).

Cytotoxicity in neurons with MnSOD and nitrotyrosine co-localization

To determine whether neurons with presumed nitrated MnSOD are prone to cell death, we used Hoechst 33342 to determine nuclear staining in cells with co-localization of MnSOD and nitrotyrosine. Nuclear condensation and fragmentation indicate cell death at 3 and 6 h in WT neurons but not in iNOSKO (Fig. 5A, 5B).

Viability in TNF- α -treated cells

Nuclear condensation and fragmentation indicating cell death, using Hoechst 33342, were used to compare surviving WT and iNOSKO neurons. The data show that WT neurons co-cultured with TNF- α -activated microglia have reduced cell viability at 3 and 6 h. The decreased cell viability was higher in WT neurons than in iNOSKO neurons similarly treated at 3 and 6 h (Fig. 6).

Discussion

Our data demonstrate that the level of TNF- α in neuronal cells containing TNF- α -primed microglia is significantly increased compared to cells containing mostly neurons treated with the same level of TNF- α . Furthermore, non-TNF- α -primed microglia were not able to cause the increase in TNF- α levels observed in TNF- α -primed microglia. Our results are consistent with the possibility that proinflammatory TNF- α and NO/ONOO⁻ synergistically induce neuronal death and that iNOS is required for significant ONOO⁻ production (Floden et al., 2005). It has been shown that increased NO can inhibit mitochondrial enzyme complex IV and can prime increased O₂^{•-}. Increased O₂^{•-} can react with NO to form ONOO⁻ and cause protein nitration, especially tyrosine 34 of MnSOD, the major superoxide removal enzyme in mitochondria. We have previously demonstrated that adriamycin induces TNF- α -mediated CNS toxicity, in part, via systemic release of cytokines and subsequent generation

of reactive oxygen and nitrogen species (RNS) in the brain (Tangpong et al., 2006). Although TNF- α levels are increased in both wild-type and iNOSKO co-cultured neuronal cells, nitration of MnSOD seems to occur only in neurons from WT mice suggesting that intracellular NO from neurons is responsible for the observed nitrated protein in WT neurons. This result excludes a significant contribution of NO generated from TNF- α -activated microglial cells in nitration of TNF- α -treated neurons and suggests that activated glial cells generate TNF- α to induce iNOS in neurons. However, the MTT reduction assays show that mitochondrial dysfunction does occur at lower doses of TNF- α in primary neuronal cells co-cultured with TNF- α -primed microglia of the WT but not in the iNOSKO neurons under identical treatment conditions. Thus, the higher cytotoxicity levels of similar TNF- α treatments are likely due to the generation of OONO⁻ from the reaction of superoxide and nitric oxide. This possibility is supported by work reported by Pfeiffer et al. (1997), which demonstrates a typical ONOO⁻ decomposition ratio by large concentrations of nitrate (NO₃⁻) and nitrite (NO₂⁻) in the neuronal media.

The co-localization of MnSOD, nitrated protein, in primary neuronal cells suggests that MnSOD may be one of the targets of TNF- α -mediated OONO-induced nitration. Due to the limited amount of primary neurons available, we did not conclusively show in this experiment that MnSOD is nitrated, but many published works (MacMillan-Crow and Thompson., 1999; Filipovic et al., 2007; Anantharaman et al., 2006; Guo et al., 2003), including our own (Tangpong et al., 2007), have amply demonstrated that MnSOD is a sensitive target for OONO⁻-induced nitration and inactivation. The role of MnSOD in protecting against oxidative stress-induced cell death has been well documented. For example, MnSOD deficiency leads to dilated cardiomyopathy, neurological injury, and neonatal lethality in MnSOD knock-out mice (Li et al., 1995; Lebovitz et al., 1996). Transfection of MnSOD cDNA into cultured cells renders the cells resistant to paraquat-, TNF- α - and adriamycin-induced cytotoxicity, and radiation-induced neoplastic transformation (Wong et al., 1989; St. Clair et al., 1991; Hirose et al., 1993; St. Clair et al., 1992). Expression of human MnSOD genes in transgenic mice protects the transgenic mice against oxygen-induced pulmonary injury, adriamycin-induced cardiac toxicity, ischemia-reperfusion-induced brain injury, trauma and neurotoxin-induced brain injury (Wispe et al., 1992; Yen et al., 1996; Keller, 1998; Klivenyi et al., 1998; Sullivan et al., 1999; Maragoes et al., 2000), and myocardial ischemia and reperfusion (Jones et al., 2003). Thus, it is highly likely that inactivation of MnSOD may contribute to the mitochondrial dysfunction and neuronal death observed following co-culture with activated glial cells.

In summary, the present study demonstrates that: 1) Treatment with low levels of TNF- α activates microglia to generate a high level of TNF- α ; 2) Neuronal iNOS is needed to produce a high level of nitrated proteins in neurons; 3) iNOS may contribute to cell death in neurons in which MnSOD is being inactivated.

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Abbreviations

TNF-α	tumor necrosis factor-alpha
CNS	central nervous system
ADR	adriamycin
RNS	reactive nitrogen species

ROS	reactive oxygen species
WT	wild-type mice
iNOSKO	deficiency of inducible nitric oxide synthase mice
MnSOD	manganese superoxide dismutase
iNOS	inducible nitric oxide synthase
NO	nitric oxide
O₂⁻	superoxide anion
ONOO⁻	peroxynitrite
ALS	amyotrophic lateral sclerosis
AD	alzheimer's disease
GFAP	anti-glial fibrillary acidic protein
MAP2	anti-microtubule associated protein 2
OX-42	microglial marker

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Figure 1. Microglia enhance TNF- α levels

TNF- α or microglial cells (separated from neurons by growing in transwells) untreated or treated with TNF- α (50 pg/mL) for 30 min and transferred to co-culture with neuronal cells for 3 and 6 h. TNF- α levels in both WT and iNOSKO primary neuronal cells were significantly increased in TNF- α -primed microglial cells (* $p < 0.0001$) compared with control, and no significance between genotypes.

Figure 2. TNF- α enhances NO production

Nitrite/Nitrate levels were measured after microglial cells were activated with TNF- α (50 pg/mL) for 30 min and transferred to co-culture with neurons for the indicated times. The levels of nitrite/nitrate were significantly increased in WT treated with activated microglial cells (* $p < 0.0001$) at 3, 6 and 24 h, and were significantly different from the iNOSKO cells (* $p < 0.0001$).

Figure 3. Differential toxicity of TNF- α in wild-type and iNOS deficient neurons TNF- α -induced Mitochondria reduction in wild-type neurons

Cell viability assays for:

A. Wild-type neurons directly treated with TNF- α at indicated concentrations. Significant difference from untreated neurons (*p<0.05 and ** p<0.01).

B. Wild-type neurons co-cultured with unactivated microglia or microglia treated with the indicated concentrations of TNF- α . Significant difference from neurons co-cultured with unactivated microglia and neurons only (*p<0.05, **0.01, *** p<0.001).

C. WT and iNOSKO neurons after being co-cultured with unactivated microglia or microglia treated with the indicated concentrations of TNF- α . Significant difference between WT and iNOSKO (*p<0.05, ** p<0.01).

Figures 4A, 4B. Localization of MnSOD and nitrotyrosine

Fluorescent microscope scanning of images of cells stained with the indicated antibody. The images were taken at 3 and 6 h after being co-cultured with 50 pg/mL TNF activated microglia. The images were also stained with Hoechst to visualize the nucleus. Images from the left two panels were merged to show the overlap of MnSOD, nitrotyrosine and nucleus. The co-localization of MnSOD and nitrotyrosine is shown only in WT cells (4A) but not in iNOSKO cells (4B) or control, neurons co-cultured with unactivated microglia (data not shown).

Figures 5A, 5B. Cytotoxicity in neurons with MnSOD and nitrotyrosine co-localization
 Fluorescent microscope scanning of images of cells stained with Hoechst to visualize the nucleus at 1, 3 and 6 h after being co-cultured with TNF α -activated microglia. Arrows

indicate nuclear condensation and fragmentation in WT neurons co-cultured with activated microglia at 3 and 6 h (5A); condensation and fragmentation in iNOSKO neurons co-cultured with activated microglia (5B).

Figure 6. Viability of TNF- α treated neurons

Neurons co-cultured with microglia activated with 50 pg/mL TNF. Viability of WT neurons co-cultured with activated microglia at the indicated time. * $p < 0.01$ and ** $p < 0.001$ compared with untreated neurons and iNOSKO neurons, respectively.