Neurotox Res (2009) 16:77–86
DOI 10.1007/s12640-009-9051-z

Mechanism for Quinolinic Acid Cytotoxicity in Human Astrocytes and Neurons

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Received: 12 December 2008 / Revised: 31 March 2009 / Accepted: 2 April 2009 / Published online: 18 April 2009

Abstract

There is growing evidence implicating the kynurenine pathway (KP) and particularly one of its metabolites, quinolinic acid (QUIN), as important contributors to neuroinflammation in several brain diseases. While QUIN has been shown to induce neuronal and astrocytic apoptosis, the exact mechanisms leading to cell death remain unclear. To determine the mechanism of QUIN-mediated excitotoxicity in human brain cells, we measured intracellular levels of nicotinamide adenine dinucleotide (NAD^+) and poly(ADP-ribose) polymerase (PARP) and extracellular lactate dehydrogenase (LDH) activities in primary cultures of human neurons and astrocytes treated with QUIN. We found that QUIN acts as a substrate for NAD? synthesis at very low concentrations (<50 nM) in both neurons and astrocytes, but is cytotoxic at sub-physiological concentrations ([150 nM) in both the cell types. We have shown that the NMDA ion channel blockers, MK801 and memantine, and the nitric oxide synthase (NOS) inhibitor, L-NAME, significantly attenuate QUIN-mediated PARP activation, NAD? depletion, and LDH release in both neurons and astrocytes. An increased mRNA and protein expression of the inducible (iNOS) and neuronal (nNOS) forms of nitric oxide synthase was also observed following exposure of both cell types to QUIN. Taken together these results suggests that QUIN-induced cytotoxic effects on neurons and astrocytes are likely to be mediated by an over activation of an NMDA-like receptor with subsequent induction of NOS and excessive nitric oxide (NO•)-mediated free radical damage. These results contribute significantly to our understanding of the pathophysiological mechanisms involved in QUIN neuro- and gliotoxicity and are relevant for the development of therapies for neuroinflammatory diseases.

Keywords: Nitric oxide, Quinolinic acid , Astrocytes , Neurons, Alzheimer's disease, Neurodegeneration

Introduction

The kynurenine pathway (KP) is the main route of L-tryptophan catabolism resulting in the production of the essential pyridine nucleotide, nicotinamide adenine dinucleotide (NAD^+) (Stone 1993). The KP also leads to the production of several neuroreactive metabolites, of which the NMDA receptor agonist, quinolinic acid (QUIN) is likely to be more important in terms of biological activity. (Heyes 1993; Stone 2001). QUIN is known to be associated with the neuropathogenesis of Alzheimer's disease (Guillemin and Brew 2002), Huntington's disease (Finkbeiner and Cuero 2006), amyotrophic lateral sclerosis (Guillemin et al. 2005a), and human immunodeficiency virus (Guillemin et al. 2005b; Heyes et al. 1991; Heyes et al. 1992). QUIN levels in the central nervous system also increase with age (Moroni et al. 1984).

QUIN is known to promote oligodendrocyte, neuronal, and astrocytic apoptosis at pathophysiological concentrations (Cammer 2002; Guillemin et al. 2005c; Kelly and Burke 1996). Although the mechanism has not been completely elucidated, it appears to be involved for a large part the formation of reactive oxygen species (ROS) possibly mediated via the NMDA receptor (Behan et al. 1999; Guillemin and Brew 2002; Kerr et al. 1998). Activation of NMDA receptors by agonists such as glutamate and QUIN opens a channel permeable to Na^+ and Ca^{2+} Ions (Guillemin et al. 2005b; Stone and Perkins 1981). An increase in intracellular Ca^{2+} has been shown to trigger numerous destructive processes, including increased nitric oxide synthase (NOS) activity, which can promote increased nitric oxide (NO•) and free-radical damage, leading to mitochondrial dysfunction

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and DNA strand breaks (Atlante et al. 1997; Behan et al. 1999; Velazquez et al. 1997). QUIN leads to the generation of ROS having been shown to induce lipid peroxidation in the rat brain (Behan et al. 1999; Santamaria et al. 2001).

NOS is a family of enzymes including the inducible isoform (iNOS) and the constitutive forms: neuronal (nNOS) and endothelial (eNOS). It has been previously shown that iNOS transcription is induced during inflammation in response to cytokine stimulation (Possel et al. 2000) and several endotoxins, including QUIN (Rya et al. 2004). Activation of nNOS also has several implications in neuroinflammation: (1) NMDA receptor-mediated excitotoxicity is reduced in response to NOS inhibition in cultured rat cortical neurons (Dawson et al. 1991); (2) nNOS knockout mice report a significant reduction in death due to NMDA receptor-mediated excitotoxicity (Ayata et al. 1997); (3) nNOS activity is increased following QUIN injection in the rat striatum (Aguilera et al. 2007; Perez- Severiano et al. 1998).

Oxidative DNA damage is known to stimulate the activity of the NAD^+ dependent nuclear DNA repair enzyme, poly(ADP-ribose) polymerase (PARP-1) (EC 2.4.2.31). PARP activation leads to DNA repair and recovery of normal cellular function. However, excessive activation of PARP by DNA strand breaks induced by ROS results in the depletion of intracellular NAD? and ATP stores culminating in cell death due to reduced energy metabolism (Braidy et al. 2008; Ha and Snyder 1999; Zhang et al. 1994).

While QUIN-mediated activation of the NMDA receptor is a well known cause of apoptosis in the neuron (Kelly and Burke 1996; Kerr et al. 1995; Stone 2001), the role of the NMDA receptor and iNOS activation in QUIN-mediated cell death in the astrocyte has not been reported.

Considering the important relationship between ROS, PARP activity, and NAD^+ levels, we measured the effect of QUIN at pathophysiological concentrations on intracellular NAD^+ levels and PARP activity in primary cultures of human astrocytes and neurons. Extracellular lactate dehydrogenase (LDH) activity was used to quantify cytotoxicity. We also tested whether NMDA receptor antagonism and NOS inhibition could protect human astrocytes from QUIN excitotoxicity. We used RT-PCR to quantify iNOS and nNOS mRNA expression in purified primary cultures of human fetal astrocytes and neurons following QUIN treatment. Immunocytochemistry was also used to detect iNOS and nNOS protein expression.

In this study we show that, paradoxically, QUIN at very low concentrations can have a cytoprotective role as a precursor for NAD^+ synthesis. However, at subphysiological concentrations it quickly becomes cytotoxic to both neurons and astrocytes. Our data suggest that the mechanism for QUIN toxicity is similar in both human astrocytes and neurons involving NMDA receptor activation and $NO^{\bullet}$ production. Understanding the mechanism through which QUIN produces its cytotoxic effect in human brain cells is therefore of potential therapeutic importance.

Materials and Methods

Reagents and Chemicals

Dulbecco's phosphate buffer solution (DBPS) and all other cell culture media and supplements were from Invitrogen (Melbourne, Australia) unless otherwise stated. Nicotinamide, bicine, β-nicotinamide adenine dinucleotide reduced form (β-NADH), 3-[-4,5-dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide (MTT), alcohol dehydrogenase (ADH), sodium pyruvate, TRIS, γ-globulins, quinolinic acid (QUIN), (+)-5-methyl-10,11-dihydro-5Hdibenzo [a,d] cyclohepten-5,10-imine maleate (MK-801), memantine, D-2-amino-5-phosphonovalerate (AP-5), and N(G)-nitro-L-arginine methylester (L-NAME), mouse mAb anti-iNOS and anti-nNOS, DAPI, and pAb anti-GFAP were obtained from Sigma-Aldrich (Castle-Hill, Australia). Phenazine methosulfate (PMS) was obtained from ICN Biochemicals (Ohio, USA). Bradford reagent was obtained from BioRad, Hercules (CA, USA). Mouse anti-MAP2 were obtained from Millipore (Melbourne, Australia). Secondary anti-mouse IgG and anti-rabbit Alexa 488 (green) or Alexa 594 (red)-conjugated antibodies were purchased from Molecular Probes (Eugene, OR). All commercial antibodies were used at the concentrations specified by the manufacturers.

Cell Cultures

Human fetal brains were obtained from 16 to 19-week-old fetuses collected following therapeutic termination with informed consent. Mixed brain cultures were prepared and maintained using a protocol previously described by Guillemin et al. (2005c).

Astrocytes were prepared from the mixed brain cell cultures using a protocol previously described by Guillemin et al. (2001). Cells were cultured in medium RPMI 1640 supplemented with 10% fetal bovine serum, 1% 1-glutamax, 1% antibacterial/antifungal, and 0.5% glucose. Cells were maintained at 37°C in a humidified atmosphere containing 95% air/5% CO_2. Cells were seeded into 24-well tissue culture plates to a density of $1x10^5$ cells 24 h prior to experimentation.

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Neurons were prepared from the same mixed brain cell cultures as previously described (Guillemin et al. 2007). Briefly, cells were plated in 24-well culture plates coated with Matrigel (1/20 in Neurobasal) and maintained in Neurobasal medium supplemented with 1% B-27 supplement, 1% Glutamax, 1% antibiotic/antifungal, 0.5% HEPES buffer, and 0.5% glucose.

Primary Brain Cells and QUIN Culture Treatments

Human astrocytes and neurons were treated with 50–1200 nM QUIN. Cell homogenates, culture supernatants, and RNA were collected after 24 h. Experiments were performed in quadruplicates using cultures derived from three different human fetal brains.

NAD(H) Microcycling Assay for the Measurement of Intracellular NAD^+ Concentrations

Intracellular NAD^+concentration was measured spectrophotometrically using the thiazolyl blue microcycling assay established by Bernofsky and Swan (1973) adapted for 96-well plate format by Grant and Kapoor (1998).

Extracellular LDH Activity as a Measurement for Cytotoxicity

The release of lactate dehydrogenase (LDH) into culture supernatant correlates with the amount of cell death and membrane damage, providing an accurate measure of cellular toxicity. LDH activity was assayed using a standard spectrophotometric technique described by Koh and Choi (1987).

PARP Assay for the Measurement of Intracellular PARP Activity

PARP activity was measured using a new operational protocol relying on the chemical quantification of NAD^+ modified from Putt et al (2005). Briefly, plated cells are washed twice with DPBS and another 500 ll was added per well. Cells were then treated with known concentrations of QUIN and incubated for 15 min. DPBS solution was then aspired and PARP lysing buffer (200 ll) was added to the cell plate. The buffer solution contained $MgCl_2$ (10 mM), Triton X-100 (1%), and NAD^+ (20 lM) in Tris buffer (50 mM, pH 8.1). The plate was then incubated for 1 h and the amount of NAD^+ consumed was measured by the NAD(H) microcycling assay using the Model 680XR microplate reader (BioRad, Hercules).

Bradford Protein Assay for the Quantification of Total Protein

NAD^+ concentration, PARP, and extracellular LDH activities were adjusted for variations in cell number using the Bradford protein assay described by Bradford (1976).

RT-PCR of iNOS, nNOS, and GAPDH mRNA Expression

The method for RT-PCR has been previously described (Guillemin et al. 2001). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The primer sequences are as follows (1) iNOS forward primer: TCCGCTATGCTGGCTACCA; reverse primer CACTCGTATTTGGGATGTTCCA. (2) nNOS forward primer: CAGCACGGCATCTGCTTTG; reverse primer CATCCCACGTCCATTCCTTTT. (3) GAPDH forward primer: CTGAGTGTAGCCCAGGATGC; reverse primer ACCACCATGGAGAAGGCTGG. The intensity of the signal was quantified using the application Adobe Photoshop (Adobe Systems Incorporated, USA).

Immunocytochemistry for the Detection of iNOS and nNOS Expression

The method for immunocytochemistry has been previously described (Guillemin et al. 2007). Cells were incubated with selected primary antibodies mAb iNOS and mAb nNOS, together with phenotypic markers (GFAP, MAP-2). Selected secondary antibodies (goat anti-mouse IgG or goat anti-rabbit coupled with Alexa 488 or Alexa 594) were used. The following controls were performed for each labelled experiment: (1) isotypic antibody controls and (2) incubation with only the secondary labelled antibody.

Data Analysis

Results obtained are presented as the means ± the standard error of measurement (SEM). One way analysis of variance (ANOVA) and post hoc Tukey's multiple comparison tests were used to determine statistical significance between treatment groups. Differences between treatment groups were considered significant if P was less than 0.05 ($P<0.05$).

Results

Effect of QUIN on Intracellular NAD^+ Concentrations and Extracellular LDH Activity in Human Astrocytes and Neurons

While QUIN is known to be excitotoxic to neurons, we chose to investigate recent evidence that QUIN may also be cytotoxic to astrocytes. Astrocytes and neurons were treated with QUIN for 24 h at increasing concentrations (50, 150, 350, 550, and 1200 nM), respectively. NAD^+ depletion was

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observed in a dose-dependent manner at concentrations above 150 nM (Fig. 1a and b). However, the intracellular NAD^+ concentration in astrocytes and neurons treated with 50 nM of QUIN was significantly greater when compared to non-treated astrocytes (Fig. 1a) and neurons (Fig. 1b). As expected the decrease in cellular NAD^+ levels correlated negatively with increasing extracellular LDH activity in a dose-dependent manner at QUIN concentrations greater than 150 nM in human astrocytes (Fig. 2a) and neurons (Fig. 2b) over 24 h.

Effect of NMDA Receptor Antagonism and nNOS Inhibition on QUIN Mediated NAD? Depletion, Extracellular LDH, and PARP Activities in Human Neurons

To determine if NMDA receptor activation and subsequent nitric oxide ($NO^{\bullet}$) production are involved in QUIN toxicity in primary human neurons, we monitored the effect of NMDA receptor antagonism and nNOS inhibition on intracellular NAD^+ levels, PARP, and extracellular LDH activities. The NMDA ion channel blocker, MK-801 (1 lM) and NOS inhibitor, L-NAME (100 lM) were able to prevent NAD? depletion in human neurons in 24 h (Fig. 3a). Significant activation of PARP was observed in neurons treated with QUIN (550 nM) for 24 h (Fig. 3b). Treatment with MK-801 (1 lM) and L-NAME (100 lM) were able to significantly reduce PARP activation and subsequent NAD? depletion in human neurons in 24 h (Fig. 3b). Extracellular LDH activity was significantly reduced following treatment with MK-801 (1 lM) and L-NAME (100 lM) in the presence of QUIN (550 nM) (Fig. 3c), corresponding to the observed preservation of intracellular NAD^+ levels (Fig. 3a) and reduced PARP activity.

Effect of NMDA Receptor Antagonism and iNOS Inhibition on QUIN-Mediated NAD^+ Depletion, Extracellular LDH, and PARP Activities in Human Astrocytes

We assessed whether a similar mechanism is involved in QUIN toxicity on primary human astrocytes. Addition of MK-801 (0.1–2 lM) attenuated QUIN-mediated NAD? depletion after 24 h (Fig. 4a). However, higher doses ([10 lM) generated a significant decrease in NAD^+

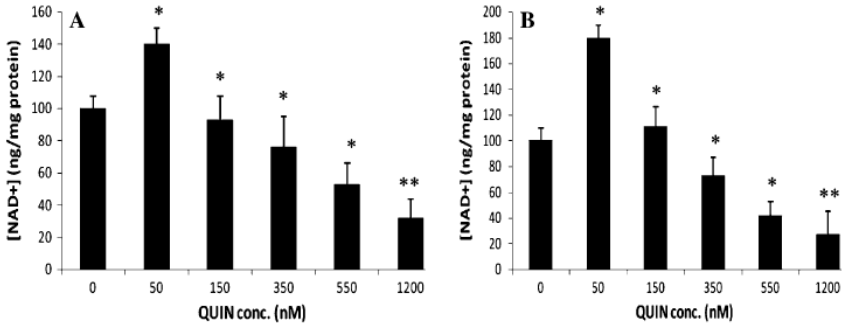

Fig. 1 QUIN treatment (0–1200 nM) on intracellular NAD^+ in **a** human astrocytes and **b** human neurons for 24 h. Significance $^*P < 0.05$, $^{**}P < 0.01$ compared to previous dose ($n = 4$ for each treatment group)

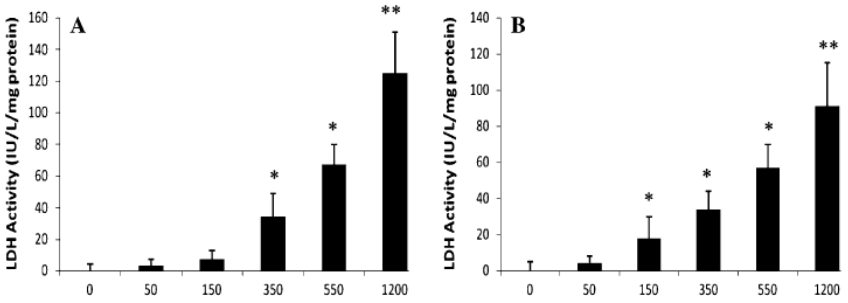

Fig. 2 QUIN treatment (0–1200 nM) on extracellular LDH activity in **a** human astrocytes and **b** human neurons for 24 h. Significance $^*P < 0.05$, $^{**}P < 0.01$ compared to previous dose ($n = 4$ for each treatment group)

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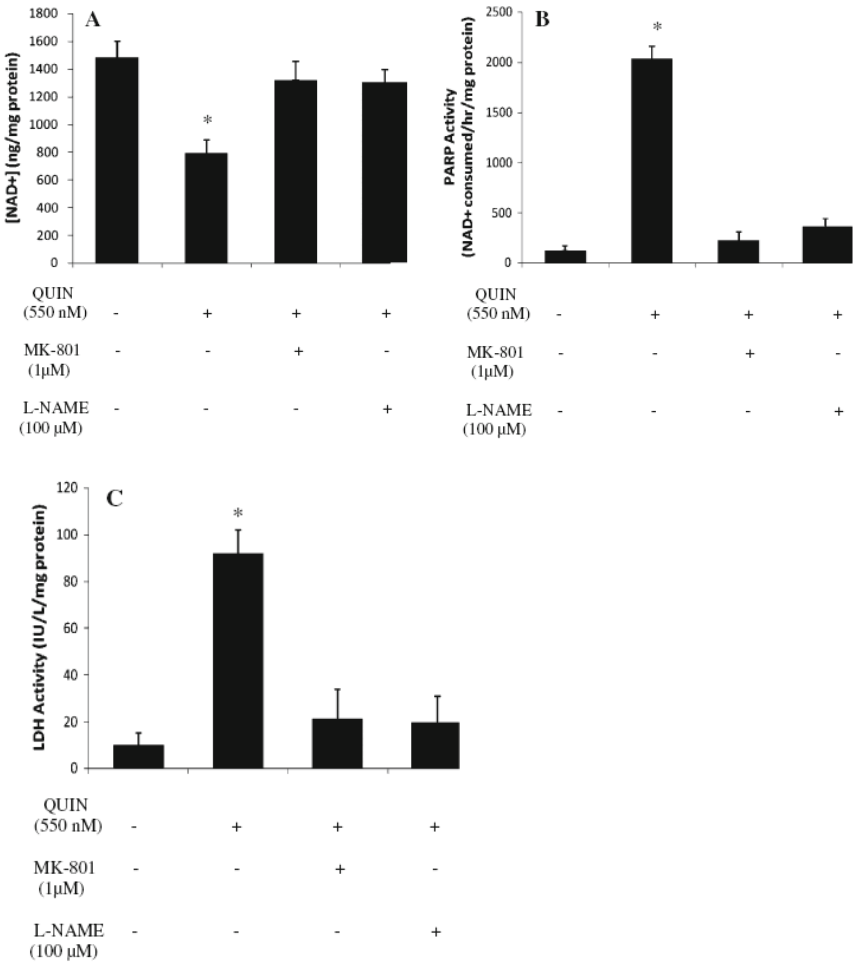

Fig. 3 Effect of NMDA receptor antagonism and nNOS inhibition on QUIN-induced changes in **a** intracellular NAD^+ levels, **b** PARP activity, and **c** extracellular LDH activity in human neurons. **a** $*P < 0.05$ compared to control ($n = 4$ for each treatment group). **b** $*P < 0.05$ compared to control ($n = 4$ for each treatment group). **c** $*P < 0.05$ compared to control ($n = 4$ for each treatment group)

compared to lower doses. Memantine, a lower affinity NMDA ion channel blocker also prevented NAD^+ depletion at higher concentrations (2–10 lM). AP-5, a competitive NMDA receptor antagonist at the glutamate site showed no significant effect on NAD? up to 10 lM; however, intracellular NAD^+ depletion was slightly ameliorated at 50 lM of treatment (Fig. 4a).

Astrocytes treated with QUIN at 550 nM for 1 h showed significantly increased PARP activity compared to the control (Fig. 4b), consistent with the previous results showing QUIN can affect NAD? concentration (Fig. 4a). Concomitant treatment of these cells with MK-801 (0.1–2 lM) significantly reduced PARP activity compared to QUIN treatment alone. Treatment with memantine (0.5–10 lM) and AP-5 (10–50 lM) also reduced PARP activity, but to a lesser extent than MK-801 (Fig. 4b).

To investigate whether QUIN toxicity was mediated via NMDA-induced NO· production, astrocytes were treated with the iNOS inhibitor L-NAME at a final concentration of 100 lM. L-NAME treatment prevented QUIN-mediated NAD^+ depletion at the cytotoxic QUIN concentrations of 550 and 1200 nM (Fig. 5a). Consistent with results for NAD^+ depletion (Fig. 5a), astrocytes treated with QUIN (550 and 1200 nM) in the presence of L-NAME (100 lM), had significantly lower PARP activity (Fig. 5b). Again, consistent with results already presented for NAD^+ (Fig. 5a) and PARP (Fig. 5b), cells treated with QUIN (550 and 1200 nM) in the presence of L-NAME (100 lM) showed significantly reduced extracellular LDH activity in culture supernatants after 24 h (Fig. 5c).

Detection of iNOS and nNOS mRNA Expression in Human Astrocytes and Neurons

Expression of the mRNA for human iNOS was studied in primary cultures of human astrocytes (Fig. 6a) and neurons (Fig. 6b) with and without QUIN (550 nM) exposure for 24 h. As previously described, iNOS was not expressed in human neurons (Aguilera et al. 2007). Based on the ratio of iNOS and nNOS expression relative to GAPDH expression, iNOS and nNOS expression was significantly higher in QUIN-treated

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astrocytes (Fig. 6c) and neurons (Fig. 6d), respectively, compared to non-treated cells.

Detection of iNOS and nNOS Expression in Human Astrocytes and Neurons

Immunocytochemical studies were performed to demonstrate that increased iNOS and nNOS expression was not limited to mRNA alone and reflects increased protein production. Higher immunoreactivity for iNOS and nNOS enzyme proteins was detected in human fetal astrocytes and neurons in the presences of QUIN (550 nM) compared to untreated cultures and cells co-treated with MK-801 (100 lM) and L-NAME (100 lM) for 24 h (Fig. 7). Double staining with MAP-2 and GFAP demonstrated that iNOS and nNOS were specifically expressed by astrocytes and neurons, respectively.

Discussion

In this study, we assessed the effects of pathophysiological concentrations of QUIN on intracellular NAD^+ and extracellular LDH activity in human astrocytes and neurons. A dose-dependent decrease in intracellular NAD^+ (Fig. 1) and a corresponding increase in extracellular LDH activity (Fig. 2) were observed in both brain cell types for concentrations above 150 nM. Our in vitro results for QUIN toxicity are in accordance with previous studies using brain cell cultures (Ting et al. 2007; Guillemin et al. 2005d; Kerr et al. 1998) and animal models (Bjorklund et al. 1984; Dihne et al. 2001).

Interestingly, a significant increase in intracellular NAD^+ was observed in human astrocytes and neurons treated with 50 nM of QUIN (physiological concentration). This indicates that extracellular QUIN can be taken up as a substrate for NAD^+ synthesis. This is supported by the previous study from Grant and Kapoor (1998) who showed that QUIN could contribute significantly to NAD^+ regeneration following acute H_2O_2-induced depletion in primary glial cells.

QUIN-induced cytotoxicity in neurons has long been known to involve over-activation of the NMDA receptor (Stone 2001). NMDA receptor activation and subsequent influx of Ca^{2+} into neurons activate nNOS and downstream enzymes, leading to the production of NO· and other free radicals able to cause DNA strand breaks and pathological activation of PARP, NAD? depletion, and cell death due to energy deprivation (Ha and Snyder 1999; Zhang et al. 1994). In this study, we have shown that QUIN at concentrations C150 nM significantly increased PARP activity (Fig. 3b) resulting in NAD depletion (Fig. 3a) and cell death, indicated by a corresponding increase in LDH activity (Fig. 3c). These results are consistent with previous work by Maldonado et al (2007), who showed that PARP activation and subsequent NAD? depletion plays an active role in neuronal cell death induced by QUIN in the rat brain.

In addition, we showed that the NMDA ion channel blocker, MK-801, and the NOS inhibitor, L-NAME, can prevent QUIN-induced

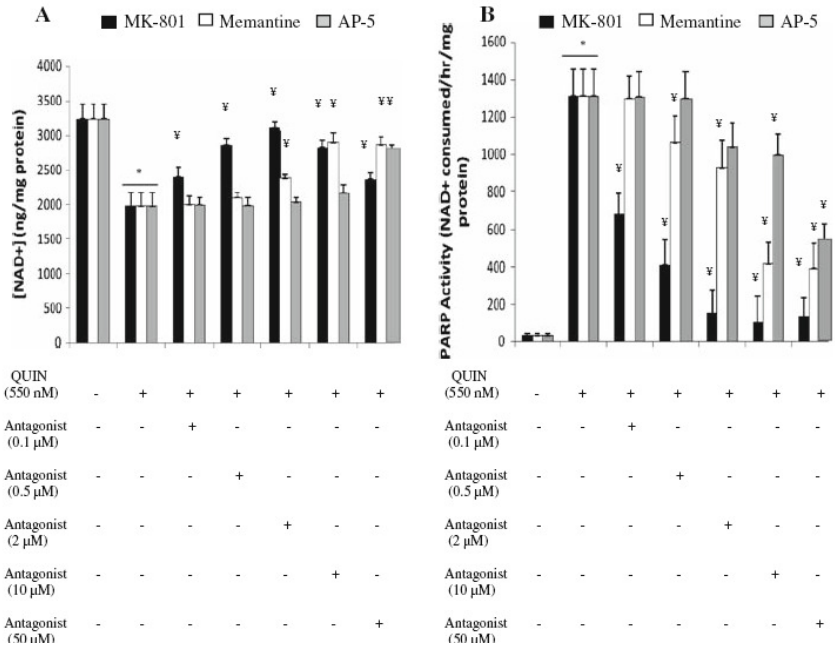

Fig. 4 Effect of NMDA receptor antagonism on QUIN-induced changes in **a** intracellular NAD^+ levels, **b** PARP activity in human astrocytes. **a** MK-801, memantine, and AP-5 (0-50 μM) on QUIN-induced NAD^+ depletion in human astrocytes for 24 h. $^*P < 0.05$ compared to control; $^¥P < 0.05$ compared to QUIN treatment alone. ($n = 4$ for each treatment group). **b** MK-801, memantine, and AP-5 (0-50 μM) on QUIN-induced PARP activation in human astrocytes for 24 h. $^*P < 0.05$ compared to control; $^¥P < 0.05$ compared to QUIN treatment alone. ($n = 4$ for each treatment group)

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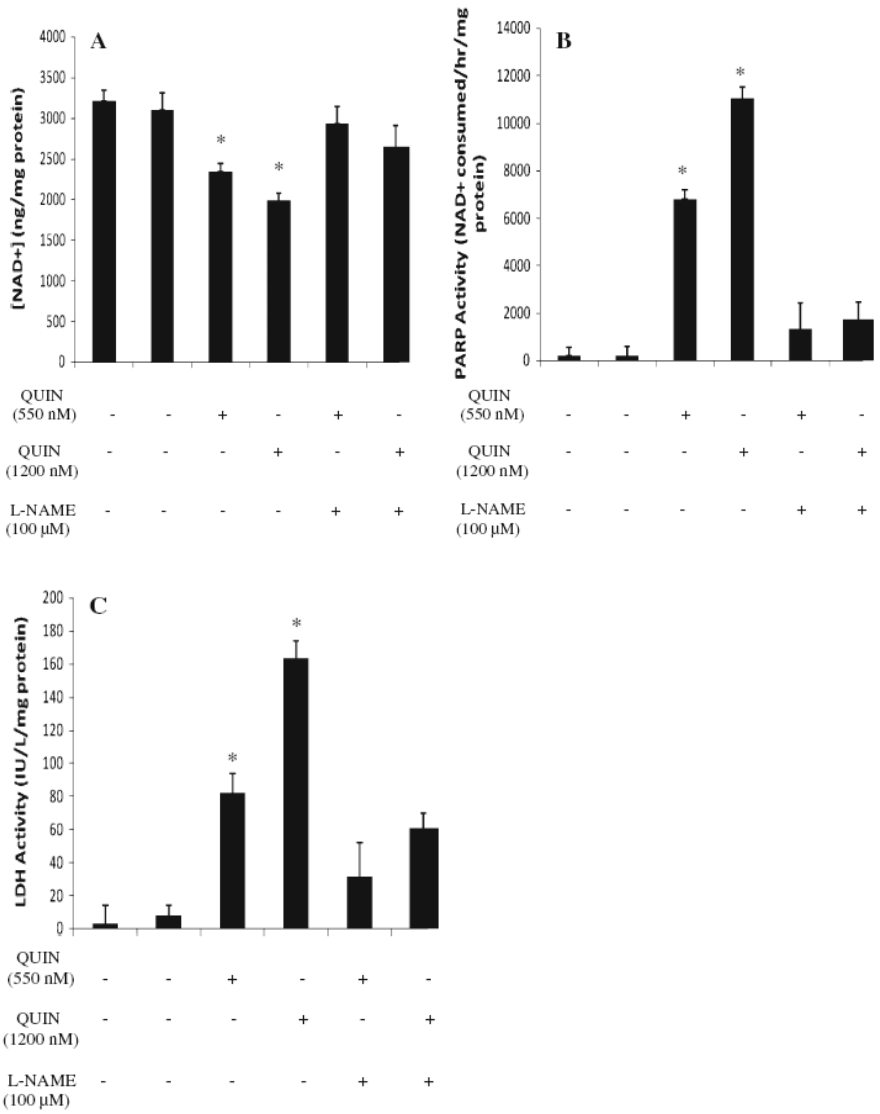

Fig. 5 Effect of iNOS inhibition on QUIN-induced changes in **a** intracellular NAD^+ levels, **b** PARP activity, and **c** extracellular LDH activity in human astrocytes. **a** L-NAME (100 µM) on QUIN-induced NAD^+ depletion in human astrocytes for 24 h. $^*P < 0.05$ compared to control ($n = 4$ for each treatment group). **b** L-NAME (100 µM) on QUIN-induced PARP activation in human astrocytes for 24 h. $^*P < 0.05$ compared to control ($n = 4$ for each treatment group). **c** L-NAME (100 µM) on QUIN-induced extracellular LDH activity in human astrocytes for 24 h. $^*P < 0.05$ compared to control ($n = 4$ for each treatment group)

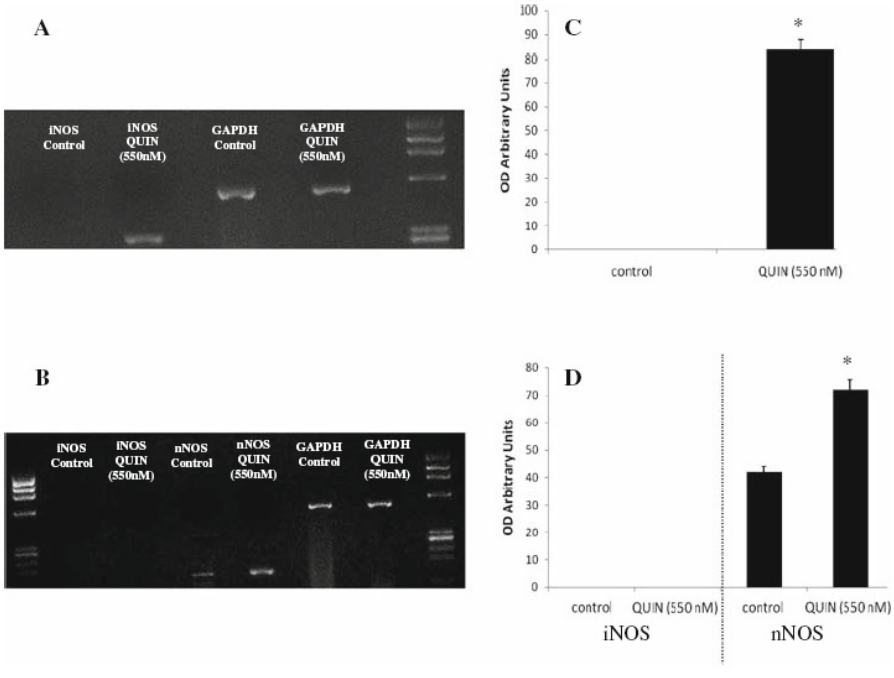

Fig. 6 Expression of iNOS, nNOS, and GAPDH mRNA in purified primary human fetal astrocytes and neurons after QUIN (550 nM) stimulation. Photograph of ethidium bromide-stained gel showing RT-PCR for iNOS (amplicon size: 220 pb), nNOS (amplicon size 210 pb), and GAPDH (amplicon size: 509 pb) in **a** astrocytes **b** neurons. Histogram showing the ratio of iNOS and nNOS expression relative to the GAPDH expression in **c** astrocytes, **d** neurons. $^*P < 0.05$ compared to control. Standard errors were ≤10%

neurotoxicity by reducing NAD^+ depletion (Fig. 3a) and PARP activation (Fig. 3b). These results are again consistent with previous studies which have shown that NMDA receptor antagonism and NOS inhibition prevent QUIN-induced toxicity in rat neurons (Stone 2001).

Although mechanisms involved in QUIN cytotoxicity on neurons are well established (Guillemin et al. 2005a, b, c, d), the biochemical pathway leading to QUIN-induced cell death in astrocytes is largely unknown. In this study we showed that QUIN cytotoxicity on astrocytes is mediated by a similar pathway as in neurons involving iNOS induction through activation of a glial NMDA-like receptor. While it is understood that the existence of functional NMDA receptors in human astrocytes is currently controversial (Conti et al. 1996; Guillemin et al. 2005b), recent work by our group has demonstrated the presence of functional NMDA receptors in primary human astrocytes (data not shown).

We observed that synthetic NMDA receptor antagonists, MK-801 and memantine, were able to successfully improve QUIN-mediated NAD? depletion and cell death. The NMDA channel blocker MK-801 and memantine dose dependently prevented QUIN-induced cell death in astrocytes (Fig. 4a) with MK-801 having a stronger effect than memantine at lower concentrations ranging from 0.1 to 2 lM, but not at higher concentrations (10–50 lM) (Fig. 4a). AP-5, an antagonist at the glutamate site of the NMDA receptor showed only a partial protective effect on NAD? at very high concentrations (50 lM) (Fig. 4a). This pattern of protection in astrocytes is consistent with a previous study using mouse neurons that showed that MK-801 and memantine were more successful at reducing QUIN toxicity than AP-5 because of their non-competitive action on the NMDA receptor (Wong et al. 1986).

Human primary astrocytes showed a significant increase in PARP activity when exposed to C150 nM QUIN. Treatment with MK-801 or memantine, and to a lesser extent AP-5, reduced PARP activation in a dose dependent manner (Fig. 4b). The involvement of $NO^{\bullet}$ in the death of
astrocytes was evident when treatment with the NOS inhibitor; L-NAME essentially blocked QUIN-induced NAD^+ depletion (Fig. 5a), PARP activation (Fig. 5b) and extracellular LDH activity (Fig. 5c). We also observed that exposure of astrocytes to QUIN for 24 h dramatically increased iNOS mRNA expression (Fig. 6a, c). Although iNOS mRNA (Fig. 6b) was not expressed in human neurons
(Aguilera et al. 2007) nNOS mRNA expression was significantly increased in QUIN-treated neurons compared to non-treated cells (Fig. 6b, d). This is further supported through increased iNOS and nNOS protein expression in QUIN-treated human astrocytes and neurons compared to non-treated cells and cells treated with NMDA receptor antagonists or a NOS inhibitor (Fig. 7).

Together, these results indicate that activation of a glial NMDA-like receptor followed by excess $NO^{\bullet}$ production, DNA damage, PARP activation, and subsequent NAD^+ depletion is a primary mechanism for QUIN-associated toxicity in human astrocytes similar to that found in our study and previously reported for neurons. Moreover, these studies suggest that nervous tissue $NO^{\bullet}$, not only serves as an essential neuronal messenger, but may also play a major role in QUIN toxicity. Previous studies have shown that PARP inhibition can prevent the depletion of intracellular NAD^+ and ATP stores, and therefore prevent cell death (Ha and Snyder 1999; Zhang et al. 1994). In addition, replenishing intracellular NAD^+ can prevent PARP-1- mediated astrocyte death in rat cultures as reported by Du et al (2003) using liposomal NAD^+ delivery into rat neurons. Identification of pathways through which QUIN promotes astrocytic and neuronal death may increase our understanding of several inflammatory brain diseases, and thus pave the way for effective and innovative therapeutic approaches.

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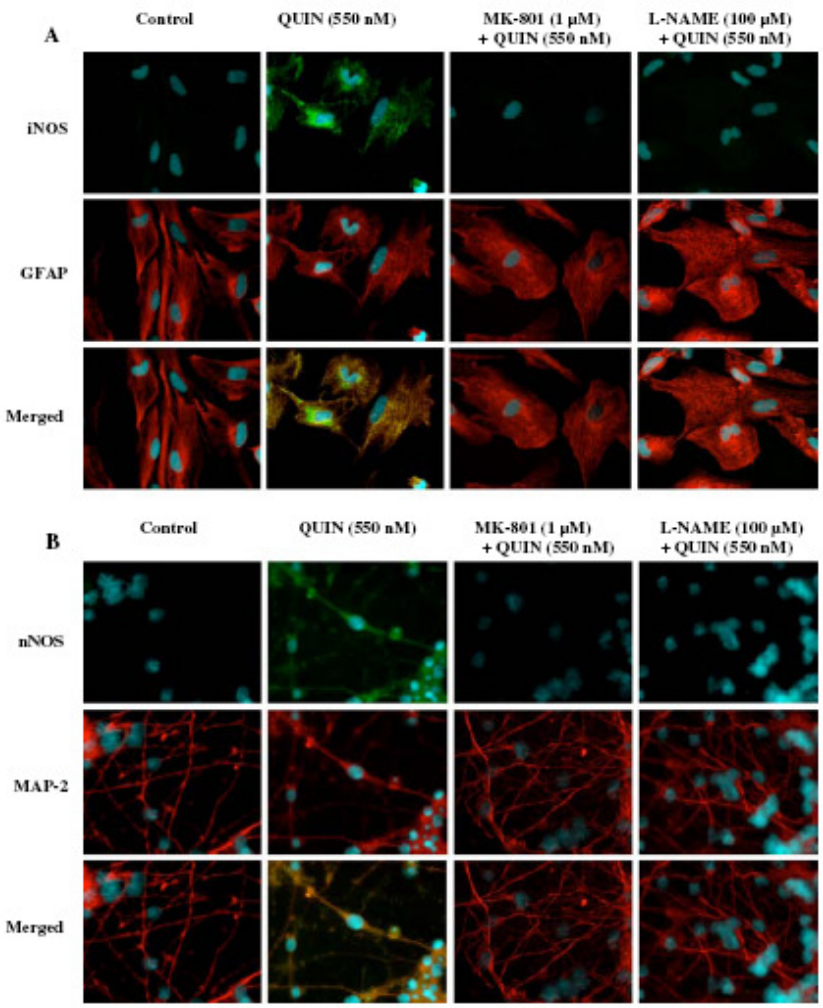

Fig. 7 Immunocytochemical detection of iNOS and nNOS in purified primary human fetal astrocytes and neurons after QUIN (550 nM) stimulation. a Staining for iNOS in human astrocytes: top, double staining for iNOS/green and DAPI/blue; center, double staining for GFAP/red and DAPI/blue; bottom, merged iNOS/green, GFAP/red, and DAPI/blue. b Staining for nNOS in human neurons: top, double staining for nNOS/green and DAPI/blue; center, double staining for MAP-2/red and DAPI/blue; bottom, merged nNOS/green, MAP-2/red, and DAPI/blue

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