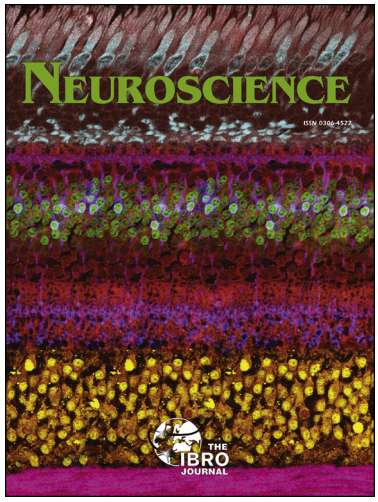

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Research paper

Early modulation of the transcription factor Nrf2 in rodent striatal slices by quinolinic acid, a toxic metabolite of the kynurenine pathway

Running title: Quinolinate modulates Nrf2

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Abstract

Nrf2 is a transcription factor involved in the orchestration of antioxidant responses. Although its pharmacological activation has been largely hypothesized as a promising tool to ameliorate the progression of neurodegenerative events, the actual knowledge about its modulation in neurotoxic paradigms remains scarce. In this study, we investigated the early profile of Nrf2 modulation in striatal slices of rodents incubated in the presence of the toxic kynurenine pathway metabolite, quinolinic acid (QUIN). Tissue slices from rats and mice were obtained and used throughout the experiments in order to compare inter-species responses. Nuclear Nrf2 protein levels and oxidative damage to lipids were compared. Time and concentration response curves of all markers were explored. Nrf2 nuclear activation was corroborated through phase 2 antioxidant proteins expression. The effects of QUIN on Nrf2 modulation and oxidative stress were also compared between slices of wild-type ($Nrf2^{+/+}$) and Nrf2 knock-out ($Nrf2^{-/-}$) mice. The possible involvement of the N-methyl-D-aspartate receptor (NMDAr) in the Nrf2 modulation and lipid peroxidation was further explored in mice striatal slices. In rat striatal slices, QUIN stimulated the Nrf2 nuclear translocation. This effect was accompanied by augmented lipid peroxidation. In the mouse striatum, QUIN *per se* exerted an induction of Nrf2 factor only at 1 h of incubation, and a concentration-response effect on lipid peroxidation after 3 h of incubation. QUIN stimulated the striatal content of phase 2 enzymes. $Nrf2^{-/-}$ mice were slightly more responsive than $Nrf2^{+/+}$ mice to the QUIN-induced oxidative damage, and completely unresponsive to the NMDAr antagonist MK-801 when tested against QUIN. Findings of this study indicate that: 1) Nrf2 is modulated in rodent striatal tissue in response to QUIN; 2) $Nrf2^{-/-}$ striatal tissue was moderately more vulnerable to oxidative damage than the Wt condition; and 3) early Nrf2 up-regulation reflects a compensatory response to the QUIN-induced oxidative stress in course as part of a general defense system, whereas Nrf2 down-regulation might contribute to more intense oxidative cell damage.

Key Words: Kynurenine pathway; Oxidative stress; Antioxidant defense; Excitotoxicity; Nrf2.

Glossary

ANOVA, Analysis of variance; ARE, Antioxidant Response Element; DMF, dimethylfumarate; γ-GCL-C, gamma-glutamylcysteine ligase-C; GPx, glutathione peroxidase; GR, glutathione reductase; HD, Huntington's disease; HO-1, heme oxygenase-1; KP, Kynurenine pathway; MDA, malondialdehyde; MK-801, dizocilpine; NMDAr, N-methyl-D-aspartate receptor; NQO-1, NAD(P)H quinone oxidoreductase 1; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; QUIN, quinolinic acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAC, S-allyl cysteine; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; tBHQ, tert-butylhydroquinone; TTC, 2,3,5-triphenyltetrazolium chloride.

1. Introduction

Neurodegenerative processes represent the major cause of neurological disorders in human beings (Coyle and Puttfarcken, 1993; Santamaría and Jimenez, 2005). Disorders coursing with neurodegeneration, such as Huntington's disease, share common key triggering factors that activate cell damage. These factors include excitotoxicity, mitochondrial energy depletion, oxidative stress and inflammation (Zádori et al., 2012). Together, these components establish a complex toxic scenario that is responsible for neuronal cell degeneration and death. Excitotoxicity, a toxic event defined as a persistent stimulation of membrane receptors in neuronal cells (Olney, 1990), is characterized by a cascade of processes comprising increased levels of intracellular calcium in response to a continuous opening of Ca^{2+} channels associated to glutamate receptors activation after sustained exposure to excitatory amino acids (reviewed by Essa et al., 2013; Mehta et al., 2013). In turn, enhanced intracellular Ca^{2+} levels trigger lethal metabolic pathways, further leading to continuous enzyme activation, enhanced reactive oxygen and nitrogen species (ROS/RNS) formation, mitochondrial dysfunction (Rami et al., 1997), and necrotic or apoptotic cell death.

Kynurenine pathway (KP), a metabolic pathway involved in tryptophan degradation to synthesize NAD^+, has gain increasing attention because some of its intermediary metabolites possess neuroactive properties, exerting either excitatory or inhibitory actions in the CNS (reviewed by Pérez-De La Cruz et al., 2007). Some KP metabolites have been involved in pathological conditions, triggering factors for degenerative events in neurological disorders (reviewed by Schwarcz et al., 2010; 2012). One of these metabolites, quinolinic acid (2,3-pyridine dicarboxylic acid or QUIN), is an endogenous glutamate agonist acting on selective populations of N-methyl-D-aspartate receptors (NMDAr) (Stone et al., 2013). QUIN has been recognized as an experimental tool to produce excitotoxicity and oxidative stress under *in vitro* and *in vivo* conditions, and its toxic pattern includes enhanced intracellular Ca^{2+} levels, GABA depletion, increased ROS formation,

decreased activity and expression of antioxidant systems, oxidative stress, increased protease activity and cell death (Braidy et al., 2009; 2010; Pérez-De La Cruz et al., 2010; Ríos and Santamaría, 1991; Rodríguez-Martínez et al., 2000). Several studies have demonstrated that QUIN toxicity can be reduced or prevented by the use of antioxidants, including ebselen (Rossato et al., 2002), L-carnitine (Silva-Adaya et al., 2008), S-allylcysteine (Pérez-Severiano et al., 2004), guanosine (Dobrachinski et al., 2012), probucol (Colle et al., 2012), caffeic acid (Kalonia et al., 2009), polyphenolic compounds (Braidy et al., 2010), and the combination of non-effective concentrations of glutamatergic modulators plus antioxidants (Dobrachinski et al., 2012). Considering that increased levels of this toxic metabolite have been reported in several neurodegenerative disorders (Schwarcz et al., 2010), its role as a pathogenic factor in the brain is a matter under continuous investigation; therefore, the study of the pro-oxidant effects and mechanisms exerted by QUIN in the CNS is of major importance for biomedical research.

The nuclear transcription factor related to NF-E2 (also known as Nrf2) constitutes a promising tool to counteract the deleterious effects of oxidative stress through the up-regulation of endogenous antioxidant genes. Nrf2 is considered a master regulator of redox homeostasis because it up-regulates the expression of more than 100 genes (phase 2 enzymes) involved in xenobiotic and ROS detoxification (Kensler et al., 2007; Itoh et al., 1999). Phase 2 enzymes share a common promoter enhancer known as Antioxidant Response Element (ARE) that is regulated by Nrf2. Among many proteins encoded by this gene are heme oxygenase-1 (HO-1), Superoxide dismutase (SOD), NAD(P)H quinone oxidoreductase 1 (NQO-1), glutathione peroxidase (GPx), glutathione reductase (GR), and γ-glutamyl cysteine ligase (γ-GCL), just to mention a few (Kensler et al., 2007; Itoh et al., 1999; Johnson et al., 2002; Lee et al., 2003).

This study was designed to investigate how Nrf2, a well-known orchestrator of antioxidant responses in mammals, is regulated by QUIN at early times of incubation. Its development

emerges from the need to offer more accurate information on this topic because: 1) QUIN is a toxic metabolite with relevance for the explanation of neurodegenerative events in neurological disorders (Schwarcz et al., 2012); and 2) to date, there is only limited information available on this emerging issue. Precisely, one of the fewest reports dealing with this issue was published by our group (Tasset et al., 2010). In that report, we described positive actions of the antioxidant and well-known Nrf2 inducer, tert-butylhydroquinone (tBHQ) in this toxic model. tBHQ exerted protective effects in different markers of oxidative damage induced by QUIN. We also described a depleting effect of QUIN on the Nrf2 levels in rat striatal slices. However, this first approach was inaccurate because we described a form of Nrf2 corresponding to 57 kDa. Recently, a report by Lau and coworkers (2013) discussed evidence pointing out that the real biologically relevant molecular weight of mammalian Nrf2 is around 95-110 kDa, and this is due to the fact that multiple acidic residues in Nrf2 promote its gaining in molecular weight. Derived from this observation, herein we characterized the effect of QUIN on the striatal levels of the ~98 kDa Nrf2 form, together with some related functional markers of oxidative stress. Therefore, striatal slices obtained of adult male rats and mice were challenged with this toxicant and further used throughout the study for comparative purposes.

2. Materials and methods

2.1. Chemicals

QUIN, MK-801, HEPES, thiobarbituric acid (TBA), malondialdehyde (MDA), 2,3,5-triphenyltetrazolium chloride (TTC) and other reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were obtained from other commercial sources. Antibodies employed along the study are described forward (in the Immunoblotting section).

2.2. Animals – rats

Adult male Wistar rats (250–300 g) were used throughout the first part of the study. Animals (N = 25) were housed five per cage in acrylic cages and provided with food and water *ad libitum*. The housing rooms at the vivarium (facilities of the Instituto Nacional de Neurología y Neurocirugía) were maintained under constant conditions of temperature (25 ± 3ºC), humidity and light cycles (12:12 light:dark schedule). All experimental manipulations were performed according to the "Guidelines for the Use of Animals in Neuroscience Research" from the Society of Neuroscience, the local Ethical Committees, and in compliance of the ARRIVE guidelines.

2.3. Animals - mice

All animal protocols with mice followed Institutional and European guidelines (86/609/EEC, 2003/65/EC European Council Directives). Experimentation with mice was also in compliance of the ARRIVE guidelines. Mice (N = 24) were housed at room temperature under a 12 h light-dark cycle. Food and water were provided *ad libitum*. Mouse genotyping was done according to previous reports (Itoh et al., 1997; Tsuchihashi et al., 2006). Six months-old male wild type C57BL/6 mice (n = 6 per group) and Nrf2-knockout littermates (n = 6 per group) were kindly provided by Dr. Antonio Cuadrado and used throughout the study.

2.4. Isolation of striatal slices and treatments

Striatal slices were collected strictly according to procedures previously described (Rojo et al., 2008). Rats and mice were decapitated and their brains were rapidly dissected out. Striata were then isolated, sectioned using a vibratome (TS1000 Leica; Heidelberg, Germany), and kept in Krebs solution until the beginning of the experiments. Five slices per probe (250-300 μm thickness) were used. Depending on the experimental design, slices were exposed to different concentrations of QUIN (25-100 μM), MK-801 (50 μM), H_2O_2 (100 μM), $FeSO_4$ (50 μM) or Krebs solution for different times of incubation in a shaking water bath at 37°C. Immediately after incubated, slices were placed on ice, collected and scheduled for measurement of different experimental parameters.

2.5. Nuclear fractions preparation

To estimate nuclear Nrf2, striatal slices were washed once with cold PBS and lysed on ice with cold buffer A (250 mM sucrose, 20 mM HEPES (pH 7.0), 0.15 mM EDTA, 0.015 mM EGTA, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM Na_3VO_4 and 1 μg/mL leupeptin plus 1% Nonidet P-40). Homogenates were centrifuged at 500 x g for 5 min. Nuclear pellets were washed in cold buffer B (10 mM HEPES (pH 8.0), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 0.1 M NaCl, 1 μg/mL leupeptin and 1 mM Na_3VO_4 plus 25% glycerol). After centrifugation at 500 x g for 5 min, nuclei were resuspended in RIPA buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4). Proteins of both fractions were resolved by SDS-PAGE and immunoblotted with the indicated antibodies (Espada et al., 2010).

2.6. Immunoblotting

Striatal slices were washed once with cold PBS and lysed on ice with lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4).

Cell lysates were pre-cleared by centrifugation and the protein concentration was quantified by Bradford's method (Bradford, 1976); then, protein extracts were resolved by SDS-PAGE using 80 μg (nuclear fraction) of protein per lane (see below the assay for separation of fractions), and transferred to Immobilon-P membranes (PVDF, Millipore). Blots were analyzed with the appropriate primary antibodies (1:1,000): anti-Nrf2, anti-Lamin B, anti-Actin, anti-Histone 1 and anti-GAPDH, all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against antioxidant enzymes (gamma-glutamylcysteine ligase-catalytic subunit (γ-GCL-C), heme oxygenase-1 (HO-1) and superoxide dismutase (SOD)) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Peroxidase-conjugated secondary antibodies (1:10,000) were used to detect the proteins of interest by an enhanced chemiluminescence kit. Secondary antibodies were goat anti-rabbit HRP from Zymed (San Francisco, CA, USA) and goat anti-mouse HRP from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.7. Lipid peroxidation

Lipid peroxidation was determined as the formation of thiobarbituric acid-reactive substances (TBARS), according to a previous report (García et al., 2008). Two hundred-μL aliquots of the homogenates obtained from striatal slices were added to 500 μL of the TBA reagent (0.75 g of TBA + 15 g of trichloroacetic acid + 2.54 mL of HCl) and incubated at 100°C for 30 min. A pink chromophore was produced in samples in direct proportion to the amount of peroxidized products. Samples were then kept on ice for 5 min and centrifuged at 3,000 x g for 15 min. The optical density from the supernatants was measured in a Thermo Spectronic Genesys 8 Spectrometer at 532 nm. Final amounts of TBARS -mostly malondialdehyde (MDA)- were calculated by interpolation of values in a constructed tetramethoxypropane standard curve, and results were calculated as nmoles of MDA per mg protein.

2.8. Statistical analysis

Results were expressed as mean values ± one S.E.M. All data were statistically analyzed using one-way analysis of variance (ANOVA) for repeated measures, followed by *post hoc* Tukey's test. All analytical procedures were performed using the scientific statistic software GraphPad Prism 5 (GraphPad Scientific, San Diego, CA, USA). Differences of $P<0.05$ were considered as statistically significant.

3. Results

3.1. QUIN induced an early increase of nuclear Nrf2 levels in rat striatal slices

First, we explored the modulation of the ~98 kDa Nrf2 form reported by Lau et al. (2012). We wanted to investigate whether this phenomenon is dependent of the presence of QUIN and if it can be modulated by tert-butylhydroquinone (tBHQ), a well-known Nrf2 modulator. For this purpose, rat striatal slices were incubated in the presence of 100 μM QUIN at 6 h (short-term effect) and/or 25 or 50 μM tBHQ. QUIN exerted a stimulatory effect on nuclear Nrf2 levels, reaching significant levels compared with control (42 % of increase; Figs. 1A and 1B). Cytoplasmic Nrf2 levels were slightly stimulated by QUIN (18 % above the control). In turn, tBHQ (25 μM) increased the nuclear and cytoplasmic Nrf2 levels *per se* (25 and 43 % above the control, respectively). Interestingly, when co-incubated with QUIN, 25 μM tBHQ stabilized Nrf2 levels to basal values, but at the 50 μM concentration, a significant stimulation in the nuclear levels of Nrf2 levels was induced by the coordinated action of these two agents (58 % above the control).

3.2. QUIN increased lipid peroxidation in a concentration-dependent manner in rat striatal slices

Since nuclear Nrf2 translocation is also known to obey pro-oxidant stimuli, in order to further know if the changes in nuclear levels of Nrf2 induced by QUIN were related to markers of oxidative cell damage, we estimated the concentration-response effect of QUIN on lipid peroxidation (Fig. 2) in rat striatal slices at 6 h of incubation. The toxin exerted a concentration-response effect on lipid peroxidation, achieving a peak with the 100 μM concentration (71 % of increase vs. control values), and this effect seems to be correlated with the increase in the levels of nuclear Nrf2 observed in Figure 1. The changes in lipid peroxidation induced by QUIN in a concentration-dependent manner clearly suggest that the pro-oxidant environment in our experiments represent a potential triggering factor for the transactivation of Nrf2.

3.3. QUIN enhanced lipid peroxidation in striatal slices of wild type (Wt) C57BL/6 mice

We then started our work with mice tissue. First, we characterized the ability of QUIN to induce oxidative damage to lipids in striatal slices of Wt mice that were incubated during 3 h in the presence of the toxin in order to establish a curve-response effect. QUIN induced a maximum peroxidative effect at 50 μM (233 % above the control). The toxin also enhanced oxidative damage to lipids at 100 μM (108 % above the control). A positive control of oxidative damage (50 μM $FeSO_4$) was included in the experiment, but the extent of lipid peroxidation induced by this condition was only moderate (Fig. 3).

3.4. QUIN augmented nuclear Nrf2 levels in an early manner in mice striatal slices

The striatal content of Nrf2 induced at different times of incubation of the slices in the presence of QUIN (50 μM) is presented in Figure 4. The time-course of Nrf2 induction by QUIN revealed a transitory and significant stimulation of this factor at short times of incubation (0.5 – 4 h), peaking at 1 h (231 % above the control), and then returning to baseline. Thus, the early and transitory increase of Nrf2 induced by QUIN followed by its partial fall could be interpreted as an early compensatory attempt of cells to counteract oxidative toxicity already in course.

3.5. QUIN induced changes in HO-1, SOD and GCL-C protein levels in striatal slices

We further investigated whether the toxin *per se* could be able to induce redox alterations characterized by increased expression of representative phase 2 antioxidant enzymes (HO-1, SOD and GCL-C) in direct correlation to its stimulatory role in the Nrf2 regulation observed in Figure 4. For this purpose, striatal slices were incubated for 3 h in the presence of QUIN, a time that was assumed to involve an early transactivation of Nrf2. QUIN (50 μM) enhanced the HO-1 levels compared to control (1.5-fold). In the case of SOD regulation, QUIN stimulated the enzyme levels by 50 % above the control. Under the same experimental conditions, QUIN induced an increase of GCL-C levels (28 %) compared with the control (Fig. 5).

3.6. MK-801 inhibited the lipid peroxidation induced by QUIN in striatal slices of C57BL/6 Wt, but not in Nrf2-/- mice

We then initiated experiments with $Nrf2^{+/+}$ (Wt) and $Nrf2^{-/-}$ mice, also investigating the possible contribution of the NMDAr to the toxic action mediated by QUIN, either in the presence or the absence of Nrf2, through the actions of MK-801. For this purpose, striatal slices from Wt and $Nrf2^{-/-}$ mice were incubated for 3 h in the presence of QUIN (50 μM) and/or MK-801 (50 μM), and then lipid peroxidation was estimated as the levels of TBARS formation (Figs. 6A and 6B). QUIN increased lipoperoxidation in slices from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice (25 and 32 % vs. control, respectively). The positive control (H_2O_2) exerted a pro-oxidant effect in the Wt and the $Nrf2^{-/-}$ conditions. In turn, MK-801 *per se* produced no effects on lipid peroxidation in mice striatal slices (data not shown). However, when combined, QUIN + MK-801 returned the levels of lipid peroxidation induced by the toxicant alone to baseline in Wt mice (Fig. 6A), but not in the $Nrf2^{-/-}$ condition (Fig. 6B). In slices from Wt mice, QUIN also induced increased protein levels of Nrf2 and HO, and MK-801 was unable to attenuate this effect (Fig. 6C). Once again, MK-801 *per se* produced no changes in these proteins.

4. Discussion

Studying the role of the KP metabolite and reported neurotoxin QUIN in the transcription factor Nrf2 regulation in the brain is relevant because variations in the levels of QUIN are known to account for the etiological explanation of Huntington's disease (HD) and other disorders; however, evidence available on this topic remains scarce. To date, some groups have described Nrf2 regulation patterns in HD experimental models. For instance, Li and coworkers (2007) overexpressed Nrf2 in astrocytes as an antioxidant gene therapy to counteract the lesion produced by the mitochondrial complex II inhibitor malonate in the striatum. In a further study, Nrf2 target genes were up-regulated in PC12 cells expressing mutant huntingtin, possibly as a protective/compensatory mechanism (van Roon-Mom et al., 2008). In parallel, these authors discovered alterations in some genes resulting in increased oxidative stress and damage. Shortly thereafter, Stack and coworkers (2010) showed that the triterpenoids CDDO-ethyl amide and CDDO-trifluoroethyl amide were able to improve the behavioral phenotype and brain pathology in the transgenic N171-82Q mouse model of Huntington's disease. Both triterpenoids up-regulated Nrf2 and induced neuroprotective genes. In the same context, the fumaric acid ester dimethylfumarate (DMF), a drug commonly employed as a therapy for relapsing-remitting multiple sclerosis, exerted neuroprotective effects via induction of Nrf2 and detoxification pathways (Ellrichmann et al., 2011). The effects of DMF in R6/2 and YAC128 HD transgenic mice revealed a significant improvement in different physiological markers (body weight, motor impairment, striatal morphology, etc.). DMF also increased the Nrf2 immunoreactivity in neuronal subpopulations. More recently, in STHdh (Q111/Q111) striatal cells, the mHtt expression resulted in reduced activity of Nrf2, whereas the activation of the Nrf2 pathway by the oxidant tBHQ was significantly impaired (Jin et al., 2013). Altogether, these studies provide biomedical evidence supporting the concept that Nrf2 transactivation and phase 2 antioxidant up-regulation in the brain

represent a neuroprotective strategy with clinical relevance for HD therapy, although the role of KP metabolites in this regulation deserves more attention.

During the first part of this study, we demonstrated that rat striatal slices exposed to QUIN are sensitive to the toxin. In contrast to our previous report (Tasset et al., 2010), the levels of Nrf2 were increased in response to QUIN. We assume that this event represents a compensatory action of the biological system to the toxicant, but not necessarily recruiting a major antioxidant strategy. Interestingly, also in rat striatal slices, QUIN exerted a concentration-response effect on an endpoint of oxidative damage, supporting our previous appreciation that, despite Nrf2 exhibited a tendency to increase after QUIN treatment, the pro-oxidant environment is propitious to exert oxidative cell damage beyond any effort to activate the endogenous antioxidant defense. Thus, compensatory mechanisms could involve adaptive responses of some specific cell types to oxidative modifications in an attempt to "reorganize" and "redirect" the redox activity if a toxic insult is present, albeit they could not be sufficient to counteract damage already in progress. Meanwhile, the consistent concentration-dependent response of the rat striatal slices to QUIN demonstrates the suitability of this preparation to explore the Keap-1/Nrf2/ARE axis using this toxic paradigm in this species.

We also explored Nrf2-associated responses in mice striatal slices. In this preparation, QUIN evoked a robust peroxidative action at 50 μM concentration, with a more moderate peroxidative action at 100 μM concentration. This result clearly establishes differences in the susceptibility of striatal tissue of these two different species if considering that concentrations typically employed by some groups (including us) for the induction of neurotoxic events in rat striatal slices are between 0.1 and 1 mM (Pérez-De La Cruz et al., 2010; Colle et al., 2012). The effect of QUIN on the striatal levels of Nrf2 in this preparation was stimulated at all times tested and reached a peak at 60 min, pointing out the differential responses to this toxic paradigm depending on the explored

species. Nonetheless, this stimulation of Nrf2 by QUIN was likely to be sufficient to induce increased levels of phase 2 enzymes, including HO-1, SOD, and in a less extent, γ-GCL-C. Noteworthy, the magnitude of HO-1 protein levels clearly corresponded to the extent of Nrf2 induction by QUIN. Although Nrf2 is known to regulate the expression of HO-1 and SOD (Tufecki et al., 2011; Zhang et al., 2012), it is also known that HO-1 can be regulated by other factors, including the transcriptional activators NF-κB and AP-1, and the transcription repressors BTB and CNC homologue 1 (Bach1), all controlling the inducible HO-1 gene expression (Paine et al., 2010). In turn, SOD and γ-GCL-C levels induced by QUIN were clearly more moderate, but still in line with an early and transitory induction of Nrf2.

Regarding the experiments in striatal slices of Wt and $Nrf2^{-/-}$ mice, our findings revealed interesting features of the early oxidative pattern exerted by QUIN: while moderate differences in the QUIN-induced lipid peroxidation were found among $Nrf2^{+/+}$ and $Nrf2^{-/-}$ slices, the $Nrf2^{-/-}$, but not the $Nrf2^{+/+}$ slices, were completely insensitive to MK-801, suggesting that Nrf2 regulation might also be subordinated to NMDAr. Altogether, these findings suggest that Nrf2 could be more relevant as a concurrent physiological redox sensor and potentially concurrent mechanism of resistance against oxidative damage than a physiologically relevant first line of defense, where NMDAr might, somehow, regulate Nrf2 expression and function. These findings also support the concept that most of the actions exerted by QUIN are mediated by NMDAr (Stone et al., 2003).

Attenuating or mitigating the deleterious actions of QUIN and other toxic KP metabolites emerges as a priority for basic and clinical research because this pathway has gain attention as a potential source of metabolites driving redox and neurochemical alterations in the CNS when the metabolic pathway is altered (Moroni, 1999; Chen and Guillemin, 2009; Zádori et al., 2012). The use of antioxidants with different profiles along several studies using QUIN as the paradigm of choice has demonstrated that its toxic effects can be reduced or even blocked by these agents

(reviewed by Pérez-De La Cruz et al., 2012). While an initial assumption on these findings has suggest that these agents are mostly acting as direct ROS scavengers, our actual perspective is changing to consider an additional, promising and not excluding mechanism: transcriptional Nrf2 regulation. Of note, one of the agents that have been successfully tested against QUIN toxicity is S-allyl cysteine (SAC), an antioxidant compound obtained from the aged garlic extract (Pérez-Severiano et al., 2004). SAC was shown to prevent the QUIN-induced oxidative damage and neurotoxicity, suggesting that oxidative stress is a major component of its toxic pattern. Whether SAC or other antioxidants already tested in the QUIN model can modulate the Nrf2 system to contribute to neuroprotection is a question to explore in further investigations. In addition, another well-known Nrf2 inducer, curcumin, has been recently tested against several markers of the toxic model induced by QUIN in the rat striatum (Carmona-Ramírez et al., 2013), thereby supporting the concept that Nrf2 modulation can constitute a key tool for the mitigation of the noxious actions of QUIN if properly stimulated, and a tool for consideration in the design of therapeutic strategies for those central and peripheral disorders exhibiting alterations in the KP metabolism.

Concluding remarks

Evidence presented herein suggests that QUIN can exert early redox modifications involving alterations in the Nrf2 modulation. These alterations depend on the time of exposure, the tested concentrations, and the extent of oxidative damage accompanying Nrf2 changes. In turn, these modifications can recruit the up-regulation of phase 2 enzymes, although this effect could merely represent a compensatory response induced by QUIN to the toxic events already in course. Two additional relevant points of this study are: 1) The moderate contribution of the $Nrf2^{-/-}$ condition to stimulate oxidative damage, albeit the striatal slices from these mutant animals were more sensitive to the toxic actions of QUIN; and 2) the possible contribution of NMDAr to the modulation of the oxidative stress and the Nrf2-mediated responses, as well as its active role in the

$Nrf2^{+/+}$, but not in the $Nrf2^{-/-}$ condition. This first approach opens more questions than unequivocal conclusions, but it serves as a platform to initiate more detailed studies on the effects of QUIN on the Nrf2 transcription factor, and its potential role as a therapeutic tool to reduce neurodegenerative events involving oxidative damage. A schematic representation of the mechanisms likely occurring in this study is summarized in Figure 7.

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Conflict of interest declaration

The authors declare no conflict of interest.

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Figure captions

Figure 1. Effects of QUIN (100 μM) and tBHQ (25 and 50 μM) on nuclear and cytoplasmic Nrf2 levels in rat striatal slices. In **A**, immunoblot for nuclear Nrf2 after 6 h of incubation with QUIN and/or tBHQ. In **B**, densitometric analysis of immunoblot from **A**. In **B**, data are expressed as mean values ± S.E.M. of n = 3 experiments per group. One way ANOVA followed by *post hoc* Tukey's test for multiple comparisons. Asterisks denote statistically significant differences (*P<0.05 and ***P<0.01) vs. control.

Figure 2. Concentration-response effect of QUIN on lipid peroxidation in rat striatal slices. Lipid peroxidation was estimated at 6 h of incubation with increasing concentrations of QUIN (25-100 μM). Data are expressed as mean values ± S.E.M. of n = 6 experiments per group. One way ANOVA followed by *post hoc* Tukey's test for comparisons against the control. Asterisks denote statistically significant differences (*P<0.05) vs. control.

Figure 3. Concentration-response effects of QUIN on lipid peroxidation in striatal slices from Wt C57BL/6 mice. Levels of lipid peroxidation generated by QUIN (25-100 μ) were compared against a positive control induced by 50 μM $FeSO_4$. Lipid peroxidation is expressed as the percent of TBARS formation vs. control line. Bars correspond to mean values ± S.E.M. of n = 6 experiments per group. One way ANOVA followed by *post hoc* Tukey's test for comparisons among treatments. Asterisks denote statistically significant differences (*P<0.05 and **P<0.01) vs. control.

Figure 4. Time-course of Nrf2 induction by QUIN (50 μM) in striatal slices of Wt C57Bl/6 mice. In **A**, immunoblots for nuclear Nrf2 levels. In **B**, densitometric analysis of immunoblots from **A**. Also in **B**, data are expressed as mean values ± S.E.M. of n = 6 experiments per group. One way ANOVA followed by *post hoc* Tukey's test for comparisons among treatments. Asterisks denote statistically significant differences (*P<0.05) vs. control (0).

Figure 5. Effect of QUIN (50 μM) on the HO-1, SOD and γ-GCL-C protein levels at 3 h of incubation in striatal slices of Wt C57BL/6 mice. Protein levels (**A** and **B**) are expressed as the ratio of protein/β-actin. Data are expressed as mean values ± S.E.M. of n = 6 experiments per group. One way ANOVA followed by *post hoc* Tukey's test for comparisons among treatments. Asterisks denote statistically significant differences (*P<0.05) vs. control.

Figure 6. Effect of the NMDAr antagonist MK-801 (50 μM) on the QUIN (50 μM)-induced lipid peroxidation in striatal slices of Wt (**A**) and $Nrf2^{-/-}$ (**B**) C57BL/6 mice at 3 h of incubation. Levels of lipid peroxidation were compared against a positive control (100 μM H_2O_2). Lipid peroxidation is expressed as the percent of TBARS formation vs. control. In **C**, the effects of QUIN and/or MK-801 on the Nrf2 and HO-1 protein levels. Data are expressed as mean ± S.E.M. of n = 6 experiments per group. One way ANOVA followed by *post hoc* Tukey's test for comparisons among treatments. Asterisks denote statistically significant differences (*P<0.05) vs. control.

Figure 7. Schematic representation of the hypothetical mechanisms involved in the Nrf2 regulation by QUIN in the striatum of rodents. QUIN, an NMDAr agonist, is acting mostly through NMDAr overactivation, but also through direct stimulation of ROS formation, thereby accounting for extracellular oxidative damage. Increased levels of intracellular Ca^{2+} induced by

opening of the NMDAr-channel complex will be responsible for persistent activation of Ca^{2+}-dependent enzymes, including proteases, phospholipases, ATPases and neuronal nitric oxide synthase (NOS). These events will create a scenario of exacerbated intracellular ROS/RNS formation, which will contribute to an altered redox status and might be responsible for an early compensatory Nrf2 nuclear translocation after its dissociation from Keap-1. This effect, however, could not be enough to induce Nrf2 transactivation in terms of a lasting stimulation of phase two enzyme synthesis for cell defense, thereby surrendering to oxidative cell damage and death.

Figure 1

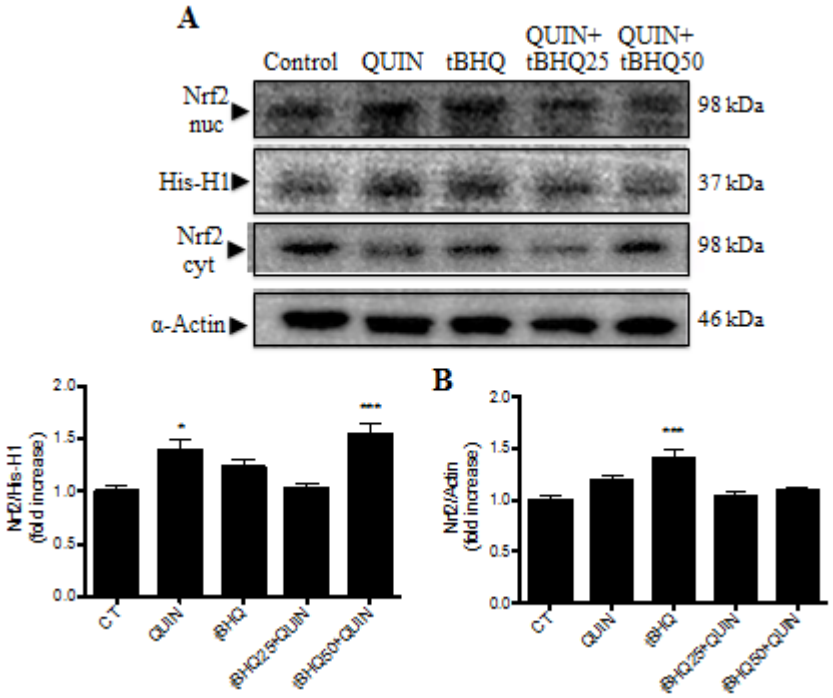
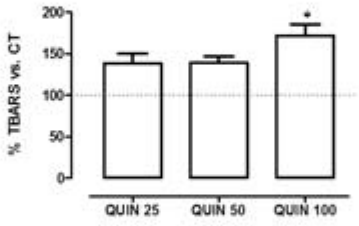


Figure 2



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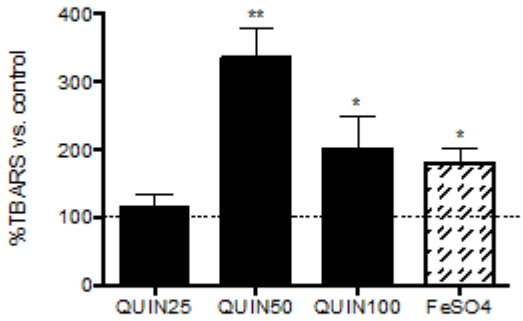


Figure 4

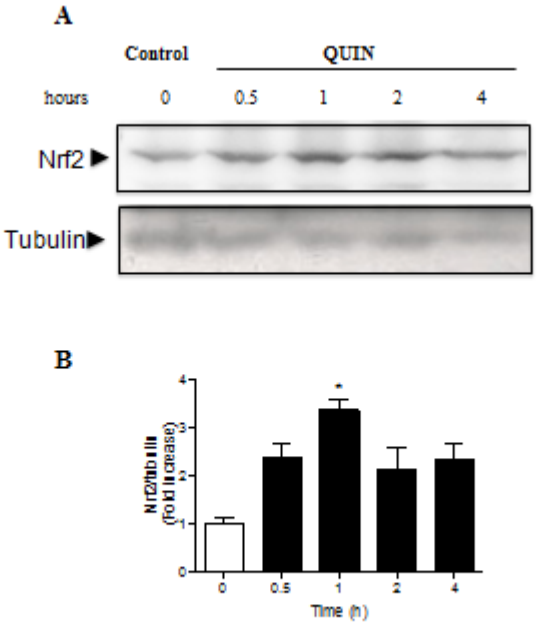


Figure 5

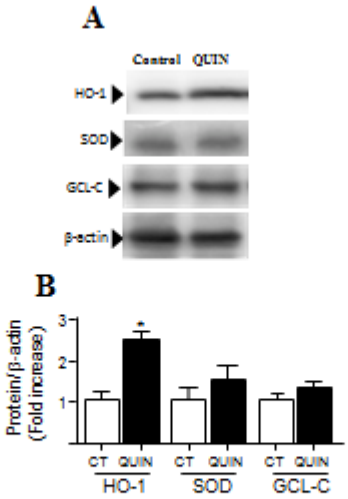


Figure 6

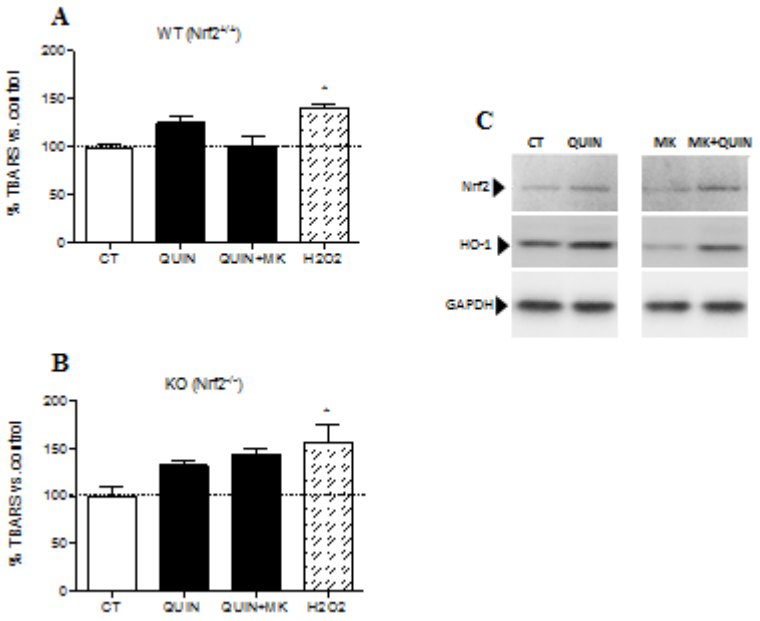
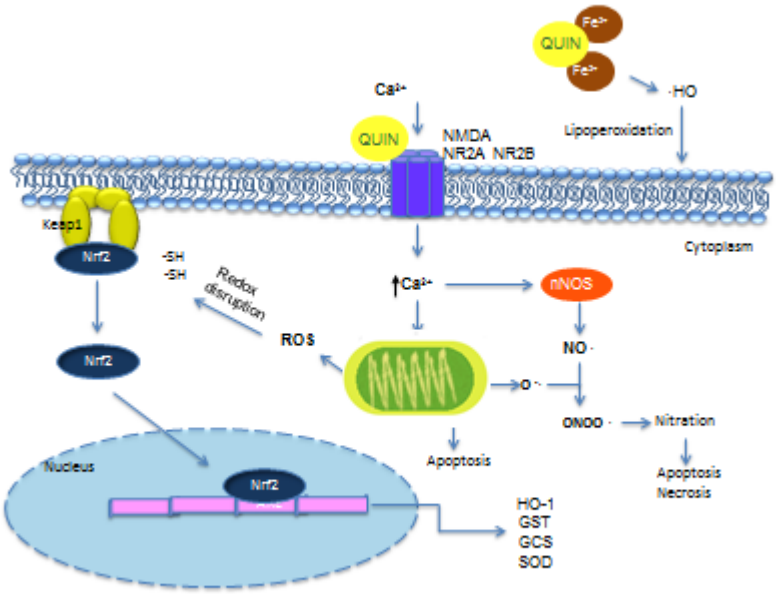
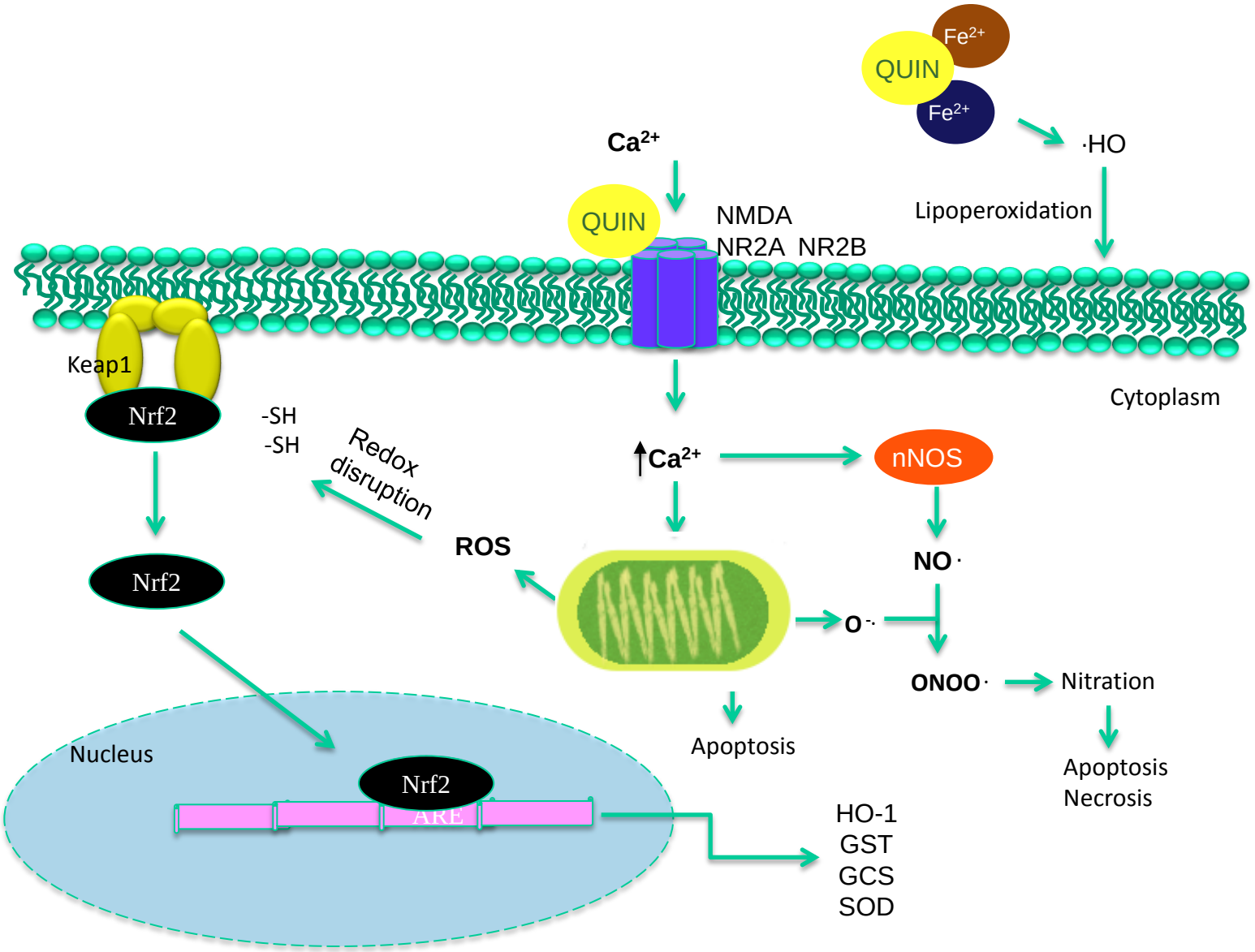


Figure 7



Graphical Abstract (for review)



Highlights

- Quinolinate increased striatal Nrf2 nuclear levels
- Quinolinate enhanced lipid peroxidation in rodent slices
- Quinolinate stimulated antioxidant enzymes expression
- Nrf2 is involved in quinolinate toxicity