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# Iron regulatory proteins increase neuronal vulnerability to hydrogen peroxide

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ABSTRACT

Iron regulatory protein (IRP)-1 and IRP2 inhibit ferritin synthesis by binding to an iron responsive element in the 5′-untranslated region of its mRNA. The present study tested the hypothesis that neurons lacking these proteins would be resistant to hydrogen peroxide ($H_2O_2$) toxicity. Wild-type cortical cultures treated with 100–300 μM $H_2O_2$ sustained widespread neuronal death, as measured by lactate dehydrogenase assay, and a significant increase in malondialdehyde. Both endpoints were reduced by over 85% in IRP2 knockout cultures. IRP1 gene deletion had a weaker and variable effect, with approximately 20% reduction in cell death at 300 μM $H_2O_2$. Ferritin expression after $H_2O_2$ treatment was increased 1.9- and 6.7-fold in IRP1 and IRP2 knockout cultures, respectively, compared with wild-type. These results suggest that iron regulatory proteins, particularly IRP2, increase neuronal vulnerability to oxidative injury. Therapies targeting IRP2 binding to ferritin mRNA may attenuate neuronal loss due to oxidative stress.


Experimental and clinical observations suggest that redox-active iron contributes to neuronal death associated with stroke, CNS trauma, and several neurodegenerative diseases [1–4]. Cells detoxify iron primarily by sequestering it in ferritin, an inducible, 24-mer heteropolymer that has the capacity to store up to 4000 ferric iron atoms in its mineral core [5]. In the CNS, ferritin is increased after ischemic and hemorrhagic stroke [6,7], traumatic injury [8], and with normal aging [9]. However, evidence to date suggests that after an acute injury this increase is delayed for at least 24 h [6–8]. Furthermore, in the substantia nigra of Parkinsonian patients, minimal or no increase has been observed despite significant iron accumulation [9–11]. These observations are consistent with the hypothesis that inadequate ferritin may contribute to the vulnerability of CNS cells to some oxidative injuries.

Ferritin synthesis is subject to both transcriptional and translational regulation, but the latter predominates in coordinating the cellular response to fluctuating levels of chelatable iron [12]. Ferritin translation is inhibited by two iron-sensing proteins, iron regulatory protein (IRP)-1 and IRP2, which bind to an iron responsive element (IRE) in the 5′-untranslated region of both H- and L-ferritin mRNA when cell iron levels are low. Although both proteins tend to detach in iron-replete cells, IRP binding analysis suggests that some ferritin mRNA likely remains inhibited even in the presence of high iron levels [13]. Pharmacologic targeting of IRP binding may therefore further increase ferritin expression, decreasing the labile iron pool and consequent oxidative stress.

A selective, high-affinity, nontoxic antagonist of IRP binding to ferritin IRE has not yet been identified. However, the detailed information that is available about the secondary and tertiary structures of the ferritin IRE would facilitate the rational design of such an antagonist if a therapeutic effect seemed likely [14]. In order to investigate the potential of this approach, we have established colonies of IRP1 and IRP2 knockout mice, and have performed a series of experiments to characterize the vulnerability of knockout cells to oxidative injury. In the present study, we tested the hypothesis that IRP1 and IRP2 knockout neurons would be less vulnerable than their wild-type counterparts to the toxicity of hydrogen peroxide ($H_2O_2$), which is catalyzed by cellular iron [15].

## Materials and methods

*Mouse breeding and genotyping.* Breeding pairs of IRP1 and IRP2 knockout mice (C57BL6/129 strain [16]) were kindly provided by Rouault and colleagues [21]. All mice used for breeding and culture preparation were the first or second generation offspring of mice heterozygous for the IRP1 or IRP2 knockout gene. In order to minimize variability due to genetic background, results from IRP1 and IRP2 knockout cultures were compared with those from wild-type cultures prepared from descendants of IRP1 or IRP2 heterozygous knockout mice, respectively. Mice were genotyped by PCR, using genomic DNA extracted from tail clippings and the following primers:

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IRP1 wild-type: forward: 5′-GAG AGG TCC TCC CTC TTG CT-3′; reverse: 5′-CCA CTC TCT CGA AGG TAG TAG-3′.
IRP2 wild-type forward: 5′-TGT TCC TGT CAG TCC TCG TG-3′; reverse: 5′-GGC CAG ACT GGT CTT CAG AG-3′.
NeoR insert forward: 5′-GAT CTC CTG TCA TCT CAC CT-3′; reverse: 5′-TCA GAA GAA CTC GTC AAG AA-3′.

NeoR insert primers were the same for IRP1 and IRP2 knockouts. Absence of wild-type IRP gene expression in mice identified as homozygous knockouts by this method was confirmed by RT-PCR, using the following primer pairs:

IRP1 forward: 5′-CCC AAA AGA CCT CAG GAC AA-3′; reverse: 5′-CCA CTC TCT CGA AGG TAG TAG-3′.
IRP2: forward: 5′-TCC GAC AGA TCT CAC AGT GG-3′; reverse: 5′-TGA GTT CCG GCT TAG CTC TC-3′.

*Cell cultures.* Cultures containing both neurons and glial cells were prepared from fetal mice (gestational age 15–17 days), as previously described in detail [17]. Plating medium contained Eagle's minimal essential medium (MEM, Gibco/Invitrogen, Grand Island, NY, USA, Product No. 11430), 5% heat inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 5% heat inactivated equine serum (Hyclone), glutamine (2 mM), and glucose (23 mM). Cultures were incubated at 37 °C in 5% $CO_2$. Two-thirds of the culture medium was exchanged twice weekly until 11 days in vitro and daily thereafter. Feeding medium was similar to plating medium, except that it contained 10% equine serum and no fetal bovine serum.

*Hydrogen peroxide exposure.* Experiments were conducted at 11–16 days in vitro. At this time interval, neurons are easily distinguished from glial cells in this culture system by their phase-bright cell bodies and extensive network of processes. Cultures were washed free of serum and placed into MEM containing 10 mM glucose (MEM10). $H_2O_2$ was diluted from a 3% stock solution immediately prior to its addition to cultures, which were then rapidly returned to the incubator for 24 h.

*Assessment of injury.* Cell death was quantified by assaying the activity of lactate dehydrogenase (LDH) released into the culture medium, which is an accurate marker of both necrotic and apoptotic death in these cultures [18,19]. Details of this method have previously been published [17,18]. Since culture LDH activity varies somewhat with its density and age, LDH values were normalized to the mean value in sister cultures treated concomitantly with 300 μM NMDA (=100), which releases essentially all neuronal LDH without injuring astrocytes [18]. The mean LDH activity in sister cultures subjected to medium exchange (sham-wash) only was subtracted from all values to quantify the signal specific to $H_2O_2$ toxicity.

Lipid peroxidation was quantified by malondialdehyde (MDA) assay. Cells were harvested in 5% trichloroacetic acid, sonicated, and centrifuged. The supernatant was collected, and a thiobarbituric acid/acetate solution was added to a final concentration of 0.3% thiobarbituric acid, 7.5% acetic acid (pH 3.5). Samples were heated in a boiling water bath for 15 min, and then were cooled to room temperature. Fluorescence was quantified using excitation wavelength 515 nm, emission wavelength 553 nm, and slit width 5. MDA concentration was extrapolated from fluorescence of control samples containing serial MDA dilutions (Sigma–Aldrich Cat. # T-1642). Protein concentration of a suspension of the pellet was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL); MDA was expressed as nanomoles/mg protein.

*Immunoblotting.* After washing with 1 ml MEM10, cells were lysed in 100 μl buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.1% Triton X-100. The lysate was collected, sonicated on ice, and centrifuged. Protein concentration of the supernatant was determined by the BCA method. Samples (30 μg total protein for ferritin and 15 μg for heme oxygenase (HO)-1) were then diluted with 4× loading buffer (Tris–Cl 240 mmol/L, β-mercaptoethanol 20%, sodium dodecyl sulfate 8%, glycerol 40%, and bromophenol blue 0.2%) and heated to 95 °C in a water bath for 3–5 min. Proteins were separated on 12% SDS–PAGE gels (Ready Gel, Bio-Rad, Hercules, CA), and were then transferred to a polyvinylidene difluoride (PVDF) transfer membrane (Immobilon-P, Millipore, Billerica, MA). After washing, nonspecific sites were blocked with 5% nonfat dry milk in a buffer containing 20 mM Tris, 500 mM NaCl, and 0.1% Tween 20 (pH 7.5) for 1 h at room temperature. Membranes were exposed to the following primary antibodies overnight at 4 °C with continuous gentle shaking: (1) rabbit anti-horse spleen ferritin, Sigma–Aldrich, Product No. F5762, 1:250; (2) rabbit anti-HO-1, Assay Designs, Ann Arbor, MI, Product No. SPA-895, 1:5000; (3) rabbit anti-actin (gel loading control), Sigma–Aldrich Product No. A2066. 1:400. Membranes were then washed and treated with secondary antibody (Pierce goat anti-rabbit IgG-HRP, Product # 1858415 1:3000) at room temperature for 1 h. Immunoreactive proteins were visualized using Super Signal West Femto Reagent (Pierce, Rockford, IL) and Kodak Gel Logic 2200. Ferritin and HO-1

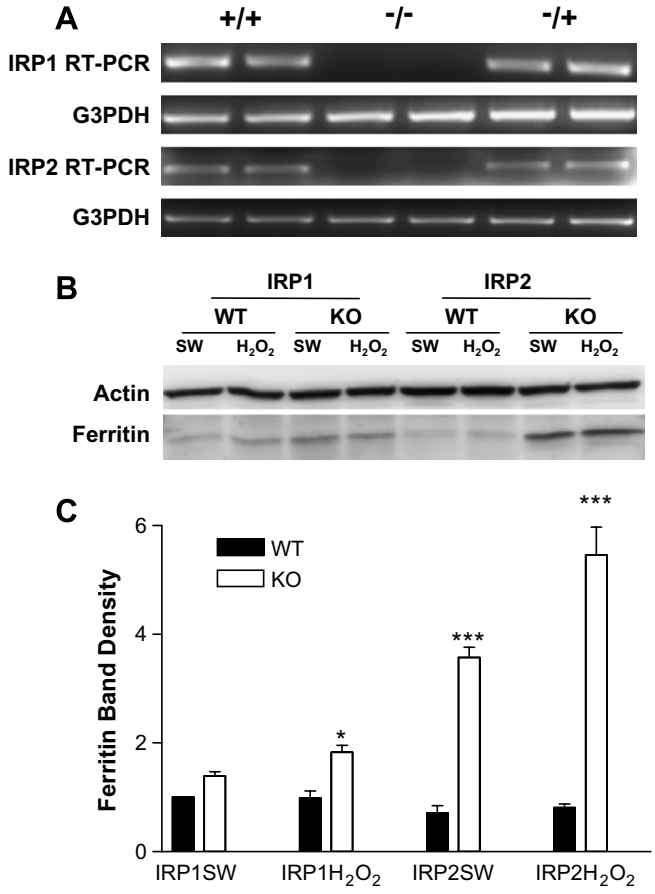


**Fig. 1.** (A) RT-PCR using wild-type IRP1 or IRP2 primers, demonstrating lack of gene expression in mice identified by genotyping protocol as knockouts (−/−) and expression in mice identified as homozygous (+/+) or heterozygous (+/−) wild-type. G3PDH: control glyceraldehyde 3-phosphate dehydrogenase primers. (B) Representative immunoblot from IRP1 or IRP2 wild-type (WT) or knockout (KO) cultures, 24 h after treatment with 300 μM $H_2O_2$ or medium exchange only (sham-wash, SW), stained with antibody to horse spleen ferritin or actin (gel loading control). (C) Mean ferritin band density ± SEM, scaled to that in IRP1 WT sham-washed cultures (=1.0). $^{*}P<0.05$, $^{***}P<0.001$ vs. density in corresponding wild-type cultures, $n=5$/condition, Bonferroni multiple comparisons test.