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A key role for heme oxygenase-1 in nitric oxide resistance in murine motor neurons and glia

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Abstract

Nitric oxide is utilized at low levels for intercellular signaling, and at high levels as a cytotoxic weapon during inflammation. Cellular NO resistance can be increased by prior exposure to sublethal NO levels to induce defense gene expression (adaptive NO resistance), which has been correlated with increased expression of heme oxygenase-1 (HO1) and was blocked by a heme oxygenase inhibitor. However, the possibility remained that other activities were affected by the inhibitor. To address this question, we conducted a genetic study of the HO1 role. We show here that primary cultures of spinal motor neurons and glia from homozygous HO1-null mice are strikingly more sensitive to NO cytotoxicity than are cells expressing HO1. Following an exposure to NO, the HO1-deficient cells were much more prone to apoptosis than were HO1-expressing cells with either one or two copies of a functional HO1 gene. These results confirm the in vivo role of HO1 as a front-line defense against NO toxicity in neuronal cells.

Keywords: Peroxynitrite; Glial cells; Mouse genetics; Adaptive NO resistance; Carbon monoxide

The free radical nitric oxide is actively synthesized by many mammalian cells and utilized for a variety of functions. At low levels, NO mediates intercellular signaling in vascular relaxation, neurotransmission [1–5], and cellular differentiation [6–9]. At high levels NO causes toxicity [10,11], and thus is employed as a weapon in the immune system [12]. NO also plays a role in neuronal injury [10,13,14] and possibly in the pathology of various neurodegenerative diseases, such as Parkinson disease, amyotrophic lateral sclerosis, and Alzheimer's disease [15–19].

Nitric oxide damages all the critical biological macromolecules. NO injures DNA by forming various lesions [11] and is a mutagen that may contribute to carcinogenesis [20]. NO and NO-dependent products such as peroxynitrite ($ONOO^-$; formed from NO reacting with superoxide) also damage lipids and proteins [19,21]. NO specifically nitrosylates protein heme-centers, resulting in mitochondrial dysfunction and the generation of additional cytotoxic free radicals [10,22]. NO-dependent nitration of tyrosine residues disrupts protein structure and function, as found in the massive neurofilament derangement in the motor neurons of patients with amyotropic lateral sclerosis [18,19].

Induction of the heme-metabolizing enzyme heme oxygenase-1 (HO1) is linked to cellular resistance to heavy metals, oxidants [23–27], and NO [28]. HO1 metabolizes the heme freed from proteins during normal turnover

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and following damage or stress. Heme oxygenase activity generates the end products CO [23,25,29], bilirubin, and iron [30]. Bilirubin has been described as a physiological antioxidant, and CO may mediate cellular protective responses [25,29,31]. In addition, HO1 diminishes the potential for oxidative damage by eliminating heme, which can participate in damaging oxidative reactions [32]. HO1 was also proposed to protect cells indirectly by releasing iron, which can cause the upregulation of ferritin synthesis and, ultimately, increased iron sequestration [33]. As a harbinger of oxidative stress related to disease states, increased HO1 levels are found in the brains of Alzheimer's disease patients [34,35]. Increased HO1 expression is also associated with spinal injury and correlates with other measures of free radical damage [36,37].

Resistance to toxic NO levels can be induced by exposure of cells to sublethal levels of NO in both immortalized mouse motor neurons and in primary motor neurons from rat spinal cord explants [28]. This adaptive resistance in the motor neuron cell line (NSC34) has been correlated with the increased expression of HO1 [28]. A key observation showed that the general heme oxygenase inhibitor Sn-protoporphyrin-IX eliminated the adaptive NO resistance [28], consistent with a role for HO1 [38]. However, this inhibitor would also act on other heme oxygenase enzymes (HO2 and HO3), and possibly on other heme-related activities such as the NO-responsive "soluble" guanylate cyclase. Thus, establishing the role of HO1 in resistance to NO requires a critical genetic experiment. Here we provide such a test, which demonstrates the profound role played specifically by HO1 in neuronal NO resistance.

Materials and methods

Isolation and maintenance of primary motor neurons. Mice (129sv × Balb-c) heterozygous for an HO1 knockout allele were mated [26] and, at day 15 of pregnancy, the dams were sacrificed and the embryos were harvested (Stage E15). Motor neuron-enriched cultures were made from each embryo (Fig. 1A) using the method of Schnaar and Schaffner [39] as previously described [28]. The procedures were carried out in Eagle's medium supplemented with D-glucose, L-glutamine, and 5% fetal bovine serum. Briefly, the meninges layers were dissected away to decrease the number of fibroblasts in the culture, and the dorsal roots were removed to eliminate the sensory neuron population. The ventral spinal cord was minced, and the cells were disaggregated [39]. The cells from minced spinal cord explants were separated by gentle centrifugation into fractions that were enriched for either motor neurons or glial cells. This procedure was repeated several times for further enrichment of motor neuronal cell types in one fraction, which was layered onto a bed of glial cells. With this culturing system the top layer is predominantly comprised of cells with a neuronal morphology, while the lower layer is comprised of predominantly glial cell types. The cells were plated on flasks coated with a mixture of laminin and poly-D-lysine at a density of 2×10^6 cells per flask and cultured for 3 days at 37 °C under 5% CO_2 in minimal Eagle's medium supplemented with D-glucose, D-glutamine, and 5% fetal bovine serum. In this system, the glial cells act as a "feeder layer"for the limited duration (⩽48 h) of the experiments [40,41].

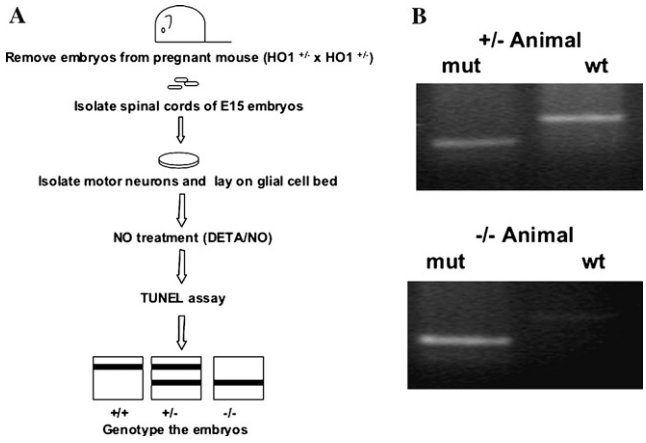

Fig. 1. Preparation and analysis of mouse embryo motor neurons. (A) Schematic of the experimental procedure used. (B) Distinct PCR products generated for the wild-type HO1 gene and the knockout allele. "mut" indicates PCR with primers specific for the mutated allele; "wt" indicates PCR with primers specific for the intact *HO1* allele.

Genotyping of embryos. PCR for genotyping was performed on DNA samples purified from each embryo (Fig. 1A) as described [42] using primers specific for the disrupted HO1 gene and the wild-type gene (Fig. 1B). The primers for detecting the wild-type HO1 gene were 5′-GGTGACAGAAGAGGCTAAG-3′ and 5′-CTGTAACTCCAC CTCCAAC-3′. For the HO1-knockout allele, the primers were 5′-TCTTGACGAGTTCTTCTGAG-3′ and 5′-ACGAAGTGACGCC ATCTGT-3′. The PCR products were resolved on 1–2% agarose gels and visualized by staining with ethidium bromide.

Growth and maintenance of NSC34 cells. NSC34 cells [43] were early passage stocks obtained from the N.R. Cashman laboratory (University of Toronto). The NSC34 line was formed by a fusion of mouse primary spinal cord neurons with mouse neuroblastoma cells. NSC34 cells differentiate in culture to cells with all the properties of primary motor neurons [3,13,27,35]. The cells were grown to 40% confluence in a humidified 5% CO_2 environment in plastic T25 flasks in Dulbecco's modified Eagle's medium (Mediatech; Logan, UT) without sodium pyruvate and supplemented with 10% heat-inactivated, fetal bovine serum.

NO treatment for NSC34 cells and primary cells. NSC34 and primary neuronal cells were exposed to a range of NO donor concentrations to determine the cut-off for sublethal exposure (see Results). Our previous work used pure NO gas [28], while the experiments described here employed diazenium-diolate NO-donor compounds [44]. These agents allowed us to expose cells to varying levels of NO, thanks to their different decay rates: for (z)-1-[2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate] (DETA-NONOate) a half-life ∼16 h, and for spermine-NONOate, a half-life of ∼40 min. The level of NO produced in the medium by decay of these compounds was measured in separate experiments (L. McLaughlin, V. Leautaud and B.D., unpublished data) using an NO-sensitive electrode (ISO-NO MarkII; World Precision Instruments, Sarasota, FL). The cells were pretreated for 1.5 h with 0.30 mM DETA-NONOate (which increased the NO concentration to 2 μM over the first 45 min, a steady-state level that was maintained over the remaining 45 min of the treatment), incubated for 2 h to allow gene expression, and then exposed for 2 h to the cytotoxic NO challenge of 0.50 mM spermine-NONOate (a steady-state level of 4.6 μM is reached within 5 min and maintained throughout the treatment) [28]. For controls, medium containing spent NO donor (after incubation for >10 half-lives) was used; these controls showed that the spent compounds had no detectable toxicity (data not shown).

TUNEL assay for apoptosis. A commercial kit (Apoptosis Detection System; Promega) was used to label the fragmented DNA in

apoptotic nuclei by means of the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) reaction. Detection employed a fluorescent label as described by the manufacturer. The cells were counterstained with propidium iodide. The non-apoptotic cells stained orange or red, while apoptotic cells stained bright yellow when observed by fluorescence microscopy. Grayscale images are provided here to depict the staining with a suitable contrast. Cells were scored as apoptotic if they exhibited a peak intensity $\geqslant$2-fold higher than the mean for untreated cells. Neurons were distinguished from the glial cell beds by their location in a higher focal plane, by their morphology, and by the differences in intensity of various stains as described [28]. Motor neurons were scored as bright cells with a long axon, with smaller nuclei that stained in the TUNEL assay more intensely. Glial cells lay in a lower focal plane and were distinguished from the neurons by their flatter appearance and the presence of thicker projections. The glial nuclei were larger, more diffuse, and stained less intensely than the neuronal nuclei. For quantification, cells (200 per sample) were counted, and the percentage of apoptotic cells was calculated. Since there was no overt difference between the homozygous wild-type and heterozygous mice (both are HO1-proficient), the data for both were pooled and compared to the results for homozygous HO1-null mice.

For non-morphological quantification, the intensity of yellow flourescence was analyzed for each cell in each field using a Compucyte LSC2 Scanning Cytometer. Using SCION software, the intensity of all the cells of a given type was averaged for each field. The fields were averaged and the values were averaged again for independent experiments. Means and standard deviations were calculated for four independent experiments.

Results

To determine if the *HO1* gene is necessary for native NO resistance in primary neurons and glia we used HO1 "knockout" ($HO1^{-/-}$) mice [26], which express no detectable HO1 mRNA or HO1 protein. To obtain motor neurons, heterozygous $HO1^{+/-}$ mice were mated and 15-day embryos were isolated. The spinal cords were dissected out from these embryos, and mixed populations of neurons and glial cells were isolated and placed in culture (Fig. 1A). These genotypes were easily discernable in a PCR assay (Fig. 1B).

The motor neuron-glial cell cultures were exposed to varying NO doses from NO-donor compounds, incubated a further 24 h in the absence of NO, and stained with propidium iodide in combination with the TUNEL assay stain for DNA degradation associated with apoptosis [45]. This double staining procedure produces images where healthy cells have a subdued brightness, while apoptotic cells are stained brightly (Fig. 2A). The neurons from $HO1^{-/-}$ animals were significantly less resistant to NO than were cells isolated from the wild-type or heterozygous animals: 24 h after the higher-level NO challenge, neurons from $HO1^{-/-}$animals suffered 90% apoptosis, compared to HO1-proficient cells showing 33% apoptosis (Figs. 2A and B). The cell death assays were performed prior to genotyping, thereby guaranteeing a blind study. These results show that the *HO1* gene is necessary for basal NO resistance in neurons.

We next wanted to determine whether the NO-hypersensitivity observed with $HO1^{-/-}$ motor neurons extends to other cell types. When spinal cords are plated, neurons remain in the top layer while the larger,

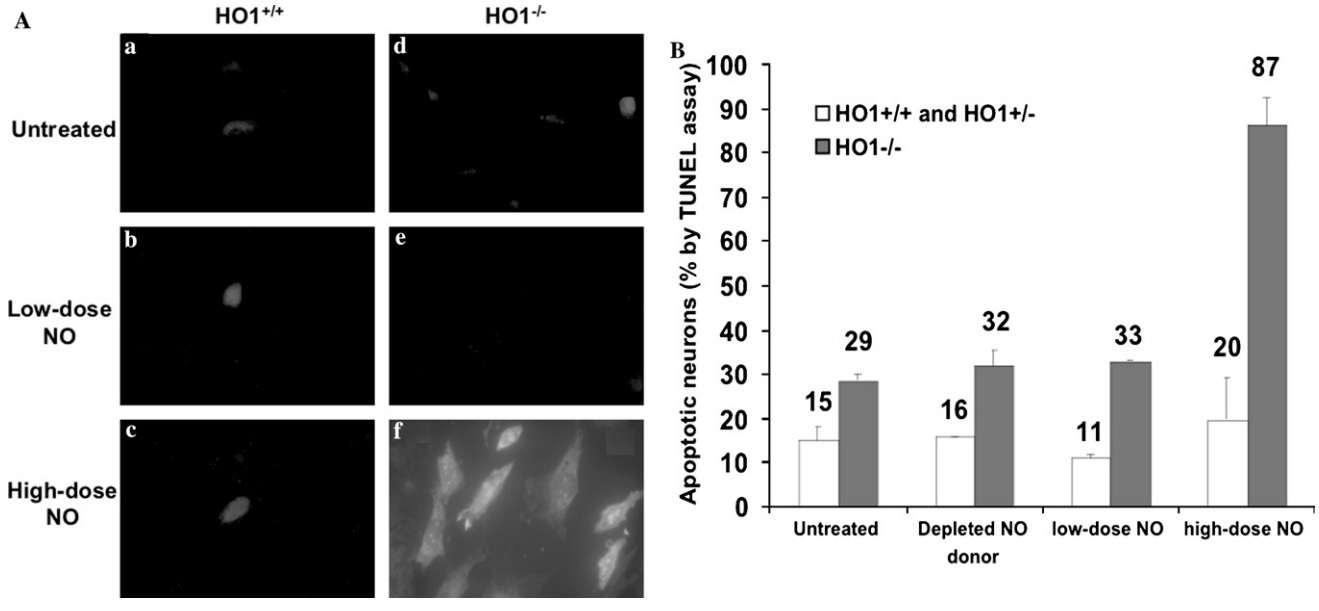

Fig. 2. TUNEL assay for NO-induced apoptosis in murine motor neurons. (A) Representative images of wild-type and $HO1^{-/-}$ neurons following NO treatment. NO treatments were for 1.5 h, with the TUNEL assay conducted 24 h later. Low-dose NO, 0.30 mM DETA-NONOate; high-dose NO, 0.50 mM spermine-NONOate. (B) Quantification of TUNEL assay data. For each point, 200 cells (as shown in A) were counted and the percentage apoptotic cells for the wild-type and heterozygous animals were pooled and compared to the $HO1^{-/-}$ null mice. Standard deviations were calculated ($n = 4$) and are indicated by error bars.

heavier glial cells comprise the bottom layer of cells. The glial cells in these cultures can be identified morphologically as having more diffuse, larger nuclei than do neurons. Glial cells also have a thicker, flatter appearance as compared to neurons, which have a smaller and more compact cell body with long projecting axons. Neurons also stain more intensely in the TUNEL assay. The microscope focus can be adjusted to capture the image in the plane containing primarily glial cells (Fig. 3A). Exposure to a high NO dose produced a significantly higher proportion of apoptosis (as monitored by the percentage of bright cells seen in the images) in the $HO1^{-/-}$ glial cells than in the HO1-containing samples (Fig. 3A). Direct cell counting confirmed the TUNEL results, which showed much greater NO sensitivity in the glia from $HO1^{-/-}$ animals than in those from wild-type or heterozygous animals (Fig. 3B). There was no significant difference in NO sensitivity between the cells from heterozygous mice compared to the wild-type (data not shown). Thus, one copy of the *HO1* gene may be sufficient to lend normal or near-normal NO resistance; further studies might reveal possible smaller effects due to gene dosage effects. When the TUNEL labeling was quantitated on a cell-by-cell basis for both neurons and glia, it was clear that cells isolated from $HO1^{-/-}$ animals displayed a more intense apoptotic signal following an NO challenge than did spinal cord cells isolated from wild-type or heterozygous animals (Fig. 3C). We also note that some apoptosis was detected even in untreated $HO1^{-/-}$ cells (both neuronal and glial), compared to $HO1^{+}$ cells (Figs. 2B and 3B). We do not know the origin of this effect, but the results suggest that HO1 also helps counteract spontaneous damage in the cultured cells, possibly related to the stress associated with handling in vitro.

Discussion

A role for HO1 in inducible resistance to NO was suggested by the NO-inducibility of the HO1 mRNA and protein in diverse cell types [24,28,34,46–48]. Supporting this hypothesis were observations that the inhibitor Sn-protoporphyrin-IX greatly sensitized rat

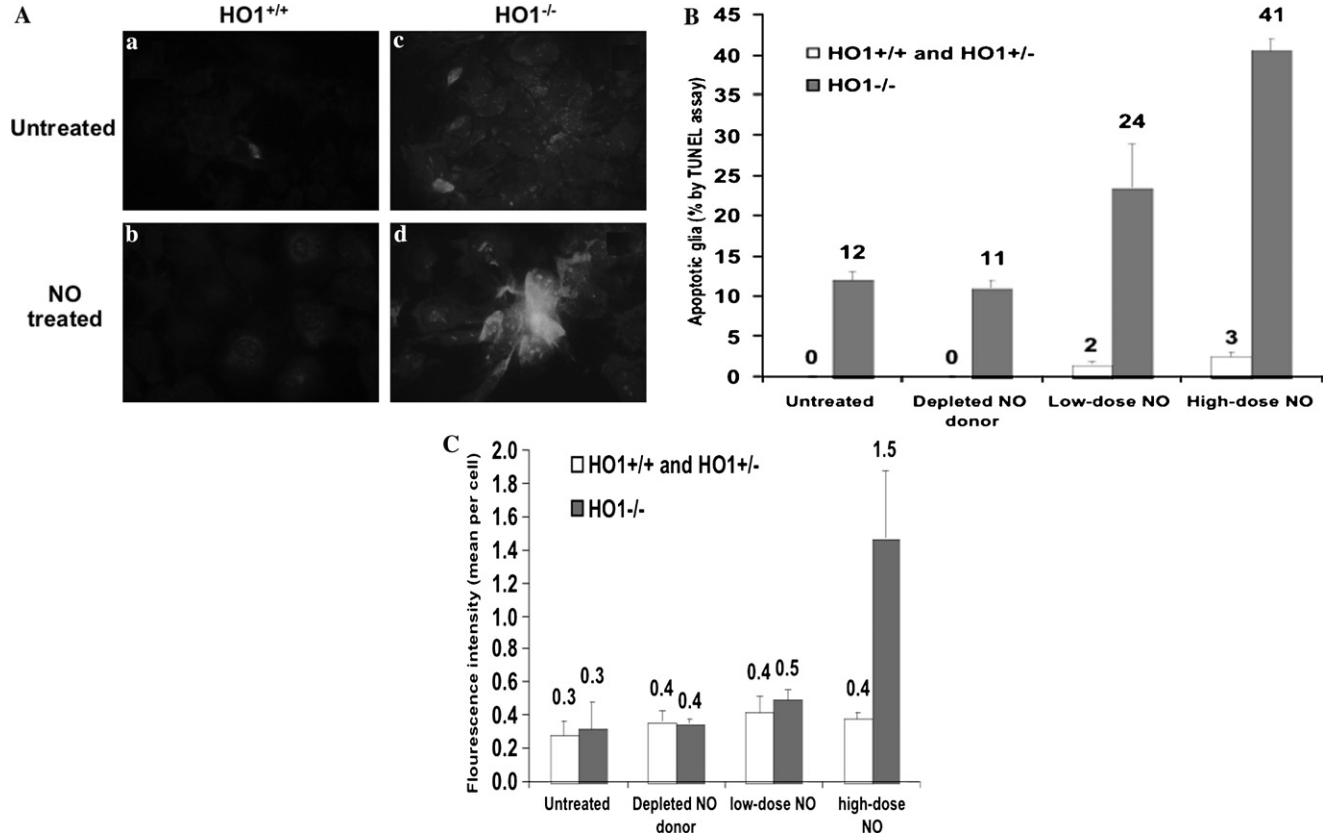

Fig. 3. TUNEL assay for NO-induced apoptosis in glial cells. (A) Representative images of wild-type and $HO1^{-/-}$ glial cells stained 24 h after control treatment or a 1.5-h challenge with 0.5 mM spermine-NONOate. Treatments as in Fig. 2. (B) Quantification of glial cell TUNEL staining. Analysis was as described for Fig. 2. (C) Cell-specific TUNEL staining. The captured images used in Fig. 2A and (A) were analyzed for the amount of fluorescence calculated for ~200 cells in each field, including both neurons and glia. The values shown are means per cell ($n = 4$) and standard deviation (indicated by error bars).

hepatocytes [46] and NSC34 neurons to an NO challenge [28]. Recently, similar experiments have correlated HO1 activity with resistance to NO and sensitization by Zn-protoporphryin-IX [32]. However, the inhibitors are heme analogs that can target all the heme oxygenase isozymes, and which might also affect other cellular functions, such as the NO-activated heme-containing guanylate cyclase [4]. Because of these uncertainties, a genetic analysis of the role of HO1 in NO resistance was clearly warranted, which we have addressed here. While the $HO1^{-/-}$ cells could be cultured as easily as those from wild-type animals, $HO1^{-/-}$ neurons and glia were both considerably more sensitive to an in vitro challenge with NO compared to HO1-expressing cells. These results provide a genetic demonstration that HO1 is a key player in NO resistance. This observation adds to the physiological functions of HO1 in resistance to free radical agents or conditions such as H_2O_2 treatment and hyperoxia [49–51], as well as its clear role in iron metabolism [52].

The specific biochemical function of HO1 in resistance to NO remains to be established. High concentrations of NO injure cells in many ways, as noted in Introduction [11,15]. If free heme (either already present or liberated from proteins during the NO exposure) is important for NO toxicity, its elimination by HO1 would help limit the damage; we are currently investigating this possibility. Another possibility is that HO1 catabolizes nitrosyl-heme, but the enzyme seems to be poorly active on this modified substrate [30]. The heme moiety of HO1 itself is subjected to nitrosylation, which inhibits the enzyme [53]. Thus, an alternative hypothesis is that new HO1 synthesis is required to offset the inhibitory effects of NO on the enzyme.

The most obvious candidates for a role in NO resistance are the products of the heme oxygenase reaction: biliverdin, iron, and carbon monoxide. Biliverdin is reduced to bilirubin, which can function as an antioxidant [54]. While a direct reaction with NO has not been documented, it is possible that bilirubin could react with a toxic NO by-product such as peroxynitrite. However, we have found that addition of bilirubin does not protect HeLa cells against NO cytotoxicity, although the resistance to H_2O_2 is increased by this treatment (T. Reiter and B. Demple, submitted). Thus, an important role for bilirubin in NO resistance seems unlikely.

Disruption of non-heme iron centers (particularly iron–sulfur centers [55]) likely releases iron that can participate in damaging redox reactions; increased amounts of bilirubin would help quench such reactions. The iron released by HO1 has been proposed [33] to activate the synthesis of ferritin through the iron-response pathway, thereby actually decreasing the overall amount of free iron available to participate in forming oxidative damage. A contrasting observation is that HO1 seems to be important for iron efflux from cells: in $HO1^{-/-}$ cells, iron accumulation leads to cytotoxicity [52]. This specific aspect of HO1 function should now be addressed in the context of NO toxicity.

The third product of the heme oxygenase reaction, CO, has recently been proposed to mediate cytoprotection by the activation of anti-inflammatory and anti-apoptotic pathways [56]. While the amount of CO generated in NO-exposed cells is unknown, and the inhibition of heme oxygenase activity by NO is problematic [53], current experiments are addressing the possible role of CO directly.

Interestingly, even without an NO challenge, there was a significantly higher rate of apoptosis observed in the $HO1^{-/-}$ cells than HO1-proficient neurons and glia. It seems possible that these effects arise from endogenous toxicants, such as the reactive by-products of normal aerobic metabolism [57]. HO1 may be required to prevent or offset the cellular damage produced by such damaging metabolites, and HO1-deficient cells as we have characterized here provide a means of addressing this question for motor neurons and glia.

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