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ROLE AND REGULATION OF FERRITIN H IN ROTENONE-MEDIATED MITOCHONDRIAL OXIDATIVE STRESS

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Abstract

Tight regulation of intracellular iron levels in response to mitochondrial dysfunction is an important mechanism that prevents oxidative stress, thereby limiting cellular damage. Here, we describe a cytoprotective response involving transcriptional activation of the ferritin H gene in response to the mitochondrial complex I inhibitor and neurotoxic compound, rotenone. Rotenone exposure increased ferritin H mRNA and protein synthesis in NIH3T3 fibroblasts and SH-SY5Y neuroblastoma cells. Transient transfection of a ferritin H promoter-luciferase reporter into NIH3T3 cells showed that ferritin H was transcriptionally activated by rotenone through an antioxidant responsive element (ARE). Chromatin immunoprecipitation assays showed that rotenone treatment enhanced binding of Nrf2 and JunD transcription factors to the ARE. In addition, rotenone induced production of reactive oxygen species (ROS), and pretreatment with N-acetylcysteine abrogated ferritin H mRNA induction by rotenone, suggesting that this response is oxidative stress-mediated. Furthermore, reduced ferritin H expression by siRNA sensitized cells to rotenone-induced apoptosis with enhanced ROS production and annexin V positive cells. Taken together, these results suggest that ferritin H transcription is activated by rotenone via an oxidative stress-mediated pathway leading to ARE activation, and may be critically important to protect cells from mitochondrial dysfunction and oxidative stress.

Keywords

ferritin; rotenone; mitochondria; iron; oxidative stress

INTRODUCTION

Iron is a vital element mandatory for metabolic processes and the function of many enzymes, including cytochrome P450s and ribonucleotide reductase, however, a surplus of free iron leads to the formation of reactive oxygen species (ROS) via the Fenton reaction, which is potentially harmful to the cells [1,2]. Iron is also involved in the catalysis of oxidation of important cellular proteins, lipids, and DNA. Thus, excess iron may lead to an increase in the oxidative stress burden of the cell in several ways [3]. Oxidative stress has been implicated in the pathogenesis of numerous conditions, including cancer, inflammation, and neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [4-6]. Therefore, cells have intricate systems to control intracellular iron levels and to detoxify ROS.

Ferritin is a nanocage protein that functions to sequester free intracellular iron that may become toxic to cells. In vertebrates, there are two ferritin subunits, heavy and light, which coassemble

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in 24 subunit bundles forming a channel that encloses iron [7]. The heavy, or H, subunit is catalytically active, and induces oxidation of ferrous iron Fe(II) to ferric iron Fe(III) and aggregation of the oxidized iron inside the core, while the light (L) subunit does not have ferroxidase activity but may serve a structural function [2,8]. In this manner, ferritin functions to protect cells against iron-mediated toxicity [9,10]. Because of its important role, ferritin is tightly regulated at both the transcriptional and translational levels. Ferritin protein levels are controlled by a well-characterized translational repression system in response to free iron levels in the cells. The 5′ untranslated region of ferritin message contains an iron responsive element (IRE), to which the iron regulatory proteins, IRP1 and IRP2, bind in low iron concentrations, thereby blocking translation when necessary [11]. In addition to iron, ferritin levels are also altered by other stimuli. TNFα was shown to stimulate ferritin H expression, but not ferritin L, through transcriptional activation via an upstream promoter region containing an NFkB site [12,13]. Our subsequent studies revealed an antioxidant responsive element (ARE) in the far upstream region of the promoter that is necessary for the transcriptional activation of the ferritin gene in response to various oxidative stressors, including H_2O_2, tBHQ (tert-butylhydroquinone), and hemin [14-16]. A similar ARE element was identified in ferritin L gene [17]. The ARE is a highly conserved enhancer element in various phase II genes involved in detoxification or with antioxidant properties, allowing for the activation of a battery of antioxidant genes including glutathione-S-transferases, NADH quinone oxidoreductase 1, and heme oxygenase 1 under chemical and oxidative stress conditions [18]. The ferritin H ARE contains a bidirectional AP1 like and AP-1/NFE2 sequence, to which basic leucine zipper (bZip) transcription factors, including JunD and NFE2-related factor 2 (Nrf2), bind [14,16]. This facilitates activation of ferritin H gene transcription under conditions of oxidative stress.

Upregulation of ferritin in response to increased iron levels and oxidative stress may be involved in preventing the cellular damage caused by the excess iron and oxidative stress that has been observed in numerous neurodegenerative diseases, like Parkinson's disease. A large body of evidence indicates a correlation between increased oxidative stress and the incidence of Parkinson's disease (PD) [4,19]. Recent studies have shown that iron chelators can block 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrapyridine (MPTP) induced dopaminergic cell death [20]. Excess free iron may be the catalyst in the production of deleterious ROS that are responsible for damage to DNA, lipids, and proteins, ultimately leading to the deletion of dopaminergic neurons [21]. Furthermore, the insults that induce neuronal degeneration are intimately connected with oxidative stress [5]. Rotenone, a mitochondrial complex I inhibitor, induces oxidative stress; however, despite its universal effect, it leads to the specific deletion of dopaminergic neurons [22], suggesting that they are highly sensitive to oxidative stress.

We hypothesized that rotenone would evoke cellular defense mechanisms through increased expression of ferritin H via an oxidative stress-pathway. In our present study, we show that ferritin H expression was increased at the transcriptional level following rotenone exposure. We further characterized that rotenone specifically activated the ferritin H ARE, and increased the binding of the oxidative stress-responsive bZip transcription factors, JunD and Nrf2, to it. Furthermore, rotenone-mediated transcriptional activation of the ferritin H gene was oxidative stress-dependent. Finally, knocking down ferritin H expression by siRNA propagated generation of ROS and sensitized cells to rotenone-mediated apoptosis, suggesting that rotenone-induced transcriptional activation of the ferritin H gene via the ARE is cytoprotective.

MATERIALS AND METHODS

Cell culture

NIH3T3 mouse fibroblasts and SH-SY5Y human neuroblastoma cells were obtained from American Type Culture Collection. NIH3T3 cells were cultured in Dulbecco's Modified

Eagle's Medium containing 1mM sodium pyruvate, 4mM L-glutamine, and 4.5g/L glucose with 10% bovine calf serum (Hyclone), and Penicillin/Streptomycin. SH-SY5Y cells were cultured in a 1:1 mixture of Eagle's Minimum Essential Medium and F12 medium supplemented with non-essential amino acids and 10% fetal bovine serum (Mediatech). These cells were incubated at 37°C and 5%CO_2 in a humidified atmosphere. Rotenone and tert-butylhydroquinone (tBHQ) were purchased from Sigma and were dissolved in DMSO. H_2O_2 and N-acetylcysteine (NAC) were purchased from Calbiochem. H_2O_2 was diluted in sterile PBS, and NAC was reconstituted in medium and adjusted to pH 7.4 with NaOH.

Plasmids and DNA transfections

pGL3-0.22kb mouse ferritin H luciferase (mFH-Luc) was constructed by digesting pGL3-4.8kb mFH-Luc with SmaI to remove the upstream 4.6 kb and then self-ligating the remaining vector and 0.22kb promoter sequence. ARE, and double mutant ARE-Luc plasmids were obtained by blunt end ligation of the oligonucleotides into the -0.22kb mFH-Luc plasmid following oligonucleotide purification by urea denaturing polyacrylamide gel electrophoresis, Sephadex G-25 column purification, and subsequent annealing. The sequences are:

wt ARE SENSE:

5′-
CATGACAAAGCACTTTTGGAGCCCAACCCCTCCAAAGGAGCAGAATGCTGAGTCACGG-3′

wt ARE ANTISENSE:

5′-
CCGTGACTCAGCATTTCTGCTCCTTTGGAGGGGTTGGGCTCCAAAAGTGCTTTGTCATG-3′

mARE SENSE:

5′-
CA**CA**ACAAAGCACTTTTGGAGCCCAACCCCTCCAAAGGAGCAGAA**CA**CTGAGTCACGG-3′

mARE ANTISENSE:

5′-
CCGTGACTCAG**TG**TTCTGCTCCTTTGGAGGGGTTGGGCTCCAAAAGTGCTTTGT**TG**TG-3′

NIH3T3 cells were transfected with 5ug of the indicated plasmid DNA via electroporation with a BioRad GenePulser XL using NIH3T3 preset conditions, 5×10^6 cells/0.2cm cuvette. Cells were then plated into 35mm dishes (10 dishes) with 5×10^5 cells, 0.5ug DNA per dish. Following a 24 hr recovery period after electroporation, cells were treated as indicated for 24 hours, and preparation of cell lysates and luminometry were performed with luciferase assay kit (Promega).

Gel retardation and ChIP Assays

Nuclear extract preparation, binding reactions, and electrophoretic mobility shifts were previously described [23]. Chromatin immunoprecipitation (ChIP) assays were performed according to a minor modification of Upstate Biology's ChIP assay protocol as previously described [14]. A total of 1×10^6 cells/100-mm plate were treated with 1 uM rotenone for 4 hrs,

followed by formaldehyde cross-linking of chromatin and preparation of lysates. (Upstate Biology). 2 ul of the following antibodies were used for immunoprecipitation: rabbit IgG (Alpha Diagnostics), anti-Nrf2 (sc-722X), anti-JunD (sc-074X, Santa Cruz Biotechnology). Quantitative PCR was performed in the presence of 0.1uCi α-^{32}P-dCTP/reaction, while using specific primers designed to amplify a 230 bp region within the mouse ferritin H promoter that contains the ARE:

Forward:

5′GGCCCCTCTGTTCTGTACAATACTAGCTC-3′

Reverse:

5′TAACCACAAAACCACAGCCCTCCAG-3′

PCR reactions were run on an 8% acrylamide gel and visualized by autoradiography.

Northern Blotting

Total RNA from NIH3T3 or SH-SY5Y cells was isolated using TRIzol (Invitrogen). 5-10 ug RNA was separated on a 1.1% agarose, formaldehyde gel, and separated RNA was transferred to a 0.45 micron nitrocellulose Protran BA85 membrane (Whatman, Schleicher & Schuell) by capillary transfer. Northern blotting was performed using an α-^{32}P-dCTP random primer labeled 0.9 kb fragment of ferritin H human cDNA as a probe.

Western blotting

Total cell lysates were subjected to electrophoresis with 15% or 12.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), followed by wet transfer of separated proteins to an Immobilon-P membrane (Millipore). Membranes were blocked for one hour in 5% skim milk in Tris buffered saline with 0.1% Tween 20, and subsequently incubated overnight at 4°C with anti-ferritin H antibody (Abcam 16875). Recombinant human ferritin H and L were purchased from Calbiochem.

^{35}S-labeling/immunoprecipitation

Exponentially growing NIH3T3 cells were treated with either rotenone or ferric ammonium citrate for the indicated times. Following treatment, medium was removed and methionine/cysteine deficient DMEM containing 10% dialyzed bovine calf serum was added. Simultaneously, 10uCi/ml of ^{35}S-methionine/cysteine (GE Healthcare) was added to each dish. Cells were incubated under normal culture conditions for 1 hr. Total cell lysates were prepared, and precleared with rabbit serum (CAPEL) and protein A agarose (Calbiochem) overnight. Total incorporation of ^{35}S was measured using TCA precipitation and scintillation counting. Input protein for immunoprecipitation was determined by adding equal counts (1×10^6cpm) for each immunoprecipitation reaction. 6 ul of anti-Ferritin antibody (DAKO, A133) and 20 ul of protein A agarose were utilized for overnight immunoprecipitation at 4°C. Finally, the resulting immunoprecipitates were subjected to SDS-PAGE as described above, the gel was dried, and exposed to film at -86° C.

SiRNA transfection

Ferritin H siRNA was purchased from DHARMACON, Inc, siGENOME duplex D-045965-01 mouse FTH, NM_010239.

Sense:

5′CAAGAAUGAUCCCCACUUAUU-3′

Antisense:

5′PUAAGUGGGGAUCAUUCUUGUU-3′

Briefly, 1×10^5 cells/35 mm dish were plated and cultured under normal conditions in serum-containing, antibiotic-free medium (2 ml) until cells reached approximately 80% confluency. 16 ul DharmaFECT3 (Dharmacon) in a final volume of 200 ul OPTI-MEM medium (Invitrogen) was added to 7.5 ul of 20 uM siRNA dissolved in 200 ul of OPTIMEM and the mix was incubated for 20 min. After formation of RNA complexes, the 400 ul mix was added to the dishes and cultured under normal conditions (the final concentration of siRNA was 60 nM). After 24 hrs, cells were split into one, 6-well plate for treatment. Whole cell extracts were obtained and subjected to Western blotting as described above to examine ferritin H protein expression. In other cases, cells were trypsinized and assessed for ROS production and apoptosis using flow cytometry.

Detection of ROS

NIH3T3 cells in 35 mm dishes were incubated with 5 uM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Molecular Probes) dissolved in molecular biology grade DMSO (Calbiochem) in phenol red-free DMEM containing 10% fetal bovine serum for 30 minutes. After loading, the unincorporated dye was washed out with phenol red-free complete medium, and sublethal concentrations of rotenone (12.5 uM and 25 uM) and H_2O_2 (50 uM) were added for 0.5 or 2 hrs. Following treatment, cells were trypsinized and resuspended in phenol red-free complete medium, and cell pellets were collected after centrifugation at 2000 rpm for 3 min. The resultant pellets were suspended in 500 ul phenol red-free complete medium for flow cytometry on a Becton-Dickinson FACSCalibur.

Detection of Apoptosis

Annexin V conjugated with AlexaFluor647 (Molecular Probes-Invitrogen) was utilized to detect rotenone-mediated apoptosis. Annexin V detects the presence of phosphatidylserine on the outside of apoptotic cells. NIH3T3 cells in 35 mm dishes were treated with 12.5 or 25 uM rotenone for 8h. Cells were trypsinized, and resulting cell pellets were stained according to manufacturer's protocol, with the following modification: 2.5 ul Annexin V AlexaFluor 647 conjugate (Molecular probes) was added to the 100 ul cell suspension. AlexaFluor 647 fluorescence (indicating Annexin V positive cells) was assessed via flow cytometry on a Becton-Dickinson FACSCalibur.

RESULTS

Rotenone induces ferritin H expression

To investigate the effect of the mitochondrial complex I inhibitor, rotenone, on ferritin H expression, we examined the levels of ferritin H mRNA following rotenone exposure by Northern blotting. As shown in Fig. 1a, treatment of NIH3T3 cells with increasing concentrations of rotenone (0-5 uM) for 24 hrs induced ferritin H message. The inductive effect of rotenone was less than that of tBHQ, an electrophilic compound that has been shown to induce various antioxidant and detoxification genes [18] including ferritin [15], therefore used as a positive control. Ferritin protein synthesis also showed a dose responsive increase following 24 hr treatment with 0.1, 0.5, and 1uM rotenone in NIH3T3 cells (Fig. 1b). In SH-SY5Y neuroblastoma cells, rotenone induced ferritin H mRNA and protein expression (Figs. 1c and 1d) similarly but at much lower concentrations (nanomolar) than those used in

experiments with NIH3T3 cells. Micromolar ranges of rotenone were used throughout the experiments in NIH3T3 cells without severe cell damage (see Fig. 6 and the legend). Our previous studies demonstrated that H_2O_2 treatment transiently activated IRP within 1-2 hrs thereby inhibiting ferritin translation prior to transcriptional activation of the ferritin H gene [15]. Since inhibition of mitochondrial complex I by rotenone has a potential to induce production of ROS and H_2O_2 [24], we next investigated whether ferritin H protein synthesis was transiently repressed following rotenone treatment. We exposed NIH3T3 cells to 1 uM rotenone for various times over a period of 24 hrs, and subsequently subjected them to ^{35}S-methionine/cysteine pulse-labeling and ferritin immunoprecipitation. As shown in Fig. 1e, increased ferritin protein was observed after 1 hr of rotenone treatment, and was maintained for 24 hrs, suggesting that transient translational repression of ferritin synthesis via IRP was not involved during rotenone treatment.

The Ferritin H gene is transcriptionally activated by rotenone through the ARE

Since we had observed increased levels of mRNA and protein synthesis following rotenone treatment, we were interested in whether or not ferritin H was transcriptionally activated. To test this, we transiently transfected NIH3T3 cells with a firefly luciferase reporter fused to 4.8kb or 0.22kb of the 5′ region of the mouse ferritin H promoter, and treated for 24 hrs with rotenone. Increasing concentrations of rotenone specifically activated the -4.8kb promoter in a dose-dependent manner, but had no effect on the -0.22kb promoter (Fig. 2a). This suggests that the increase in ferritin H mRNA results from transcriptional activation.

The -4.8kb region of the mouse ferritin H promoter that was activated by rotenone contains an antioxidant responsive element (ARE), located 4.1kb upstream of the transcription start site [23]. Next we asked whether or not the ARE was involved in transcriptional activation of ferritin H by rotenone. We cloned the wt-ARE or double mt-ARE, in which both the AP-1 like and AP-1/NFE2 sites contain critical mutations that abrogate transcription factor binding [15,16], into the minimum 0.22kb promoter reporter, and employed them for transient transfection assays in NIH3T3 cells. Fig. 2b shows that insertion of the wt ARE was sufficient for promoter activation by rotenone treatment, while introduction of mutations in the AP-1 like and AP-1/NFE2 sites abrogated rotenone mediated promoter activation. This suggests that not only is the ARE activated by rotenone treatment, but that the AP-1 binding sequences are also critical for rotenone-mediated transcriptional activation of the ferritin H gene.

Binding of Nrf2 and JunD to the ferritin H ARE is enhanced by rotenone treatment

Given that the mutations of the AP-1 like and AP-1/NFE2 sites blocked transcriptional activation of ferritin H in our transient transfection assays, we investigated whether transcription factor binding to the ARE was altered following rotenone treatment. First, gel retardation assays demonstrate that total protein binding to the ARE was increased by rotenone treatment as well as tBHQ in NIH3T3 cells (Fig. 3a). To assess the role of Nrf2, a major b-zip transcription factor responsible for regulation of the ARE in various phase II genes [25], and JunD, another b-zip family member that regulates the ferritin H ARE [16], we performed ferritin H ARE ChIP assay. As shown in Fig. 3b, *in vivo* binding of Nrf2 and JunD to the ARE was increased following rotenone treatment, suggesting that they are involved in the transcriptional activation of the ferritin H gene in response to rotenone treatment.

ROS production is involved in rotenone-mediated ferritin H induction

Given the results of rotenone-mediated ferritin H ARE activation in this study, we then hypothesized that rotenone induces ferritin H in an oxidative stress dependent manner. To test this hypothesis, we assessed rotenone's propensity to produce ROS, using the dye CM-H_2DCFDA, which is taken up by cells and is non-fluorescent in its acetylated, reduced form. Once localized in the cell, intracellular esterases deacetylate the dye, allowing for its oxidation

by ROS. The oxidized dye exhibits a shift in its emission spectra to the fluorescein range. Treatment of NIH3T3 cells with 5 uM rotenone for 0.5 hr resulted in a significant increase in the percent fluorescent positive cells, indicating that rotenone induces production of ROS and has the potential to cause oxidative stress (Fig. 4a). To reveal the role of oxidative stress in the induction of ferritin H by rotenone, we assessed the ferritin H mRNA levels of NIH3T3 cells treated with vehicle, rotenone, or rotenone following pre-administration of N-acetylcysteine (NAC), which is known to prevent production of reactive oxygen species by raising levels of glutathione. Indeed, NAC pretreatment abrogated the increase in ferritin H mRNA by rotenone treatment, but NAC treatment alone had negligible effects on ferritin mRNA levels (Fig. 4b). Taken together, these results suggest that rotenone activates the ferritin H transcription by an oxidative stress-dependent mechanism.

Ferritin H knockdown increases ROS production and sensitizes cells to rotenone-induced apoptosis

Our observation that oxidative stress is required for the induction of ferritin H by rotenone, led us to assess the role of ferritin H in protecting cells from rotenone induced oxidative stress and apoptosis. To determine whether ferritin H is cytoprotective against rotenone mediated oxidative damage, we utilized ferritin H siRNA to transiently decrease ferritin expression. Ferritin H siRNA (FH) decreased the expression of ferritin H protein in NIH3T3 cells by 50% compared to non-targeting siRNA (NT) (Fig 5a). Following either Non-targeted (NT) or FH siRNA transfection, cells were treated with sublethal concentrations of rotenone and examined for ROS production. As shown in Fig. 5b, FH siRNA NIH3T3 transfectants had increased ROS production following rotenone treatment compared to NT siRNA transfectants. We then asked if ferritin H knockdown cells are more susceptible to cytotoxicity induced by rotenone. To this end, rotenone-induced apoptosis was detected using fluorescently labeled annexin V, a protein that binds externalized phosphatidylserines. FH siRNA transfected cells had higher levels of annexin V staining, indicating that a greater percentage of the ferritin H knockdown cells were undergoing apoptosis (Fig. 6). Taken together, these results suggest that decreased ferritin H expression increased ROS production and sensitized cells to rotenone, and that a deficient ferritin H response to rotenone-induced oxidative stress caused cell death.

DISCUSSION

Maintenance of iron homeostasis is critically important in preventing oxidative cell damage via the Fenton reaction. Ferritin is a major protein involved in the regulation of intracellular free iron levels [10]. It sequesters iron within its shell, thereby blocking iron from participating in reactions that generate free radicals [1,26]. In some instances where cellular oxidative load is increased, cells may be more susceptible to iron mediated production of ROS and damage. We, along with others, demonstrated that ferritin is upregulated in response to a battery of different oxidative stressors [14,15,17]. Overexpression of ferritin H in cells reduced free iron levels and increased cellular resistance to H_2O_2 toxicity [27,28]. Deficiencies in ferritin lead to cellular profiles of oxidative stress and iron accumulation [9].

Oxidative stress and iron also have both been implicated in the pathogenesis of Parkinson's disease. Increased iron concentrations and oxidative damage have been observed in damaged regions of the substantia nigra in both human cases of Parkinson's disease (PD) and in animal models [29,30]. Taken together with rotenone-induced experimental models of Parkinson's disease [22], we hypothesized that rotenone may increase levels of ferritin H. In fact, we observed that ferritin H is induced following rotenone exposure at the mRNA and protein synthesis levels in NIH3T3 fibroblasts and SH-SY5Y neuroblastoma cells (Fig. 1). Our previous studies demonstrated that H_2O_2 produces a transient activation of IRP binding to the IRE, thus conferring a temporary reduction in ferritin H synthesis [15]. Our results in this study

demonstrated that ferritin H synthesis was not reduced at any time point following rotenone exposure, suggesting that IRP was not activated by rotenone. Like rotenone, tBHQ has a potential to produce reactive oxygen species [31] but did not activate IRP (unpublished data). This may potentially be due to the differences in the ROS produced by each stressor. Rotenone and tBHQ produce ROS indirectly by leakage of electrons from the electron transport chain and redox cycling, respectively [32]. H2O2 on the other hand, a ROS precursor, may directly interact with IRP, leading to its activation.

We were interested in the mechanism of ferritin increase by rotenone. Upregulation of ferritin following chronic MPTP administration in mice was demonstrated by cDNA microarray [33], but little is known about the mechanism of transcriptional activation of ferritin, or the signaling pathway responsible for the inductive effect of neurotoxicants. We showed that the ferritin H gene is transcriptionally activated by rotenone in a dose dependent manner through an ARE (Fig. 2). Furthermore, this activation was mediated through the ARE. Wild type ARE insertion alone was sufficient for rotenone-mediated activation similar to that observed by the 4.8kb promoter. In addition, mutation of the AP-1 like and AP-1/NFE2 binding sites abrogated the activation by rotenone (Fig. 2), suggesting the importance of these AP1 sites in the activation of transcription of the ferritin H gene. Indeed, enhanced total protein binding to the ARE was observed following rotenone treatment, in which increased Nrf2 and JunD binding to the ARE was detected by ChIP assay (Fig. 3). This led us to propose that oxidative stress may be downstream of the complex I inhibition by rotenone in the cascade of events that lead to ferritin H transcriptional activation. Confirming our hypothesis, addition of the glutathione precursor, NAC, blocked rotenone-mediated ferritin H mRNA induction (Fig. 4), suggesting that oxidative stress is a necessary cue in ferritin H activation by rotenone. Several recent studies have reported that in some conditions, especially hypoxia, rotenone reduces mitochondrial ROS generation [34-36]. To date there is no clear consensus about the effect of rotenone on mitochondrial ROS generation under various conditions; however, such contradictory reports support the need for further investigation into the intricacies of mechanisms of mitochondrial ROS generation and suppression. Recently, two studies demonstrated a protective function of Nrf2 in mitochondrial dysfunction and oxidative stress caused by the complex II inhibitor, 3-nitropropionic acid [37,38]. These findings, along with our results in this study suggest that ferritin H may be a target gene in the response to mitochondrial dysfunction and oxidative stress produced by rotenone insult.

To investigate the cytoprotective role of ferritin against rotenone toxicity, we examined the effect of ferritin H knockdown on cellular sensitivity to rotenone by siRNA transfection. Ferritin H siRNA transfection caused increased production of ROS and apoptosis in response to sublethal concentrations of rotenone treatment compared to non-targeting siRNA transfected cells (Fig. 5a). In this experiment we observed that knocking down ferritin H expression resulted in induction of a slower-migrating ferritin in NIH3T3 cells (Fig. 5a). This may be ferritin L (mouse ferritin L protein migrates slower than ferritin H in SDS-PAGE— H and L ferritins from mouse migrate in the reverse order of that observed for human ferritin H and L [28,39].) because a recent study reported by Cozzi et. al., showed that ferritin H knockdown increased ferritin L expression in HeLa cells [9]. In this study the increased ferritin L expression did not affect iron availability [9]. These results suggest that alterations in ferritin H expression may directly contribute to the altered iron metabolism and increased oxidative stress present during the disease progression of PD. In fact, it was reported that overexpression of ferritin H prior to MPTP exposure in a mouse model of PD conferred resistance to the neurotoxic insult [20]. This seems to be consistent with the present study showing that ferritin H knockdown resulted in an increased percentage apoptotic cells following rotenone treatment (Fig. 6). However, it should also be noted that prolonged ferritin accumulation in the same mouse PD model has recently been shown to contribute to a progressive age-related neurodegeneration

[40]. In this study the authors discussed that iron-saturated ferritin in the aged brain may release more iron during ferritin turnover, which would be neurotoxic rather than neuroprotective.

In brain tissue from individuals afflicted with Parkinson's Disease, the Fe(II)/Fe(III) ratio is 1:2 compared to 2:1 in a normal substantia nigra pars compacta [41]. This indicates that an increased amount of Fenton reactions occur during the progression of PD. Also, regions of iron accumulation colocalize with those of neuronal death [42]. Furthermore, iron chelators prevent alpha-synuclein translocation and mitochondrial aggregation, two hallmark events in the pathogenesis of PD [43]. In addition to ferritin, a number of other metal-regulatory/antioxidant genes have been implicated in PD. For instance, metallothionein overexpression was shown to be cytoprotective against neurotoxic insult [44]. Like ferritin H, metallothionein also contains an ARE and is regulated by both oxidative stress and iron [45]. It seems likely that a common ARE-mediated mechanism of phase II gene activation is a critical cytoprotective response to neurotoxicants. Ferritin H, as a major iron sequestering protein, may be an important component among the battery of activated genes. Our results in this study may shed light on the potential role of ferritin regulation in the pathogenesis of PD, as well as providing information as a potential target for chemoprevention.

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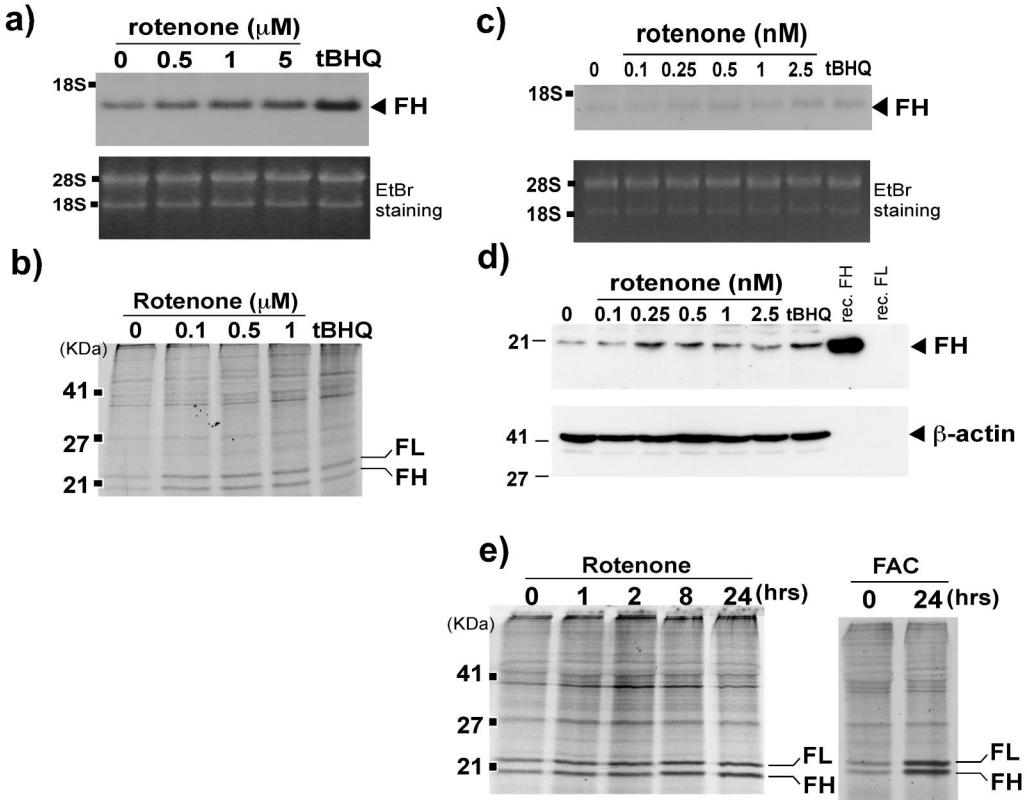

Figure 1. Effect of rotenone treatment on ferritin H expression

a) NIH3T3 cells were treated with either 0, 0.5, 1 or 5 uM rotenone or 10 uM tBHQ for 24 hrs. Total RNA was isolated and subjected to Northern blotting with a ferritin H cDNA probe. The resulting ferritin H band is indicated. To assess equal loading and integrity of total RNA, ethidium bromide staining is shown below. The positions of 18S and 28S ribosomal RNA are also indicated. A representative Northern blot result of 5 independent experiments is shown. b) NIH3T3 cells were treated with 0, 0.1, 0.5, or 1 uM rotenone or 10 uM tBHQ for 24 hrs and subjected to in vivo 1 hr pulse labeling of newly translated proteins with ^{35}S-methionine/cysteine labeling. 1×10^6 TCA insoluble counts were subjected to immunoprecipitation with anti-ferritin antibody and subsequently separated by SDS-PAGE. A representative image from 3 independent experiments is shown. c, d) SH-SY5Y cells were treated with 0.1-2.5 nM rotenone or 10 uM tBHQ for 24 hrs, and c) total RNA was isolated and subjected to Northern blotting with a ferritin H cDNA probe or d) preparation of whole cell lysates and Western blotting with the anti-ferritin H antibody (Abcam 16875). e) NIH3T3 cells were incubated with 1uM Rotenone for 0, 1, 2, 8, and 24 hrs, and subsequently subjected to in vivo labeling with ^{35}S-methionine/cysteine, immunoprecipitation with anti-ferritin antibody and SDS-PAGE. NIH3T3 cells were treated with 5ug/ml ferric ammonium citrate (FAC) as a positive control for ferritin protein induction and ^{35}S-labeled at 0 and 24 hrs. The resulting ferritin H (FH) and ferritin L (FL) bands are indicated.

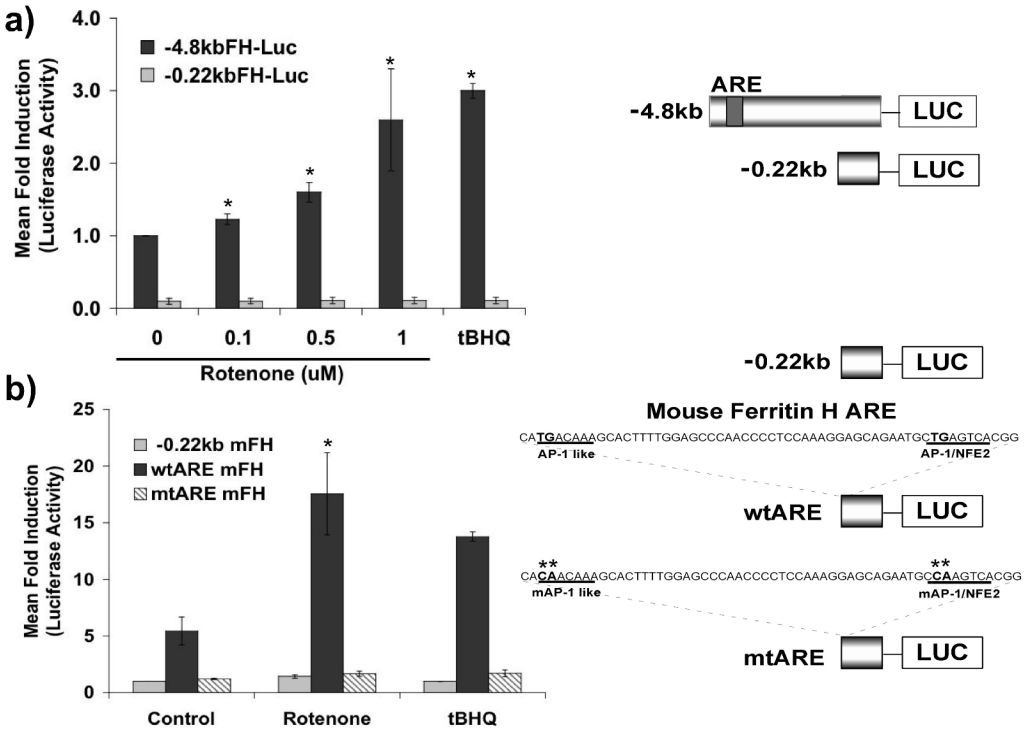

Figure 2. Transcriptional regulation of the ferritin H gene by rotenone treatment

a) NIH3T3 cells were transfected via electroporation with 0.5 ug each of either -0.22kbFH or -4.8kbFH firefly luciferase promoter reporter construct. After a 24 hrs recovery period, cells were treated with 0, 0.1, 0.5, or 1.0 uM rotenone, or 10 μM tBHQ and incubated for 24 hrs. Cell lysates were collected and assessed for transcriptional activation via luminometry. -4.8kbFH-Luc without rotenone treatment was set to 1 to calculate mean fold induction. Standard errors of means (S.E.M.) are shown, where n=5 independent experiments. b) NIH3T3 cells were transfected with -0.22kbFH, wt ARE insertion, or mt ARE insertion fused to a firefly luciferase reporter construct. Cells were treated for 24 hrs with either 1.0 μM rotenone or 10 μM tBHQ. Samples were normalized for recovery and transfection efficiency differences using a co-transfected internal control pRL-EF (elongation factor-renilla luciferase plasmid). The -0.22kbFH-Luc control value was set to 1 to calculate mean fold induction. S.E.M. shown, n=4 independent experiments. Asterisks denote statistical significance compared to a) 0uM rotenone, or b) control (no treatment), defined as $p<0.001$ by Student's t-test.

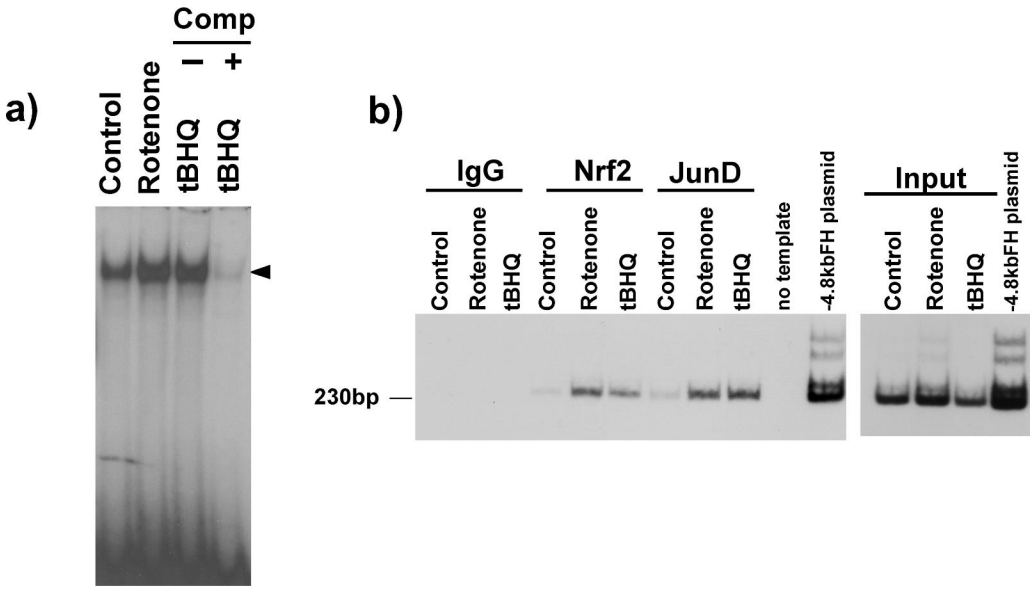

Figure 3. Nrf2, JunD transcription factor binding to the ARE after rotenone treatment

a) 50 ug of nuclear extracts from NIH3T3 cells treated with 1uM Rotenone or 10 uM tBHQ for 4hr, or untreated cells (control) were subjected to gel retardation assay using a probe for the AP-1/NFE2 site ferritin H ARE. Addition of 50X excess cold probe competitor to the right lane is indicated by Comp +. b) NIH3T3 cells untreated (control) or treated with 1 uM Rotenone or 10 uM tBHQ for 4hr, were used for ferritin H ARE ChIP assay. Primers specific to a region of the mouse ferritin H promoter that contains the ARE were employed for PCR with the input DNA or the DNA obtained following immunoprecipitation with either rabbit IgG, Nrf2 specific antibody or a JunD specific antibody. The resulting 230 bp product is shown. -4.8kbFH plasmid indicates the use of the mouse 4.8kbFH plasmid DNA as template as a positive control, and no template as a negative control. Representative images are shown of 3 and 4 independent experiments for a) and b), respectively.

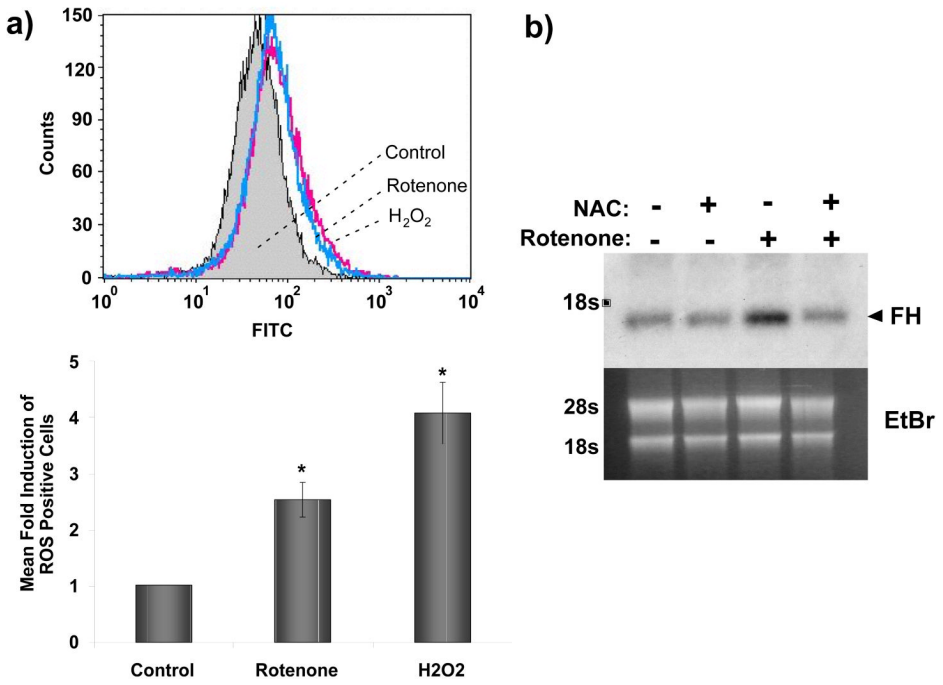

Figure 4. Involvement of ROS production in rotenone-mediated ferritin H mRNA induction

a) NIH3T3 cells were preloaded with the ROS sensitive dye, CM-H2DCFDA for 0.5 hr, and then treated with 5 uM rotenone or 50 uM H2O2 or left untreated (Control) for 0.5 hr. Levels of ROS, as indicated by FITC fluorescence, were assessed via flow cytometry. A representative histogram of ROS levels showing number of counts vs. relative FITC fluorescence is shown on the top. Mean fold induction of the number of ROS positive cells from 3 independent experiments was calculated by setting the control levels to 1. Asterisks denote statistical significance compared to control, $p<0.001$ by Student's t-test. b) NIH3T3 cells were treated with 0 or 1 uM rotenone for 24 hr (indicated by - or + respectively, lower row, marked rotenone), following a 2 hr pre-treatment with 0 or 10 mM NAC (indicated by - or + respectively, upper row, marked NAC). Resulting total RNA was subjected to Northern blotting with a ferritin H cDNA probe. Ethidium bromide RNA staining is shown below for loading and RNA integrity. The positions of 18S and 28S RNA bands are indicated. A representative image of 3 independent experiments is shown.

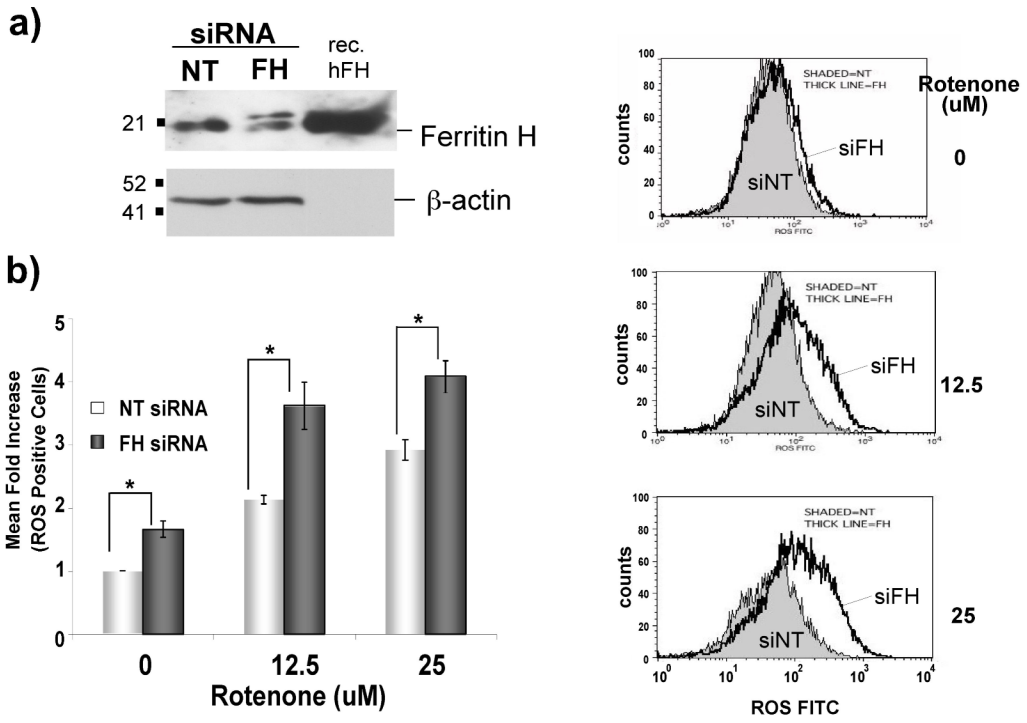

Figure 5. The effect of ferritin H knockdown on ROS production

a) NIH3T3 cells were transfected with non-targeted (NT) siRNA or ferritin H (FH) siRNA. Resulting ferritin H protein levels were assessed by Western blotting with anti-ferritin H antibody. The corresponding β-actin expression is shown as a loading control. A representative result of Western blotting is shown. Recombinant human ferritin H (rec hFH) was included as a Western blotting control. b) NT and FH siRNA transfectants were treated with sublethal concentrations of rotenone (12.5, and 25 uM) for 0.5 hr following loading for 0.5 hr with CM-H2DCFDA, and ROS levels were analyzed by flow cytometry. Representative histograms with counts (y-axis) vs. ROS FITC fluorescence (x-axis) are shown at right. The shaded area represents the histogram of NT siRNA transfectants (siNT), the thick black line is the FH siRNA transfectants (siFH). The mean fold increase in ROS positive cells between NT and FH siRNA transfectants is summarized at left. The difference between NT and FH siRNA transfectants left untreated was set to 1.0. Standard error is shown for n=3 independent experiments. Asterisks denote statistical significance compared to control, $p<0.005$ by Student's t-test.

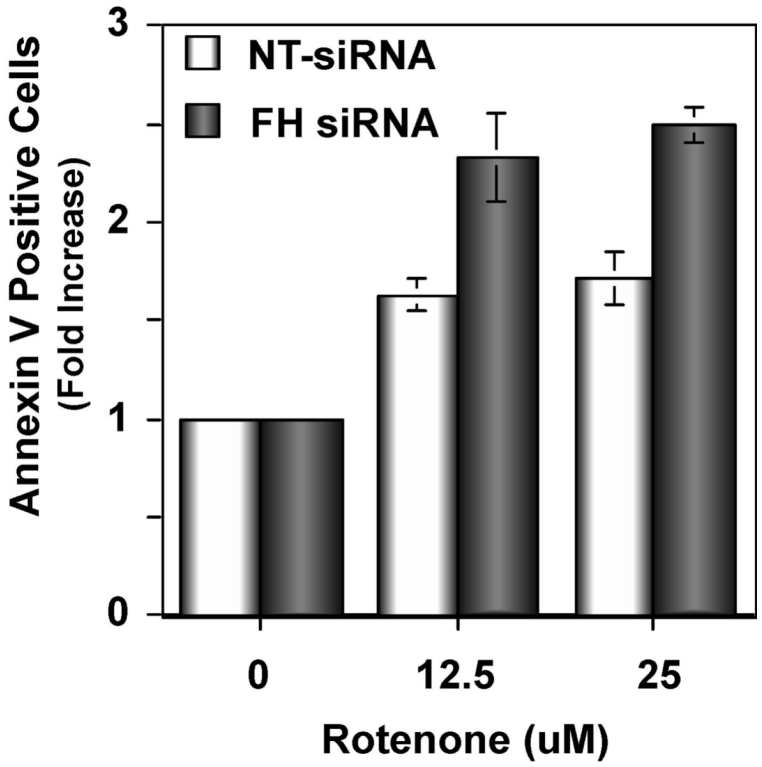

Figure 6. The Effect of ferritin H knockdown on rotenone-induced apoptosis

NIH3T3 cells were transfected with non-targeted (NT) siRNA or ferritin H (FH) siRNA as described in Fig. 5 and the section of experimental procedures. NT and FH siRNA transfectants were then treated with 0, 12.5, or 25 uM rotenone for 8 hrs, and apoptosis was quantified using flow cytometry to measure the fluorescence of AlexaFluor 647 conjugated to Annexin V. The mean fold increase in percent Annexin V positive cells was determined by setting NT and FH siRNA transfectants left untreated was set to 1.0 in each experiment. Means of Annexin staining-positive cells were 9.2% (rotenone 0), 15.0% (rotenone 12.5 uM), 16.0% (rotenone 25uM) in NT siRNA transfected cells, and 8.9% (rotenone 0), 20.4% (rotenone 12.5uM), 22.5% (rotenone 25uM) in FHsiRNA transfected cells. Standard error is shown for n=3 independent experiments.