Reactive Oxygen Species Regulate Ceruloplasmin by a Novel mRNA Decay Mechanism Involving Its 3′-Untranslated Region

IMPLICATIONS IN NEURODEGENERATIVE DISEASES

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Ceruloplasmin (Cp), a copper-containing protein, plays a significant role in body iron homeostasis as aceruloplasminemia patients and Cp knock-out mice exhibit iron overload in several tissues including liver and brain. Several other functions as oxidant, as antioxidant, and in nitric oxide metabolism are also attributed to Cp. Despite its role in iron oxidation and other biological oxidation reactions the regulation of Cp by reactive oxygen species (ROS) remains unexplored. Cp is synthesized in liver as a secretory protein and predominantly as a glycosylphosphatidylinositol-anchored membrane-bound form in astroglia. In this study we demonstrated that Cp expression is decreased by an mRNA decay mechanism in response to extracellular (H2O2) or intracellular oxidative stress (by mitochondrial chain blockers rotenone or antimycin A) in both hepatic and astroglial cells. The promotion of Cp mRNA decay is conferred by its 3′-untranslated region (UTR). When chloramphenicol acetyltransferase (CAT) gene was transfected as a chimera with Cp 3′-UTR in hepatic or astroglial cells, in response to either H2O2, rotenone, or antimycin A, the expression of CAT transcript was decreased, whereas expression of a 3′-UTR-less CAT transcript remained unaffected. RNA gel shift assay showed significant reduction in 3′-UTR-binding protein complex by ROS in both cell types that was reversed by the antioxidant N-acetylcysteine suggesting that ROS affects RNA-protein complex formation to promote Cp mRNA decay. Our finding is not only the first demonstration of regulation of Cp by ROS by a novel post-transcriptional mechanism but also provides a mechanism of iron deposition in neurodegenerative diseases.

Iron plays an essential role in many biological processes because of its ability to participate in electron transfer reactions as a protein-bound redox element. However, in excess, iron is highly toxic in the presence of reactive oxygen species (ROS) because of its ability to form highly reactive hydroxyl radicals (1) and is implicated in several pathological conditions including hepatic injury-related cancer and neurodegenerative diseases e.g. Parkinson and Alzheimer diseases (2, 3). Iron homeostasis is thus usually regulated by ROS to avoid iron-mediated injury (4). Ceruloplasmin (Cp), a copper-containing 132-kDa acute phase protein mainly synthesized in liver as a secretory form, regulates body iron homeostasis by its capacity as a ferroxidase (5, 6). In the central nervous system of humans and other mammals, Cp is expressed in astroglial cells as a GPI-anchored membrane-bound form (7, 8). As a major ferroxidase in plasma, Cp catalyzes the conversion of Fe2+ to Fe3+ for binding to apotransferrin (9). The role of Cp in iron homeostasis has been confirmed by findings of abnormal iron metabolism in patients with hereditary Cp deficiency (10) and in mice with targeted disruption of the Cp gene (11). These findings, together with early organ culture and animal studies (9, 12), suggest that Cp is required for efficient iron release from cells and tissues. In contrast, Cp also has been shown to mediate inward iron flux in several cell culture systems including hepatic, erythroid (13, 14), and glioblastoma cells (15, 16). Iron deposition in brain of aceruloplasminemia patients and related neurodegeneration strongly indicate its role as a neuroprotector in the central nervous system by regulating iron transport. It was suggested that GPI-Cp in astrocytes releases iron (17) and later confirmed using purified astrocytes from Cp knock-out mice (18). GPI-Cp co-localizes on the astrocyte cell surface with ferroportin (IREG1), a ferrous iron transporter. A recent study showed that the ferroxidase activity of GPI-Cp is required for the stability of ferroportin, providing a molecular mechanism of iron deposition in brain in the absence of or in conditions of reduced Cp (19). Thus, any reduction of Cp may affect cellular release of iron and cause oxidative damage in the presence of ROS.

Besides its role in iron homeostasis, Cp is also reported to have other functions including participation in several biological oxidation reactions that include a role in copper transport, coagulation, angiogenesis, defense against oxidant stress as an antioxidant, and low density lipoprotein oxidation (20). Cp was described as an antioxidant because of its ability to inhibit the...
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oxidation of lipids (21) as well as for its ability to scavenge superoxide (O$_2^·$) and sequester free copper ions (22). The ferroxidase activity may also contribute to the antioxidant capacity of Cp because conversion of Fe$^{2+}$ to Fe$^{3+}$ may reduce the oxidant capacity of iron by inhibition of the Fenton reaction. In contrast, several other studies have shown that Cp contains pro-oxidant activity, is able to oxidize low density lipoprotein in the presence of vascular cells such as endothelial or smooth muscle cells or monocytes, and is implicated in atherosclerosis (20, 23, 24). Recently its role as a nitrite oxidase has also been established (25).

Therefore, considering the role of Cp as a principal regulator of body iron homeostasis as well as an important participant in many biological oxidation reactions (20) we explored the possibility of Cp regulation by ROS. The increased generation of ROS in hepatic and brain cells by environmental toxins and during other pathological conditions is of particular interest as any regulation of Cp by ROS would be critical for maintaining iron status in these two very important organs. Thus, to understand the regulation of Cp by ROS, human hepatic HepG2 cells, rat glial C6 cells, or human astroglial U373MG cells were subjected to ROS treatment either by addition of H$_2$O$_2$ or by mitochondrial electron transport chain blockers, i.e. rotenone or antimycin A. We demonstrate here that ROS treatment decreases the synthesis of both the secretory form of Cp in hepatic cells and membrane-bound GPI-Cp in astroglial cells by a novel post-transcriptional mechanism involving its 3'-UTR. We also found that Cp 3'-UTR-binding protein complex is decreased by ROS apparently to cause increased decay of Cp mRNA. Our study is the first demonstration that Cp expression is down-regulated by ROS of extra- or intracellular origin, which may explain the iron deposition and related damage by increased ROS generation in neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Reagents—Rabbit polyclonal anti-human Cp IgG, anti-human transferrin (Tf) IgG were obtained from Accurate Chemical (Westbury, NY), and peroxidase-conjugated anti-rabbit IgG was from Bio-Rad. Other reagents were obtained from Sigma-Aldrich if not mentioned otherwise.

Cell Lines and Culture Conditions—Human hepatocarcinoma HepG2 cells were from the American Type Culture Collection, and human U373MG (glioblastoma-astrocytoma) and rat C6 glial cells were obtained from the National Centre for Cell Sciences, Pune, India. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine. Cells at 50–60% confluence were used in all experiments. Cells were maintained in a humidified atmosphere containing 5% CO$_2$ at 37 °C in a chamber (Forma-Scientific). Cell viability and toxicity were assessed using the trypan blue exclusion test and lactate dehydrogenase cytotoxicity assay using a detection kit (TaKaRa), respectively.

Intracellular ROS Measurement in Cell Culture—Fluorescence microscopy was used to detect intracellular ROS generation with DCF-DA as the probe. Cells were incubated with either medium, antimycin A, or rotenone and to detect ROS production further incubated with 5 µM DCF-DA in Dulbecco’s modified Eagle’s medium for 30 min at 37 °C in the dark as described before (26). To quantify the number of cells that are DCF fluorescent 10 different fields were considered for each experiment, and every experiment was performed separately for three times.

Immunoblot Analysis of Cp and Tf—Conditioned medium from HepG2 cells or cell extracts from U373MG or C6 cells were subjected to 7% SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated with anti-human Cp IgG (1:10,000) as primary antibody and then with peroxidase-conjugated secondary antibody (1:5000). Similarly conditioned medium from HepG2 cells was subjected to immunoblot analysis using anti-human Tf IgG (1:5000) and secondary antibody (1:5000). Cp and Tf were detected by chemiluminescence using ECL (Amersham Biosciences).

RNA Blot Analysis—Total RNA was isolated from cells using TriPure reagent (Roche Applied Science). Total RNA (20 µg) was denatured in formamide/formaldehyde, electrophoresed through a 1% agarose gel containing 6% formaldehyde, and then blotted onto nylon membranes (Schleicher & Schuell). After cross-linking by ultraviolet irradiation (Stratalinker, Stratagene), the membranes were hybridized to a 646-bp BstXI/BamHI restriction fragment (positions 984–1629 of the open reading frame) of a human Cp cDNA or glyceraldehyde-3-phosphate dehydrogenase cDNA (13) and labeled by random priming with [α-$^32$P]dCTP using a New England Biolabs kit. For mRNA decay assay, HepG2 cultures were treated for 2 h with medium and H$_2$O$_2$ (100 µM), then actinomycin D (7.5 µg/ml) was added, and cells were harvested after 0, 2, 4, and 8 h of antibiotic addition. Total cellular RNA was isolated, and 20 µg of RNA was fractionated on an agarose gel, transferred to nylon membrane, and hybridized with human Cp cDNA.

Reverse Transcription-PCR—Reverse transcription (RT)-PCR was performed on 4 µg of total RNA extracted from transfected HepG2 cells with TriPure reagent in a final volume of 50 µl using the Titan One Tube RT-PCR system (Roche Applied Science) using the following primers (CAT gene: forward, 5'-CAC TGG ATA TAC CAC CGT-3'; reverse, 5'-GCT ATC AGC ACC-3'; luciferase gene: forward, 5'-GCT GGA GAG CAA CTG CAT AAG-3'; reverse, 5'-CAT CAG AAA CAG CTC TTC TTC-3'). PCRs were carried out for 23 amplification cycles. The products were analyzed on a 1% agarose gel.

Cloning of Cp 3'-UTR in pcAT-3 Control Vector—The 247-bp Cp 3'-UTR was originally cloned from a genomic library as described earlier (27) and was subcloned into the XbaI site on the 3'-end of the CAT gene in pcAT-3 control vector (Promega). The clone was confirmed by sequence analysis. For in vitro transcription assays the same was subcloned into pcDNA3 downstream of T7 polymerase between BamHI and XbaI.

Cloning of Iron-responsive Element (IRE)-containing Transferin Receptor (TfR) 1 3'-UTR—A 188-bp fragment containing the proximal two iron-responsive elements of the total five from the 3'-UTR of TfR1 was PCR-amplified from genomic DNA by using the forward (5'-CAG GAA AGC TTC TTA...
Preparations of Cytosolic Extracts—Cytosolic extracts were prepared from HepG2, C6, and U373MG cells. Cells were washed twice with ice-cold 1× phosphate-buffered saline, pelleted down at 1000 × g for 5 min, and resuspended in lysis buffer containing 50 mM Tris-Cl (pH 7.6), 50 mM NaCl, 1 mM phenylmethylsulfonl fluoride, 0.5 mM dithiothreitol, and 1× protein inhibitor mixture (Roche Applied Science). After that cells were subjected to four to five freeze-thaw cycles and passed through a 30-gauge needle for 10–12 times on ice. Finally the samples were centrifuged at 15,000 × g for 30 min at 4 °C. Supernatants were collected, and protein estimation was carried out by protein assay kit (Bio-Rad).

In Vitro Transcription of Cp and TfR1 3′-UTR—Following linearization with BglII and XbaI, the 247-nucleotide region of Cp was decreased with increasing concentrations of H2O2. Similarly pcDNA3 plasmid construct containing 188 nucleotides of TfR1 3′-UTR was incubated with cytoplasmic extracts (10 μg) in 10 mM Tris-Cl buffer (pH 7.6), 15 mM KCl, 5 mM MgCl2, 0.1 mM dithiothreitol, 10 units of RNasin, and 0.2 mg/ml yeast tRNA in a volume of 20 μl. After incubation for 30 min in ice, 1 unit of RNase T1 was added for 10 min followed by 5 mg of heparin/ml for 10 min. In competition experiments, a 3×, 10×, and 30× molar excess of Cp 3′-UTR and a 10× and 30× molar excess of Cp exon 5 were added prior to the labeled probe. RNA-protein complexes were electrophoresed on 5% nondenaturing polyacrylamide gels in 0.5× TBE (Tris borate-EDTA) as the running buffer at 4 °C at 150 V that were prerun for 30–60 min. The gels were dried and subjected to autoradiography.

RNA Gel Shift Assay—[32P]UTP-labeled Cp 3′-UTR or IRE-containing TfR1 3′-UTR was incubated with cytoplasmic extracts (10 μg) in 10 mM Tris-Cl buffer (pH 7.6), 15 mM KCl, 5 mM MgCl2, 0.1 mM dithiothreitol, 10 units of RNasin, and 0.2 mg/ml yeast tRNA in a volume of 20 μl. After incubation for 30 min in ice, 1 unit of RNase T1 was added for 10 min followed by 5 mg of heparin/ml for 10 min. In competition experiments, a 3×, 10×, and 30× molar excess of Cp 3′-UTR and a 10× and 30× molar excess of Cp exon 5 were added prior to the radio-labeled probe. RNA-protein complexes were electrophoresed on 5% nondenaturing polyacrylamide gels in 0.5× TBE (Tris borate-EDTA) as the running buffer at 4 °C at 150 V that were prerun for 30–60 min. The gels were dried and subjected to autoradiography.

Statistical Analysis—All experiments were performed at least three times with similar results, and representative experiments are shown. Densitometric results are normalized with respect to internal controls and are expressed relative to the results in untreated control.

RESULTS

Extracellular and Intracellular ROS Regulate Cp Synthesis—To determine whether ROS has any role in Cp regulation, HepG2 cells were treated with H2O2 (10–200 μM) for 16 h, and immunoblot analysis of the conditioned media was performed. The steady state synthesis and secretion of the 132-kDa intact Cp was decreased with increased concentrations of H2O2 (Fig. 1A). Densitometric analysis revealed an approximately 50% decrease by 100 μM H2O2 treatment in the conditioned media after 16 h. Interestingly in response to H2O2 (0–200 μM) treatment a marginal but consistent increase (about 20%) in transferrin synthesis was detected by immunoblot analysis (Fig. 1B) indicating specificity of regulation of Cp by H2O2. When HepG2 cells were exposed to H2O2 (100 μM) the decrease in Cp synthesis was detected as early as 6 h, and the optimal decrease was found after 12 h of the treatment (Fig. 1C). To show that H2O2 treatment up to 200 μM is not deleterious to cells we performed a cell viability assay using the trypan blue exclusion test. The result shows that the exposure of HepG2 cells to 200 μM H2O2 is not detrimental to the cells (data not shown) in good agreement with previous finding (29).

Antimycin A or rotenone treatment generates intracellular ROS by blocking the mitochondrial electron transport chain (30). To find whether intracellular ROS has any role in Cp synthesis, we initially tested intracellular ROS formation by mitochondrial electron transport chain blocker antimycin A or rotenone. Treatment of HepG2 with antimycin A (100 ng/ml) or rotenone (1 μM) induced intracellular ROS as detected by fluorescence microscopy using ROS-sensitive fluorescent dye DCF-DA. After 1 h of treatment 4–5-fold more cells showed DCF fluorescence than untreated cells (Fig. 2A), but the treatment did not cause cellular toxicity as measured by lactate dehydrogenase assay even after 16 h (data not shown). When conditioned media were tested for Cp status, Western blot analysis showed an approximately 55% decrease of Cp by antimycin A and an approximately 52% decrease by rotenone after 12-h treatment (Fig. 2B), whereas a marginal but consistent increase of Tf synthesis (20–30%) was detected by Western blot analysis (Fig. 2C). These results show that Cp synthesis decreased specifically in response to both extra- and intracellular ROS.

ROS Decrease Cp mRNA by Decay Mechanism—To determine the mechanism of decrease of Cp synthesis by ROS we determined Cp mRNA levels by Northern analysis. When HepG2 cells were treated with H2O2 (Fig. 3A) both of the 4.5- and 3.7-kb alternative spliced transcripts of Cp (31) were decreased with increasing concentration of H2O2. Similarly antimycin A or rotenone treatment also decreased Cp transcripts by 45–55% (Fig. 3B). A similar decrease in both Cp protein and Cp mRNA indicated that the decrease of Cp protein is due to a decrease in Cp mRNA level by ROS. Because Cp mRNA is very stable with a half-life of more than 10 h (13), we hypothesized that H2O2 treatment would decrease Cp mRNA by decreasing its half-life. To test that, HepG2 cells were pre-treated with H2O2 (100 μM) for 2 h, and then transcriptional inhibitor actinomycin D was added. After 0, 2, 4, and 8 h of actinomycin D treatment total RNA was isolated, and RNA blot analysis was performed using a Cp mRNA-specific probe as described earlier (13). The result shows (Fig. 4) a considerable increase in the rate of Cp mRNA decay in HepG2 cells treated with H2O2. Cp mRNA decreased normally by about 10% after 8 h of actinomycin D addition, but Cp mRNA isolated from H2O2-treated cells decreased about 45% after 8 h of actinomycin D treatment. A similar decrease of half-life of Cp mRNA was also obtained by antimycin A and rotenone treatment (data not shown).
shown). To verify that actinomycin D is fully effective, the half-life of transferrin receptor 1 was also measured (supplemental Fig. 1); the half-life detected was about 2.2 h as reported earlier (32).

Role of 3'-UTR in Cp mRNA Decay—The 3'-UTR is known to influence the expression of respective genes by contributing to the stability/decay of the mRNA (33). To determine whether Cp 3'-UTR has any such role in ROS-mediated Cp mRNA decay, 247-nucleotide-long 3'-UTR was cloned downstream of the CAT gene in pCAT-3 control vector (pCAT-Cp-3'-UTR) and transfected into HepG2 cells. Similarly pCAT-3 control vector (without Cp 3'-UTR) was also transfected into HepG2 cells. In both cases pGL3-basic vector containing luciferase gene was co-transfected to determine transfection efficiency. Transfected HepG2 cells were treated with H2O2 for 12 h, and RT-PCR was performed from isolated total RNA by CAT-specific primers. When cells transfected with CAT gene containing Cp 3'-UTR were treated with H2O2, a dose-dependent decrease in CAT transcript was detected (Fig. 5A), whereas no change in CAT expression was found in the absence of Cp 3'-UTR (Fig. 5B). All the PCRs were performed within the linear range of gene expressions standardized previously (supplemental Fig. 2). Similarly when pCAT-Cp-3'-UTR was transfected into HepG2 cells and cells were treated with either antimycin A or rotenone, a decrease in CAT transcript was detected in the presence of Cp 3'-UTR (Fig. 6A), but expression of CAT remained unaltered in the absence of Cp 3'-UTR (Fig. 6B). These results show that Cp 3'-UTR contributes to the mRNA decay mechanism of Cp in response to extra- or intracellular ROS.

Involvement of 3'-UTR-binding Protein in ROS-mediated Cp mRNA Decay—3'-UTR-binding proteins are often involved in mRNA stability/decay mechanisms (33). We hypothesized that Cp 3'-UTR confers stability to the transcript by binding with one or more proteins and that ROS might decrease the binding of the protein(s) to 3'-UTR to cause instability of the transcript. Therefore we performed an RNA gel shift assay using the whole 247-nucleotide-long 3'-UTR. Cytosolic extracts were prepared from H2O2-treated HepG2 cells (0–100 μM), and an RNA gel shift assay was performed with in vitro transcribed 32P-labeled Cp 3'-UTR. When cytosolic extract from untreated HepG2 cells was incubated with the radiolabeled RNA probe, a major RNA-protein complex was observed (Fig. 7A). Less binding was seen with cytosolic extracts of HepG2 cells treated with increasing concentrations of H2O2 (Fig. 7A). Cold competition with Cp 3'-UTR and Cp exon 5 shows the specificity of the binding as increasing amounts of Cp 3'-UTR decreased the specific binding in a dose-dependent manner, whereas RNA generated from Cp exon 5 showed little effect even at a 30-fold molar excess (Fig. 7B). A similar decrease in 3'-UTR-protein complex was

FIGURE 1. Effect of H2O2 on Cp expression in HepG2 cells. A, HepG2 cells at 50–60% confluence were incubated with increasing concentrations of H2O2 (0–200 μM) for 16 h. Conditioned media were subjected to immunoblot analysis using polyclonal rabbit anti-human Cp IgG. B, similarly HepG2 cells were incubated with increasing concentrations of H2O2 (0–200 μM) for 16 h, and conditioned media were subjected to immunoblot analysis using polyclonal rabbit anti-human Tf IgG. C, immunoblot analysis for Cp was performed with conditioned media from HepG2 cells treated with 100 μM H2O2 for different periods of time. Results are represented as the mean of three observations and normalized to control, arbitrarily chosen as 1 unit. Data are expressed as means ± S.D.
was performed with the cytosolic extracts. No significant alteration of complex formation was detected after hypoxic exposure of cells (Fig. 7E), but more than a 2-fold increase was detected in Cp protein expression (supplemental Fig. 4) as reported earlier (34).

ROS Regulates GPI-anchored Cp Synthesis in Glial Cells by a Similar Mechanism—A GPI-anchored membrane-bound form of Cp was detected predominantly in astroglial cells (8) that is implicated in iron release (17, 18). Thus, the decreased synthesis of glial Cp might result in iron accumulation in glia and subsequently affect both the glia and neuronal function. So we tested whether GPI-Cp is also regulated by ROS. When rat C6 and human U373MG cells were treated with H_{2}O_{2} and Western blot analyses were performed in cell lysates, more than a 50% decrease in the steady state synthesis of GPI-Cp was found in C6 cells after 12 h of H_{2}O_{2} treatment (Fig. 8A). Similarly a dose-dependent decrease of GPI-Cp was detected in U373MG cells treated with H_{2}O_{2} (Fig. 8B). When U373MG cells were treated with antimycin A or rotenone as described before an approximately 50% decrease by antimycin A and an approximately 40% decrease of Cp synthesis by rotenone were detected by Western analysis (Fig. 8B). To determine the role of Cp 3'-UTR in regulating the ROS-mediated decrease in GPI-Cp in glial cells, pCAT-Cp-3'-UTR construct and pCAT vector were transfected into U373MG cells. The transfected cells were subjected to H_{2}O_{2} (100 μM), antimycin A (100 ng/ml), or rotenone (1 μM) treatment, and RT-PCR was performed using CAT-specific primers from total RNA. About 45–50% decreases in CAT transcript were found in response to H_{2}O_{2}, antimycin A, or rotenone treatment (Fig. 8A). Similarly a dose-dependent decrease of CAT transcript was found in U373MG cells treated with H_{2}O_{2} (Fig. 8B).

observed by antimycin A or rotenone treatment (Fig. 7C). When cytosolic extracts from H_{2}O_{2} (0–100 μM), antimycin A-, or rotenone-treated HepG2 cells were incubated with 3'-UTR of TfR1 containing two proximal IREs no decrease in iron regulatory protein (IRP)-IRE interaction was detected (supplemental Fig. 3) indicating that a decrease of 3'-UTR-binding protein complex in response to ROS is specific for Cp, whereas the iron chelator deferrioxamine showed strong induction of the IRE-IRP interaction. To confirm the role of ROS in regulating the interaction between Cp 3'-UTR and cytosolic protein, we pretreated HepG2 cells with a 5 μM concentration of antioxidant N-acetylcysteine for 30 min before the addition of H_{2}O_{2}. The complete protection of the RNA-protein complex by the antioxidant N-acetylcysteine (Fig. 7D) strongly suggests that ROS regulates Cp mRNA stability by regulating the binding of cytosolic protein to its 3'-UTR. We have previously reported that Cp expression is regulated by hypoxia (34). To determine whether there is any effect of hypoxia on Cp 3'-UTR-binding protein complex HepG2 cells were subjected to hypoxic condition (1% O_{2}) for 4 h, and RNA gel shift analysis determined the binding of cytosolic protein to its 3'-UTR in regulating the ROS-mediated decrease in Cp expression in HepG2 cells. Antimycin A (Ant A) and rotenone (Rot), Subconfluent HepG2 cells were treated with antimycin A (100 ng/ml) and rotenone (1 μM) for 1 h. The generation of ROS was measured by a fluorescence microscope using DCF-DA as a substrate and quantified as described under “Experimental Procedures.” HepG2 cells were treated with antimycin A and rotenone for 12 h, and Western analysis was performed (upper panels) in conditioned media using ceruloplasmin antibody (8) or transferrin antibody (C) as described earlier. The lower panels show densitometric analyses of the blots as the mean of three observations normalized to control (Cont). Data are expressed as means ± S.D.
from antimycin A- or rotenone-treated U373MG cells were incubated with radiolabeled Cp 3'UTR transcript, substantial decreases in RNA-protein complex formation were detected compared with untreated cellular cytosolic extract (Fig. 8F). All together these results confirm that either extra- or intracellular ROS formation decreases Cp synthesis both in hepatic and glial cells by a 3'UTR-mediated mRNA decay mechanism.

**FIGURE 3.** Effect of ROS on Cp mRNA. A, by H₂O₂. Total RNA was isolated from H₂O₂ (0–100 μM)-treated HepG2 cells (12 h), and Cp mRNA expression was determined by Northern blot analysis (upper panel) using a radiolabeled 646-bp Cp cDNA fragment as a probe. The blot was reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as a loading control (bottom panel). B, by intracellular ROS. Upper panel, HepG2 cells were treated with antimycin A (100 ng/ml) and rotenone (1 μM) for 12 h, total RNA was isolated, and Northern analysis was performed using a Cp cDNA fragment (upper panel). The blot was reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA as described before (middle panel). The bottom panel shows densitometric analysis of the experiment as the mean of three observations after being normalized to glyceraldehyde-3-phosphate dehydrogenase. Data are expressed as means ± S.D.

**FIGURE 4.** Determination of Cp mRNA stability in response to H₂O₂. Initially HepG2 cells were treated for 2 h with medium alone (A) and 100 μM H₂O₂ (B). Then actinomycin D (7.5 μg/ml) was added in both the H₂O₂-treated and untreated cells. Total cellular RNA was isolated at 0, 2, 4, and 8 h after actinomycin D treatment, and Northern analysis was performed using a 646-bp Cp cDNA fragment. 28 S ribosomal RNA depicted using ultraviolet light was used as a loading control. C, the rate of Cp mRNA decay of untreated and H₂O₂-treated cells was determined after normalization with 28 S ribosomal RNA by densitometry. Data are expressed as means ± S.D.
Iron is extremely essential for maintaining normal physiological function but potentially toxic due to its ability to form hydroxyl radicals that could damage cellular constituents (1).

Therefore, iron homeostasis is very tightly regulated in normal conditions. The role of multicopper oxidase Cp is well established in mammalian iron homeostasis (5–9). In this study we revealed a novel negative regulation of Cp synthesis by ROS in human hepatic HepG2 cells as well as rat C6 glial and human astroglial U373MG cells by an mRNA decay mechanism involving Cp 3′-UTR. The hepatic cells are long known to express the secretory form of Cp (35), whereas Cp is reported to be predominantly expressed as a GPI-anchored membrane-bound form in glial cells (8, 17). We found that both the secretory form of Cp in HepG2 and membrane-bound GPI-Cp form in glial cells are decreased by ROS suggesting that this previously unreported regulation of Cp is not form-specific, tissue-specific, or species-specific. We further demonstrated that ROS generated either intracellularly by inhibition of the mitochondrial electron transport chain as may happen by environmental toxins or extracellularly as may be generated by NADPH oxidases of macrophages, neutrophils, or microglial cells could decrease Cp synthesis. The study further revealed the involvement of the 3′-UTR in ROS-mediated regulation of Cp as verified by conferring a similar promotion of mRNA decay using a heterologous reporter where addition of Cp 3′-UTR downstream of CAT gene caused decay of CAT mRNA both in hepatic and astroglial cells (Figs. 5, 6, and 8). We further demonstrated that in response to ROS a decrease in binding of a yet unidentified protein to 3′-UTR makes it apparently susceptible to endonuclease-mediated cleavage (Figs. 7 and 8). The complete blocking of the reduction of RNA-protein complex by antioxidant N-acetylcysteine shows that the actual role of ROS is to regulate the complex formation of the protein with the Cp 3′-UTR (Fig. 7D). Although multicopper oxidases are present across evolution and well documented as important regulators of iron homeostasis, so far to our knowledge no other studies have demonstrated that any of these multicopper oxidase is regulated by ROS.

In aceruloplasminemia patients, secondary iron overload is detected in liver ascribing its role in cellular iron release (10); this has also been confirmed in Cp knock-out mice (11). To release iron from hepatic cells the ferroxidase activity of Cp is required to load iron into apotransferrin (5, 9). The formation of iron-loaded holotransferrin generates, from the perspective of cells, a negative, free ferrous iron concentration gradient, thereby releasing iron from cells (36). Thus, release of iron from cells is affected because iron loading into apotransferrin is inhibited due to the absence of Cp. Similarly any event that down-regulates Cp synthesis would also inhibit hepatic iron release. Excessive alcohol consumption, hepatitis C infection, and xenobiotic metabolism with an increased ROS generation in liver with simultaneous iron deposition have been reported (37). Our finding of a decreased Cp level in response to ROS could well explain deposition of iron in liver found in these pathological conditions (37). Undetectable serum Cp in hepatitis C viral infection can also be explained from our observation (38).

The effect of ROS generation on iron homeostasis has been demonstrated previously as ROS was found to regulate binding of the cellular iron sensor IRP1 to IREs present in mRNA of several iron homeostasis genes including TfR1 implicating increased cellular iron uptake during inflammation or activation of neutrophils or macrophages (39, 40). Interestingly a dif-
FIGURE 6. Intracellular ROS confers instability to transcript mediated by Cp 3'-UTR. A. 2 μg of pCAT-Cp-3'UTR (upper panel) was transfected into HepG2 cells along with 1 μg of pGL3 as a loading control (Cont), and cells were treated with antimycin A (Ant A; 100 ng/ml) and rotenone (Rot; 1 μM). After 12h of treatment, total RNA was isolated, RT-PCR was performed for 23 cycles using CAT-specific (upper panel) and luciferase-specific (middle panels) primers, and PCR products were determined in a 1% agarose gel. The lower panel shows comparative analyses of expressions of CAT and luciferase transcripts. B, a similar experiment was done with similar conditions after transfection of HepG2 cells with pCAT and pGL3 vector. PCR products were verified in a 1% agarose gel for transcripts of CAT (upper panel) and luciferase (middle panel). The lower panel shows comparative expressions of CAT and luciferase transcripts. Data are expressed as means ± S.D.
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study demonstrated that a single bolus addition of $\mathrm{H}_2\mathrm{O}_2$ (100 M) in the perfused rat liver failed to activate IRP1-IRE interaction (41), but a steady state generation of $\mathrm{H}_2\mathrm{O}_2$ was effective suggesting that the effect of ROS on iron homeostasis is also implicated in hepatic cells. Our failure to detect any substantial increase in IRP-IRE interaction might be due to a lesser proportion of IRP1 present in HepG2 cell than in rat liver cells as mainly IRP1-IRE interaction is sensitive to ROS (41). Deferoxamine caused a substantial increase in IRP-IRE interaction (supplemental Fig. 3) because the iron chelator can activate both IRP1 and IRP2. An increase in cellular ROS generation was also shown previously to increase heme oxygenase-1 content suggesting heme oxygenase-1-mediated heme degradation during ROS generation (42). The resultant increase in the intracellular labile iron pool was confirmed by EPR analysis (42). A simultaneous increase in cellular ferritin synthesis is probably required to protect cells from iron-mediated cellular damage by storing the excess intracellular iron (42). Another possibility to avoid this iron-mediated injury is to release iron mediated by Cp by loading iron into apotransferrin extracellularly. But in that case an increase in transferrin by ROS (Figs. 1B and 2C) and differential response to extracellular and intracellular ROS generation and to IRP1-IRE interaction was detected in B6 fibroblasts: in response to $\mathrm{H}_2\mathrm{O}_2$ treatment binding of IRP1 to IRE was faster than that in response to mitochondrial electron transport chain blocker antimycin A treatment (40). Faster generation of intracellular ROS by antimycin A treatment than by IRP1-IRE interaction suggests that ROS influences IRP1-IRE interaction probably by activating a signaling mechanism but not by directly interacting with the Fe-S cluster of IRP1 (40). Our study shows that the regulation of Cp 3′-UTR-binding protein is similar to the regulation of both extracellular $\mathrm{H}_2\mathrm{O}_2$ or intracellular ROS generation by antimycin A and rotenone treatment in both HepG2 and glial cells (Figs. 7 and 8) suggesting that a different mechanism might be operating than the role of ROS in IRP1-IRE interaction. Interestingly we could not detect any substantial increase in IRP-IRE interaction with IRE-containing Tfr1 3′-UTR either by extracellular or intracellular ROS (supplemental Fig. 3), but substantial decreases in ROS-mediated Cp 3′-UTR-binding protein complex suggest that it is specific and different from IRP1-ROS interaction. A previous

FIGURE 7. RNA gel shift analysis to detect proteins that bind to Cp 3′-UTR and the effect of ROS. In vitro transcribed [32P]-labeled Cp 3′-UTR transcript (10 fmol) was incubated with cytosolic extracts (10 M) from HepG2 cells at 4 °C for 25 min. RNA-protein complexes were resolved by a 5% non-denaturing polyacrylamide gel and detected by autoradiography. A, cytosolic extracts were isolated from HepG2 cells after 4 h of H2O2 treatment (0, 50, and 100 M, lanes 1, 2, and 3, respectively). Only probe was run in lane 4. B, specificity of the binding was determined with a 3×, 10×, and 30× molar excess of cold Cp 3′-UTR transcript and a 30× and 100× molar excess of RNA corresponding to Cp exon 5. The cold transcripts were added 2 min before the addition of radiolabeled probe. C, cytosolic extracts from HepG2 cells were isolated after no treatment (None), antimycin A (Ant A; 100 ng/ml) treatment, and rotenone (Rot; 1 M) treatment for 4 h, and an RNA gel shift assay was performed. D, HepG2 cells were pretreated with antioxidant N-acetylcysteine (NAC; 5 M) for 30 min, the medium was replaced with fresh medium before H2O2 treatment for 4 h, and an RNA gel shift assay was performed using cytosolic extract. E, an RNA gel shift analysis was performed with cytosolic extracts isolated from untreated (None), hypoxia- (Hyp; 1% O2), or H2O2 (100 M)-treated HepG2 cells (4 h).
mitogen-activated protein kinase pathway (44). Although there are several examples in the literature for ROS-mediated stabil-
ity of mRNA, not many examples of the ROS-mediated mRNA decay process have been reported. In a recent study, ROS was shown to promote SIRT1 mRNA decay by triggering the disso-
ciation of the HuR-SIRT1 mRNA complex, thus reducing SIRT1 abundance and the resultant lowering of cell survival (45). HuR usually interacts with the AU-rich element present in the 3'-UTR of several genes to regulate mRNA stability, but the absence of any recognizable AU-rich element in Cp 3'-UTR indicates involvement of HuR is highly unlikely in ROS-mediated regulation of Cp.

The regulation of Cp was previously reported mostly at the transcriptional level in response to various stimuli like hypoxia (34), iron deficiency (34), redox-active copper (46), insulin (47), and hyperoxia (48) and at the translational level by interferon-γ (27). In the context of Cp gene regulation, the current report is the first to describe its regulation by an mRNA stability mechanism involving its 3'-UTR. The presence of the AU-rich element or stem-loop structure like IRE is often reported in the 3'-UTR of genes regulated by mRNA decay/stability mecha-
nism (33). The absence of any AU-rich element or IRE in Cp 3'-UTR opens the intriguing possibility of finding a novel response element involving the mRNA stability/decay mecha-
nism in mammalian cells. Here we hypothesize that a redox
protein normally remains bound to the 3'-UTR and provides stability to the Cp transcript in both hepatic and glial cells. In response to ROS this redox-sensitive protein may undergo oxi-
dative modification and eventually leaves the 3'-UTR. As a result, the unoccupied 3'-UTR becomes a better substrate for endonuclease cleavage. The region of Cp 3'-UTR responsible for binding the protein and the mechanism by which ROS affect the binding of this protein remain to be determined.

The implications of our finding could also explain iron accu-
mulation and related injury in neurodegenerative diseases. The accumulation of iron is suggested to contribute in neurodegenerative diseases like Parkinson and Alzheimer diseases (3). Generation of ROS in neuronal and glial cells by inflammation, injury, or environmental toxins like pesticides (such as rote-
none) is implicated in the development of these neurodegen-

FIGURE 8. Effect of ROS in Cp expression in astrocytic cells. A, effect of H2O2 on Cp expression in C6 glial cells. Semiconfluent C6 glial cells were treated with 100 μM H2O2 for 12 h. Western analyses of cell lysates isolated from untreated and treated cells were performed for Cp (upper panel) and α-actin (bottom panel). B, effect of ROS on Cp expression in human astroglia U373MG cells. Semiconfluent U373MG cells were either treated with media alone (control (C)), H2O2 (30 and 100 μM), antimycin A (AntA; 100 ng/ml), or rotenone (Rot; 1 μM) for 12 h, and Western analyses of Cp (upper panel) and α-actin (middle panel) were performed using cell extracts. Relative expressions of Cp and α-actin are depicted in the lower panel. C, U373MG cells were transfected with pCAT-Cp-3'-UTR construct and pGL3 vector. Then cells were treated with media alone, H2O2 (100 μM), antimycin A (100 ng/ml), or rotenone (1 μM). After 12 h, total RNA was isolated, and semiquantitative RT-PCR was performed using CAT-specific primers (upper panel) or luciferase-specific primers (middle panel). Relative expressions of CAT and luciferase expressions were determined by densitometric analysis (bottom panel). D, similarly pCAT and pGL3 were transfected, and relative expressions of CAT transcript (upper panel), luciferase transcript (middle panel), and both transcripts (lower panel) were determined after treatment similar to that described in C. Cont, control. E, C6 glial cells were treated with H2O2 (0, 30, and 100 μM) for 4 h, cytosolic extracts were prepared, and an RNA gel shift assay was performed using in vitro transcribed 32P-labeled Cp 3'-UTR. F, U373MG cells were treated with only medium, antimycin A (100 ng/ml), and rotenone (1 μM) for 4 h. An RNA gel shift assay was performed using cytosolic extracts and radiolabeled Cp 3'-UTR probe. Data are expressed as means ± S.D.
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