Journal of Cellular Biochemistry 82:660–665 (2001)

Iron-Induced Oxidative Stress Up-Regulates Calreticulin Levels in Intestinal Epithelial (Caco-2) Cells

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Abstract Calreticulin, a molecular chaperone involved in the folding of endoplasmic reticulum synthesized proteins, is also a shock protein induced by heat, food deprivation, and chemical stress. Mobilferrin, a cytosolic isoform of calreticulin, has been proposed to be an iron carrier for iron recently incoming into intestinal cells. To test the hypothesis that iron could affect calreticulin expression, we investigated the possible associations of calreticulin with iron metabolism. To that end, using Caco-2 cells as a model of intestinal epithelium, the mass and mRNA levels of calreticulin were evaluated as a function of the iron concentration in the culture media. Increasing the iron content in the culture from 1 to 20 μM produced an increase in calreticulin mRNA and a two-fold increase in calreticulin. Increasing iron also induced oxidative damage to proteins, as assessed by the formation of 4-hydroxy-2-nonenal adducts. Co-culture of cells with the antioxidants quercetin, dimethyltiourea and N-acetyl cysteine abolished both the iron-induced oxidative damage and the iron-induced increase in calreticulin. We postulate that the iron-induced expression of calreticulin is part of the cellular response to oxidative stress generated by iron. J. Cell. Biochem. 82: 660–665, 2001.

Key words: iron homeostasis; calreticulin; oxidative stress; antioxidants; Caco-2 cells

Calreticulin is a highly conserved multifunctional calcium binding protein involved in the folding of nascent proteins by binding to N-linked monoglucosylated oligosaccharides [Krause and Michalak, 1997; Michalak et al., 1999]. Calreticulin resides in the endoplasmic reticulum (ER), however, its cytosolic and nuclear localization has also been reported [Sonhteimer et al., 1995; Holaska et al., 2001]. Lately, calreticulin has been recognized as a stress protein. Its synthesis is induced by heat-shock [Conway et al., 1995; Nguyen et al., 1996; Szewczenko-Pawlikowski et al., 1997], heavy metals [Nguyen et al., 1996], and amino acid starvation [Plakidou-Dymock and McGivan, 1994; Heal and McGivan, 2001]. Moreover, over-expression of calreticulin protected NIH/3T3 cells against chemically induced oxidation [Liu et al., 1997] suggesting that calreticulin might have a role in the cellular response to oxidative stress.

Iron is a trace element that is both essential and potentially toxic to cells. Its toxicity derives from the catalytic production of free radicals through the Fenton reaction [Okada, 1996; McCord, 1998]. A connection between iron metabolism and calreticulin is given by mobilferrin, a cytosolic protein involved in the intracellular transport of iron during intestinal iron absorption [Conrad et al., 1993]. The amino-terminal amino acid sequence of rat mobilferrin is 100% homologous to rat calreticulin, both proteins have the same apparent molecular weight and the same isoelectric point, and antibodies raised against one protein cross-react with the other [Conrad et al., 1993]. Therefore, mobilferrin is most probably an isoform of calreticulin. Because calreticulin is a stress-response protein and its cytosolic form mobilferrin is a putative iron transporter, we decided to characterize the possible associations between calreticulin and cellular iron levels.

Grant sponsor: Fondo de Ciencia y Tecnología (FONDECYT); Grant number: 1970465; Grant sponsor: Departamento de Investigación y Desarrollo (DID), University of Chile; Grant sponsor: Cátedra Presidencial en Ciencia to MTN.

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Received 22 January 2001; Accepted 11 April 2001

To that end, the expression of calreticulin as a function of the concentration of intracellular iron was determined in Caco-2 cells, a culture model of human intestinal epithelia [Hillgreen et al., 1995]. We found that physiological concentrations of iron induced an increase in calreticulin mRNA and in calreticulin. Furthermore, the iron-mediated increase in calreticulin mass was abolished by quercetin, an indication that the effect of iron was mediated by oxidative stress. We propose that the increase in calreticulin mass induced by iron is part of the cellular response to iron-mediated oxidative stress.

METHODS

Reagents

Rabbit anti-human calreticulin was from Affinity Bioreagents (Neshanic Station, NJ). 1,3-dimethyl-2-thiourea (DU), N-acetyl-L-cysteine (NAC), quercetin, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin, A, 3-[N-morpholino]propanesulfonate (MOPS), and salts were from Sigma Chemical Company, St. Louis, MO. Fetal bovine serum, Dulbecco's-modified Eagle medium, and low-iron Iscove medium, were from Gibco BRL (Grand Island, NY). ^{55}Fe, in the ferric chloride form, were from New England Nuclear (Boston, MA). Culture plasticware and Transwell bicameral inserts were from Costar (Cambridge, MA). To eliminate contaminant iron, all buffer solutions were filtered through Chelex-100 (Sigma Chemical Company, St Louis, MO).

Cell Culture

Caco-2 cells, from the American Type Culture Collection (# HTB37, Rockville, MD), were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum. Culture medium was changed every 2–3 days. Cells grown for 5–7 days were used.

Cells Containing Varied Levels of Iron

Cells were loaded to equilibrium with varied concentrations of ^{55}Fe as described [Tapia et al., 1996]. Briefly, cells were seeded at 10^5 cells/25 cm^2 flask and were incubated in low iron medium (10% iron-deficient serum in Iscove medium) supplemented with varied concentrations (0.5–10 μM) of ^{55}Fe as the Fe-nitrilotriacetic acid (1:2.2, mol:mol) complex. After one week in culture the cells were confluent ($2–3 \times 10^6$ cells/flask). The cells were trypsinized and seeded again at 10^5 cells/25 cm^2 flask. At the end of the second week of culture the cells were equilibrated in ^{55}Fe, and the intracellular iron concentration was estimated from their ^{55}Fe radioactivity [Tapia et al., 1996]. Cell extracts were prepared by treating cells with lysis buffer (100 μl/1×10^6 cells of 10 mM MOPS, pH 7.5, 3 mM $MgCl_2$, 40 mM KCl, 1 mM PMSF, 10 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.7 μg/ml pepstatin A, 5% glycerol, 1 mM dithiothreitol, 0.5% Triton X-100). The mixture was incubated for 15 min on ice and centrifuged for 10 min at 1,000*g*. The supernatant was stored at −80°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) of Calreticulin mRNA

Total RNA was prepared from cells with low or high levels of intracellular iron using TRIZOL® (Gibco). The extracted RNA was precipitated with isopropanol and washed with 70% ethanol. The purity and yield of total RNA were determined spectrophotometrically [Ausubel et al., 1995]. Five microgram of total RNA were reverse-transcribed in a total volume of 20 μl containing 2.5 μg oligo (dT)15 (Promega, Madison, WI), 1 mM dNTPs (Promega), 1 mM DTT, 200 U Superscript II (Gibco). First-strand cDNAs were obtained after 50 min at 42°C. The samples were then heated at 70°C for 10 min to terminate the reverse transcription. The final reaction was treated with 2 U of RNAse H at 25°C for 10 min and finally chilled in ice or stored at −20°C until use. The cDNA, were amplified by PCR using Taq polymerase (Gibco). The primers used, obtained from the human calreticulin sequence (GeneBank # M84739), were 5′calreticulin: GTCTACTTCAAGGAGCAGTTTCTGGACGG and 3′ calreticulin: TCCAAACT AT TAGGAAACAGCTTCACATAGCCG. Actin cDNA was used as an internal control using the primers 5′ actin: GTCGTCGACAACGGCTCCGGCATGTG and 3′ actin: GCCAGCCAGGTCCAGACGCAGGATGG. PCR was carried out in a final volume of 25 μl containing 1.3 U *Taq* polymerase (Gibco), 200 μM dNTPs, 0.025–1.1 μM of each of the 5′ and 3′ sequence specific primers in a buffer containing 1 mM $MgCl_2$ and various quantities of reverse-transcribed total RNA. The samples were denatured initially at 94°C for 3 min and amplification with denaturation at 94°C for 1 min, annealing at

58°C for 1 min and extension at 72°C for 75 sec, for varying cycle numbers. The final cycle was followed by 10 min extension step at 72°C. The absence of contaminants was routinely checked by RT-PCR assays of negative controls samples in which the RNA samples were replaced by sterile water or the Superscript II was not added. The PCR products were resolved in 1.5% agarose gels and visualized with ethidium bromide [Ausubel et al., 1995], using as standard a 100-bp ladder.

Immunodetection of Calreticulin

Proteins were separated by SDS–polyacrylamide gel electrophoresis using the Laemmli method [Bollag and Edelstein, 1991]. Gel load was standardized to equal amount of protein as determined by the Lowry protein assay. Calreticulin was detected by Western blot analysis [Harlow and Lane, 1988], using as primary antibody a polyclonal anti-calreticulin antibody (Affinity Bioreagents, dilution 1:5,000), and a peroxidase-based secondary antibody (Pierce, www.piercenet.com). Subsequently, protein bands were detected by hydrogen peroxide-catalyzed oxidation of luminol (SuperSignal kit, Pierce) and autoradiography. The antibody recognized a protein band of about 55 kDa apparent mass that was not evident when either the primary or the secondary antibody were omitted from the procedure. To control for gel load, the blotted membranes were acid-stripped after calreticulin detection and were re-blotted with anti-actin antibody (sc-8432, Santa Cruz Biotechnology, Santa Cruz, CA). The chemoluminiscence autoradiograms were imaged on a Perkin-Elmer scanner equipped with Photo-Paint software (Corel Systems). When stated, band intensities were determined densitometrically using SigmaScan software (Jandel Scientific, San Rafael, CA).

Antioxidant Treatment

Caco-2 cells were seeded and cultured under standard conditions (DMEM supplemented with 10% fetal bovine serum) for three days. The medium was then changed to either low (1 μM) or high (10–20 μM) iron. After two days in culture, the media was alternatively supplemented with either quercetin, DU or NAC and the cells were cultured for an extra 1–2 days, after which calreticulin levels were assayed by Western blot analysis.

Determination of Oxidative Damage

Evaluating HNE-modified proteins assessed oxidative damage to proteins. HNE is an α,β-unsaturated aldehyde product of the peroxidation of ω6-unsaturated fatty acids that form adducts with amino acid residues such as histidine, lysine, and cysteine. HNE was detected in a Western blot assay, using monoclonal antibody HNEJ-2, which recognizes HNE-histidyl adducts [Toyokuni et al., 1995] and a chemoluminiscence kit (ECL, Amersham).

Statistical Analysis

Variables were tested in triplicates, and the experiments were repeated at least two times. Variability among experiments was <20%. One-way ANOVA was used to test differences in means, and post-hoc *t*-test was used for comparisons. Differences were considered significant if $P < 0.05$.

RESULTS

Iron Up-Regulates Calreticulin Expression

To investigate if the level of calreticulin responded to changes in the levels of intracellular iron, Caco-2 cells grown to different levels of intracellular iron were analyzed for calreticulin and calreticulin transcript (Fig. 1). For the experiment shown in Figure 1A, the intracellular iron concentrations of 20±3, 45±5, 158±13 and 224±31 μM, were obtained incubating Caco-2 cells in media containing 0.5, 2, 5 and 10 μM, respectively [Tapia et al., 1996]. It is noteworthy that these iron concentrations are well within physiological limits [Williams and Moreton, 1980]. Western blot analysis of cell extracts revealed that calreticulin levels increased as a function of intracellular iron (Fig. 1A). Cells containing up to 45 μM intracellular iron presented basal levels of calreticulin while an increase in calreticulin mass was observed in cells containing iron concentrations of 158 μM or higher. Densitometric analysis of the calreticulin bands showed relative densities of 1.0±0.1, 1.3±0.1, 2.3 + 0.2, and 2.2±0.2, for the intracellular iron concentrations of 20, 45, 158 and 224 μM, respectively. The difference in calreticulin levels between the low iron (20 and 45 μM) cells and the high iron (158 and 224 μM) cells was significant ($P < 0.005$). We determined whether the iron-mediated increase in calreticulin mass corresponded with changes

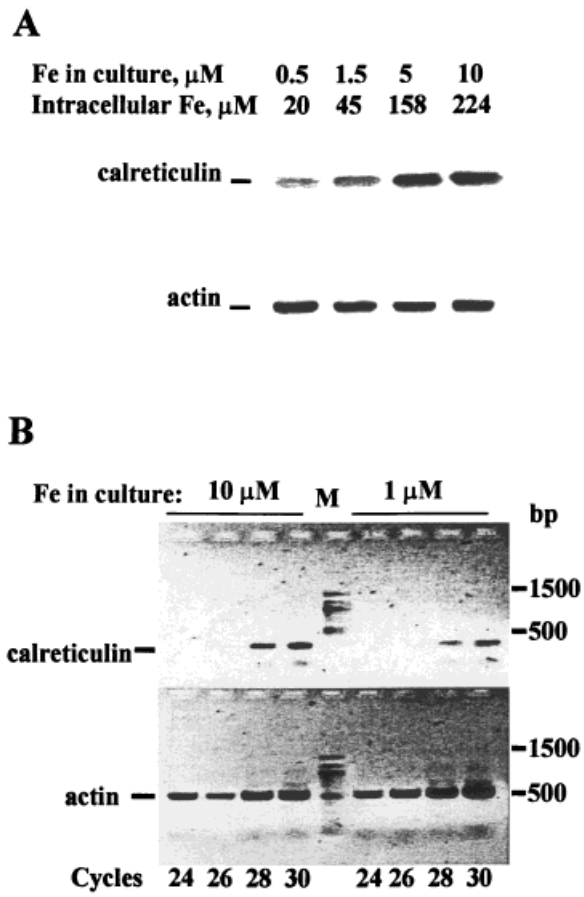

Fig. 1. Increasing cellular iron levels increase calreticulin protein and mRNA. **A:** Caco-2 cells were cultured in media containing 0.5, 1.5, 5, and 10 μM iron, which resulted in intracellular iron concentrations of 20, 45, 158, or 224 μM, respectively. Calreticulin from these cells was detected by a Western blot assay. The immunodetection of actin was used as a control for gel load. **B:** Representative ethidium bromide-stained agarose gel of PRC products. The PCR was also performed with actin for quantification. 12.5 ng of the total RNA from cells grown in either 1 or 10 μM iron was reverse transcribed and calreticulin and actin cDNAs were amplified for 24, 26, 28,and 30 cycles PCR. Lanes show PCR reactions terminated at the indicated number of PCR cycles, demonstrating that the PCR was analyzed during the linear phase of amplification. Lane M contains size markers. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and photograph under UV light.

in calreticulin transcript levels (Fig. 1B). Similar to the effect observed on protein levels, iron induced a moderate increase in calreticulin transcripts. Therefore, iron increased the transcription of the calreticulin gene.

Iron is a known oxidant that in excess generates hydroxyl free radicals under intracellular conditions [Okada, 1996; McCord, 1998]. Therefore we reasoned that the iron-induced increase in calreticulin could be due to an oxidative signal generated by iron. To test this hypothesis, we determined the iron-induced increase in calreticulin levels in the presence of quercetin, a flavonoid that protect fibroblasts, neurons, epithelial cells, and endothelial cells against oxidative damage [Skaper et al., 1997; Yamashita et al., 1999]. Cells were cultured in media containing 10 μM iron and varied concentrations of quercetin (range 15–120 μM). calreticulin levels were then determined in cell extracts by Western blotting (Fig. 2A). As observed before, 10 μM iron in the culture medium induced a marked increase in calreticulin expression, when compared with cells cultured in 1 μM iron. Quercetin (60 and 120 μM) afforded a complete protection of the iron-induced decrease in calreticulin expression. Under low iron conditions, hydrogen peroxide, but not quercetin, induced the expression of calreticulin (Fig. 2B).

To evaluate the potential oxidative damage produced by the physiological concentrations of iron used in this study, we measured the formation of 4-OH-2-nonenal (HNE)-histidine

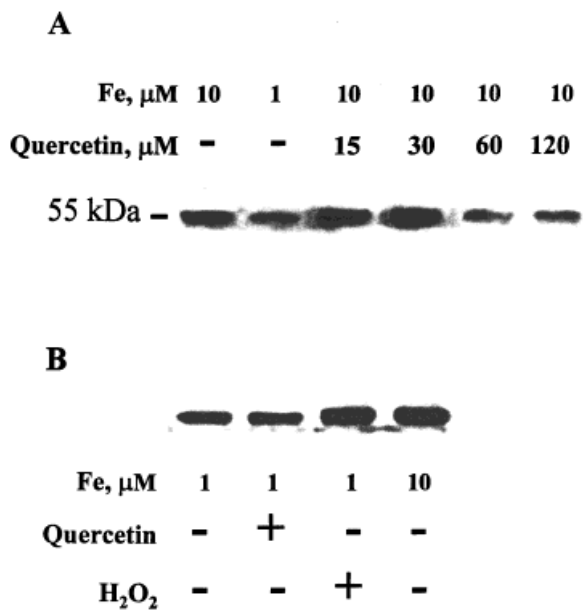

Fig. 2. Antioxidants diminish iron-induced calreticulin levels. **A:** Caco-2 cells were cultured for 1 day in either low (1 μM) or high (10 μM) iron medium. The high iron was supplemented with varied concentrations of the antioxidant quercetin (range 0–120 μM). Cell extracts were then prepared and calreticulin levels were assayed by Western blot analysis. **B:** Quercetin (60 μM) did not further decrease calreticulin expression in cells grown in low iron medium, while, under similar conditions, 100 μM hydrogen peroxide induced calreticulin expression.

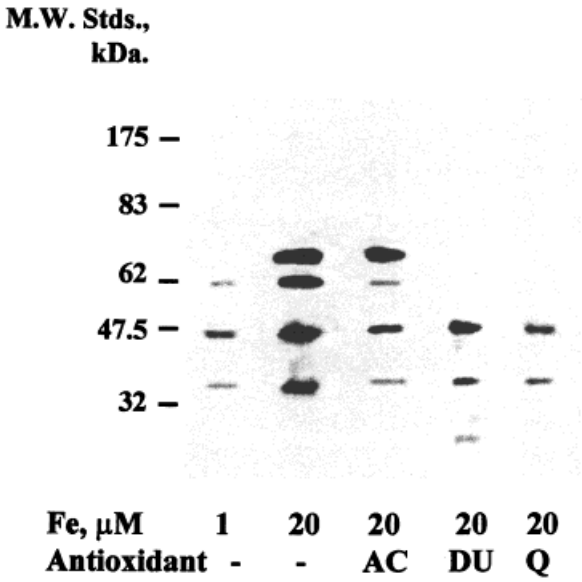

Fig. 3. Iron induces the oxidative modification of proteins. Caco-2 cells were incubated for 24 h in either low (1 μM) or high (20 μM) iron media. The 20 μM iron media was alternatively supplemented with one of the following antioxidants added to duplicate wells: 60 μM quercetin (Q), 10 mM dimethyltiourea (DU) or 10 mM N-acetyl-L-cysteine (AC). The formation of HNE-protein adducts was assayed by Western blot analysis using monoclonal antibody HNEJ-2. Shown is one of three similar experiments.

adducts, since HNE adducts has been shown to accurately describe oxidative damage in proteins [Toyokuni et al., 1995]. Proteins from cells cultured in 20 μM iron showed increased content of HNE-histidine adducts when compared with proteins coming from cells cultured in 1 μM iron (Fig. 3). Moreover, quercetin, DU, and NAC, protected the cells from iron-induced oxidative damage. Hence, the physiological concentrations of iron used in this study indeed induce oxidative damage to proteins that was largely abolished by antioxidants.

DISCUSSION

Recent studies have recognized calreticulin as a protein induced by heat, calcium, heavy metals, and amino acid starvation [Plakidou-Dymock and McGivan, 1994; Conway et al., 1995; Nguyen et al., 1996; Szewczenko-Pawlikowski et al., 1997; Heal and McGivan, 2001]. Indirect evidence suggests that calreticulin may be also an oxidative stress-induced protein since overexpression of calreticulin protected renal epithelial cells against lipid peroxidation induced by the pro-oxidant reagent *tert*-butyl-hydroperoxide [Liu et al., 1997]. Because iron is an inductor of oxidative stress, we set to investigate the possible association of calreticulin with iron metabolism. We found that physiological increases in intracellular iron induced a two-fold increase in calreticulin. Semi-quantitative RT-PCR of total cell RNA also showed an increase in calreticulin transcripts with increasing iron concentrations, an indication that iron affected the transcriptional regulation of calreticulin. To our knowledge, this is the first report that links increases in calreticulin expression with increases in cellular iron levels.

The iron-induced increase in calreticulin expression was most probably mediated by reactive oxygen species since it was very effectively blocked by the antioxidant quercetin. It is possible that increased intracellular iron produce an oxidative signal that results in the transcriptional activation of the calreticulin gene. A possible mediator for the iron-induced oxidative signal is the AP-1/Jun NH2-terminal kinase pathway, which is activated by the oxidant H_2O_2 and inhibited by quercetin [Yamashita et al., 1999; Yokoo and Kitamura, 1997].

The question arises on the physiological meaning of iron-induced calreticulin synthesis. Since calreticulin is a homologue of the cytosolic iron-binding protein mobilferrin [Conrad et al., 1993], it is possible that the increase in calreticulin with increasing levels of iron in the culture medium responds to the cell need for more iron-binding capacity, to ameliorate the iron-induced oxidative stress. The main cellular locus for this protective role of calreticulin should be the ER, where the larger amount of calreticulin is found. As far as we know, no report is available on the content of iron in the ER. Nevertheless, an ATP-driven iron pump has been described in the nuclear membrane [Gurgueira and Meneghini, 1996]. In principle, the action of this pump should produce iron accumulation in both nucleus and ER, because of the continuity of the nuclear and ER membranes. Thus, the iron-induced increase in calreticulin synthesis observed in this report could protect the ER, cytosol, and nucleus environment from iron-mediated oxidative damage.

In summary, we found that physiological increases in cell iron induced a two-fold increase in calreticulin mass. We also found that physiological concentrations of iron generated

HNE-protein adducts, a clear indication of oxidative damage. Since the iron-induced increase in calreticulin mass was blocked by antioxidants, the increase in calreticulin mass was most probably mediated by oxidative stress. We propose that the iron-induced increase in calreticulin mass is part of the cellular response to oxidative stress generated by iron.

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