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Toxicology

Cadmium-induced aggregation of iron regulatory protein-1

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ABSTRACT

Iron regulatory protein-1 (IRP-1) is central to regulation of iron homeostasis, and has been shown to be sensitive to Cd^{2+} in vitro. Although Cd^{2+} induces disulfide-bond formation in many proteins, the critical cysteine residues for iron binding in IRP-1 were shown not to be involved in Cd-induced IRP-1 aggregation in vitro. Here we show that Cd^{2+} causes polymerization and aggregation of IRP-1 in vitro and in vivo, and decreases in a dose-dependent manner both its RNA-binding and aconitase enzymatic activities, as well as its cytosolic expression. We have used two-dimensional electrophoresis to demonstrate thiol-dependent self-association of purified recombinant IRP-1 treated with Cd^{2+}, as well as self-association in Cd^{2+}-exposed mesangial cells. Circular dichroism spectra confirm significant conformational changes in the purified protein upon Cd^{2+} exposure. Following Cd^{2+} treatment, there is increased translocation of inactive IRP-1 to the actin cytoskeletal fraction, and this translocation is diminished by both antioxidant (BHA) treatment and inhibition of CaMK-II. These changes differ from those elicited by manipulation of iron levels. Cadmium-induced translocation of proteins to cellular compartments, and particularly to the cytoskeleton, is becoming a recognized event in Cd^{2+} toxicity. Polymer-dependent translocation of IRP-1 in Cd^{2+}-exposed cells may underlie effects of Cd^{2+} on iron homeostasis.

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1. Introduction

Iron regulatory protein-1 (IRP-1) regulates cellular $Fe^{2+/3+}$ homeostasis through binding to iron-responsive elements (IREs) in the mRNA's coding for a number of proteins such as ferritin, transferrin receptor (TfR), divalent metal transporter 1, and aminolevulinate synthase (Eisenstein, 2000; Theil, 2000). When the protein contains an intact 4Fe–4S cubane cluster as a cofactor, it lacks IRE binding activity and assumes enzymatic activity as a cytosolic aconitase (c-aconitase), a homologue of mitochondrial aconitase (m-aconitase) (Kaptain et al., 1991). Three of the iron atoms in the cluster are coordinated to protein thiol residues while the fourth more labile iron is involved in substrate binding. Loss of the labile iron eliminates enzymatic activity and is generally thought of as a switch for engaging IRE binding (Kühn, 2003). More complete disruption of the cluster may expose protein thiols for intramolecular disulfide bond formation that renders the protein inactive in both functions (Kühn, 2003).

The kidney plays an important role regulating iron homeostasis. Current data support a model of transferrin-bound Fe^{3+} being filtered at the glomerulus and reabsorbed by the proximal tubules (Smith and Thévenod, 2009). Proximal tubule cells reabsorb 75 to 90% of the citrate that enters the glomerular filtrate, and this reabsorbed citrate likely is metabolized by c-aconitase to isocitrate, which can then be converted by cytosolic isocitrate dehydrogenase to 2-oxoglutarate with production of NADPH (Zhang et al., 2007). In the mouse, IRP-1/c-aconitase is most highly expressed in kidney. Animals that lack IRP-1 are unable to repress ferritin synthesis fully in the kidney under conditions of iron deficiency, demonstrating that IRP-1 contributes significantly to regulation of iron metabolism in the kidney, although IRP-2 levels increase in a compensatory manner (Meyron-Holtz et al., 2004).

Cadmium (Cd) is a non-essential, toxic metal affecting multiple aspects of cell function, including activation of cell signaling, metabolic functions, and cellular redox status. Because Cd exists as the Cd^{2+} ion in aqueous media until bound to a thiol group under biological conditions, it has been proposed to increase oxidative stress not by redox cycling, but rather by interfering with antioxidant defenses (Waisberg et al., 2003). Another contributing factor may be interference with

homeostasis of redox-active metals such as Fe^{2+}/Fe^{3+}, allowing their participation in the Fenton reaction. IRP-1 is a redox-sensitive protein and the mechanism of its regulation by reactive oxygen species (ROS) is compartment-specific and complex (Anderson et al., 2012; Mueller, 2005). Superoxide inactivates aconitase activity by directly targeting the [4Fe–4S] cluster, but does not change IRE binding properties. Aconitase activity and IRP-1 protein level were decreased in superoxide dismutase-knockout mice. On the other hand, extracellular hydrogen peroxide activates IRE binding at the expense of aconitase activity. Nitric oxide induces down-regulation of IRP-1 at the protein level and has an impact on IRE-binding (Stys et al., 2011). Cadmium has been shown to increase ROS (Liu and Templeton, 2008) and could potentially influence IRP-1 through ROS indirectly, by post-translational modification of IRP-1 thiol group(s), or by direct interaction of Cd^{2+} with thiols, but potential mechanisms by which Cd^{2+} might influence IRP-1 function through thiol modifications has not been documented.

IRP-1 has been considered as a target of Cd^{2+}. Oshiro et al. (2002) used reducing conditions with EDTA to remove the labile iron from the 4Fe–4S cluster of purified bovine liver IRP-1 and replaced it by titration with various metals. Cadmium decreased IRE binding and increased aconitase activity. Incubation of PCL hepatoma cells with 150 μM Cd-nitrilotriacetate had similar effects in subsequent analyses of cytosol (Oshiro et al., 2002), suggesting that Cd^{2+} modulates IRP-1 by incorporating into the fourth labile iron position of the 4Fe–4S cluster. Martelli and Moulis (2004) confirmed that Cd^{2+} deceases IRE binding activity in a dose-dependent manner in vitro using purified recombinant IRP-1, although with modest decreases in aconitase activity. Reduction with 2-mercaptoethanol (2-ME) cannot fully restore the binding, suggesting irreversible loss of binding activity. Replacement with serine of the three cysteine residues (C437, C503 and C506) (Martelli and Moulis, 2004) involved in iron binding does not affect IRE binding, nor the interference by Cd^{2+} with IRE binding indicating that, despite Cd's propensity for thiol binding, the cysteine residues involved in iron binding are not sites of Cd^{2+} binding. The loss of binding activity with purified protein is especially obvious at higher concentrations of Cd^{2+} at or above 500 μM, and is due to the precipitation of IRP-1 apoprotein (Martelli and Moulis, 2004). Reversal of precipitation with glutathione equimolar to Cd^{2+} was attributed to chelation of Cd^{2+} from the protein metal center. Although, such a high concentration of Cd^{2+} was observed in certain cells such as proximal tubular kidney cells, precipitated or insoluble IRP-1 was not found in vivo, and it was probably degraded inside the cell (Moulis, 2010). The reason for the difference in effects on aconitase activity in the two studies (increased in Oshiro et al. (2002); decreased in Martelli and Moulis (2004)) is unknown, but the presence of EDTA in the aconitase assay of the earlier study (Oshiro et al., 2002) may be involved, as EDTA was shown to prevent precipitation of IRP-1 by Zn^{2+} (Martelli and Moulis, 2004). A more recent study found that Cd^{2+} decreases the IRP-1 protein level and, consistently, TfR mRNA levels in HeLa cells (Rousselet and Moulis, 2008); the mechanism of increased IRP-1 turnover by Cd^{2+} was not studied.

These studies prompted us to look at regulation of IRP-1 by oxidative stress in rat renal mesangial cells (RMC). Here we report studies on the mechanism of effects of Cd^{2+} and Fe^{3+} on IRP-1 in RMC, and on recombinant IRP-1 (rIRP-1) purified from *E. coli*. We show that Cd^{2+} decreases IRP-1 activities and induces aggregation through intermolecular disulfide-bond formation both in vitro and in vivo. In addition, aggregated IRP-1 associates with the cytoskeletal fraction in RMC. For comparison, Fe^{3+} can also down-regulate IRP-1 in conditions of serum starvation, with increased aconitase and decreased IRE-binding activities, but the decreased protein level is found in both cytosolic and cytoskeletal fractions.

2. Materials and methods

2.1. Cell culture

Rat mesangial cell cultures were established and cultured as previously described (Wang et al., 1996). Cells were grown in RPMI-1640 medium with 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C and used between passages 7 and 15. Overnight cultures were starved in RPMI 1640 with 0.2% FBS for 48 h to render them quiescent. Cells were treated with various concentration $CdCl_2$ or ferric ammonium citrate (FAC) in serum-free medium, and parallel SF controls were included. For inhibitor studies, cells were pre-treated with 10 μM KN-93 or 50 μM butylated hydroxyanisole (BHA) in serum-free media for 1 h followed by $CdCl_2$ co-treatment for 6 h.

2.2. Cell fractionation

Total cell lysate was prepared by washing cells three times with ice-cold PBS prior to lysis in extraction buffer containing 10 mM HEPES, pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 1 mM DTT with 0.2% Nonidet P-40 with protease inhibitors. Lysates were then sonicated twice for 5 s and centrifuged at $10,000 \times g$ for 10 min. The supernatant was designated crude cytoplasmic extract.

For preparation of cytosol and cytoskeletal-enriched fractionations, cells were lysed with 10 mM Tris–HCl, pH 7.4, containing 2 mM $MgCl_2$, 138 mM KCl, and 0.2% Triton X-100 with protease inhibitors. The lysates were centrifuged at $10,000 \times g$ for 15 min and supernatants were collected as the cytosolic fraction. The pellet was washed once with PBS and resuspended in 5 mM Tris–HCl, pH 8.0, with 0.2 mM $CaCl_2$ and 200 μM ATP, sonicated three times at 5 s and centrifuged at $10,000 \times g$ for 5 min. The supernatants contained the cytoskeletal fraction.

Subcellular fractions were isolated by differential detergent fractionation (Biederbick et al., 2006; Popovic and Templeton, 2012). Cells were washed with ice-cold PBS and pelleted at $500 \times g$ for 5 min. The cell pellets were resuspended with 0.007% digitonin in 5 mM Tris–HCl, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$ and protease inhibitors. The suspensions were incubated for 8 min on ice and centrifuged at $1800 \times g$ for 8 min. The supernatant was further centrifuged at $15,000 \times g$ for 20 min, and the second supernatant was taken as the cytosolic fraction. The pellet was washed twice with ice-cold PBS and resuspended in 20 mM Tris–HCl, pH 7.4, containing 2 mM $MgCl_2$, 138 mM KCl, 0.5% Triton X-100 with protease inhibitors, and incubated on ice for 30 min. The suspension was centrifuged at $8000 \times g$ for 10 min and the supernatant was designated the membrane fraction. The detergent-insoluble pellet was washed three times with ice-cold PBS and resuspended in 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, with 0.5% sodium deoxycholate and 1% SDS. The suspension was sonicated twice for 5 s and boiled for 5 min. Samples were centrifuged at $16,000 \times g$ for 5 min and the supernatant was taken as the cytoskeletal fraction. Cytosolic fraction was shown to be free of the mitochondrial marker apoptosis inducing factor (AIF) and the membrane protein trasferrin receptor (TfR); these two proteins were localized in the membrane fraction, which is free of the cytosolic marker GAPDH (see Fig. 6A).

2.3. Purification of recombinant IRP-1

Human recombinant IRP-1 (rIRP-1) was purified from *E. coli* transformed with pT7-His-IRP-1 plasmid, isolated with Ni^{2+}-nitrilotriacetate agarose beads (Qiagen, Mississauga, ON, Canada) and eluted in buffer N (24 mM HEPES, pH 7.6, with 150 mM potassium acetate, 1.5 mM $MgCl_2$, and 5% glycerol) with