Oxalomalate, a competitive inhibitor of aconitase, modulates the RNA-binding activity of iron-regulatory proteins

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We investigated the effect of oxalomalate (OMA, α-hydroxy-β-oxalosuccinic acid), a competitive inhibitor of aconitase, on the RNA-binding activity of the iron-regulatory proteins (IRP1 and IRP2) that control the post-transcriptional expression of various proteins involved in iron metabolism. The RNA-binding activity of IRP was evaluated by electrophoretic mobility-shift assay of cell lysates from 3T3-L1 mouse fibroblasts, SH-SY5Y human cells and mouse livers incubated *in vitro* with OMA, with and without 2-mercaptoethanol (2-ME). Analogous experiments were performed *in vivo* by prolonged incubation (72 h) of 3T3-L1 cells with OMA, and by injecting young mice with equimolar concentrations of oxaloacetate and glyoxylate, which are the precursors of OMA synthesis. OMA remarkably decreased the binding activity of IRP1 and, when present, of IRP2, in all samples analysed. In addition, the recovery of IRP1 by 2-ME in the presence of OMA was constantly lower versus control values. These findings suggest that the severe decrease in IRP1 RNA-binding activity depends on: (i) linking of OMA to the active site of aconitase, which prevents the switch to IRP1 and explains resistance to the reducing agents, and (ii) possible interaction of OMA with some functional amino acid residues in IRP that are responsible for binding to the specific mRNA sequences involved in the regulation of iron metabolism.

Key words: α-hydroxy-β-oxalosuccinic acid, iron metabolism, RNA-binding protein.

INTRODUCTION

Oxalomalate (OMA), a tricarboxylic acid (α-hydroxy-β-oxalosuccinic acid) formed *in vitro* and *in vivo* by condensation of oxaloacetate with glyoxylate, has long been known to be a powerful competitive inhibitor of aconitase [1–7]. This enzyme, which is required for the first step of the citric acid cycle, attracted renewed attention with the discovery of the structural identity between cytosolic aconitase and the iron-regulatory protein (IRP) 1. IRP1 and a structurally related protein, IRP2, are RNA-binding proteins that, by binding to a different site of a stem-loop region of the iron-responsive element (IRE), control the post-transcriptional expression of proteins involved in iron metabolism (see [8–13] for reviews).

Because OMA inhibits cytosolic aconitase, it is feasible that it also affects the IRE-binding activity of IRP1. A preliminary study showed that OMA added *in vitro* to lysates of a confluent culture of 3T3-L1 fibroblasts reduced the RNA-binding capacity of IRP1 [14]. This finding suggested that OMA plays a role in the regulation of iron metabolism and raised questions about the mechanisms by which OMA could modify the IRE-binding activity of IRP1. In an attempt to clarify these issues, we have analysed *in vitro* the IRE-binding activity of IRP1 in cell lysates of 3T3-L1 mouse fibroblasts, of SH-SY5Y cells derived from human nervous tissue and of mouse liver, with and without OMA, under reducing and non-reducing conditions. We also performed experiments *in vivo* on 3T3-L1 fibroblasts cultured with OMA for different times, and on young mice treated with equimolar concentrations of oxaloacetate and glyoxylate, known to be precursors of OMA synthesis. OMA caused a remarkable decrease in the IRE-binding activity of IRP1 and, when present, of IRP2, which is reported to lack aconitase activity [15].

EXPERIMENTAL

Cell cultures and treatment

Low-passage-number mouse 3T3-L1 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml) and L-glutamine (2 mM). SH-SY5Y human neuroblastoma cells were grown in Ham's F12 nutrient mixture/Eagle's minimal essential medium (1:1) supplemented with 10% foetal bovine serum, 1% non-essential amino acids, penicillin (50 units/ml), streptomycin (50 μg/ml) and L-glutamine (1 mM). The cells were grown at 37 °C in a 5% CO_2 atmosphere.

For studies on iron-repleted cells, confluent cultures of 3T3-L1 cells were treated for 18 h with 20 μg/ml of ferric ammonium citrate. The chelation of intracellular iron was achieved on 18-h treatment with 100 μM desferrioxamine mesylate (Desferal; Ciba-Geigy, Varese, Italy). Oxalomalic acid trisodium salt was prepared as described in [2] or purchased from Sigma (St. Louis, MO, U.S.A.). Oxaloacetic acid and glyoxylic acid sodium salt were produced by Fluka Chemika (Buchs, Switzerland).

For experiments *in vivo*, confluent cultures of 3T3-L1 cells were incubated at 37 °C with 5 mM OMA for 24, 48 and 72 h, after which RNA–protein complexes were evaluated by electrophoretic mobility-shift assay (see below).

Abbreviations used: OMA, oxalomalic acid; IRP, iron-regulatory protein; IRE, iron-responsive element; Desferal, desferrioxamine mesylate; 2-ME, 2-mercaptoethanol.

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Preparation of cytosolic extracts

3T3-L1 and SH-SY5Y cells were washed and scraped off with PBS containing 1 mM EDTA. To obtain cytosolic extracts, cells were lysed at 4 °C in lysis buffer containing 10 mM Hepes, pH 7.5, 3 mM $MgCl_2$, 40 mM KCl, 5 % glycerol, 1 mM dithiothreitol and 0.2 % Nonidet P-40. After lysis, samples were diluted 3-fold with lysis buffer without Nonidet P-40. Nuclei and mitochondria were removed by centrifugation for 30 min at 100000 ***g*** in a Beckman L8-70 ultracentrifuge, and the supernatants were frozen in small aliquots at −80 °C. The protein concentration was determined by a Bio-Rad protein assay used according to the supplier's manual (Bio-Rad Laboratories, Milan, Italy).

OMA treatment *in vitro*

Cytosolic lysates (5 μg) from 3T3-L1 and SH-SY5Y cells and from mouse liver (see below) were incubated with various concentrations of OMA (0.5, 1, 2.5 and 5 mM) at the times indicated in each experiment. The reaction volume was adjusted to about 19 μl with lysis buffer with Nonidet P-40 diluted to 0.07 %. In alternative reaction mixtures 2 % 2-mercaptoethanol (2-ME) was added 10 min after incubation with OMA. In all samples the binding reaction was started by the addition of 0.2 ng of radiolabelled RNA, giving a final volume of 20 μl, and left for 30 min at room temperature (for further details see the section on electrophoretic mobility-shift assay, below).

Treatment *in vivo* of mice and preparation of liver extracts

Male Swiss mice (Mario Negri Sud, Chieti, Italy) weighing about 30–40 g were used. The mice were starved for 20 h (water *ad libitum*) and then injected subcutaneously with aliquots of a solution that yielded 92.6 mg of neutralized oxaloacetic acid and 50.3 mg of sodium glyoxylate per 100 g of body weight. Other mice were injected with a half dose and the controls were injected with physiological solution. After 1 h all the mice were killed by CO_2 and the livers were removed, washed in physiological solution and 500 mg were homogenized at 4 °C in 2 ml of lysis buffer supplemented with 350 mM sucrose, 2.5 μg/ml leupeptin and 0.5 mM PMSF. The homogenates were centrifuged at 100000 ***g*** for 30 min at 4 °C in a Beckman L8-70 ultracentrifuge and the supernatants were frozen in small aliquots at −80 °C. The protein concentration was determined by the Bio-Rad protein assay used according to the supplier's manual.

Electrophoretic mobility-shift assay

Plasmid pSPT-fer, containing the sequence corresponding to the IRE of the H-chain of human ferritin mRNA, was kindly provided by Professor G. Cairo (University of Milan, Milan, Italy). This plasmid was linearized at the *Bam*H1 site and transcribed *in vitro* with T7 RNA polymerase (Promega, Madison, WI, U.S.A.). The transcription reaction was performed at 38.5 °C for 1 h with 200 ng of plasmid DNA in the presence of 50 μCi of [α-^{32}P]CTP (800 Ci/mmol; Amersham Corporation, Milan, Italy) and 0.5 mM ATP, GTP and UTP (Promega) in a 20-μl reaction volume. The DNA template was digested with 10 units of RNase-free DNase 1 for 10 min at 37 °C. Free nucleotides were removed on a Sephadex G-50 column (Boehringer-Mannheim, Milan, Italy).

For electrophoretic mobility-shift analysis, 5 μg of protein extracts were mixed with 0.2 ng of *in vitro*-transcribed ^{32}P-labelled IRE RNA with or without unlabelled competitor RNA. The reaction was performed, as reported above, in lysis buffer with Nonidet P-40 adjusted to 0.07 %, in a final volume of 20 μl for 30 min at room temperature. Then, to degrade unbound probe, the reaction mixture was incubated with 1 unit of RNase T1 (Calbiochem, La Jolla, CA, U.S.A.) for 10 min, and non-specific RNA–protein interaction was displaced by the addition of 5 mg/ml heparin for 10 min. A 6 % non-denaturing polyacrylamide gel was pre-electrophoresed for 20 min at 200 V. Then, after the addition of 10 μl of loading buffer containing 30 mM Tris/HCl, pH 7.5, 40 % sucrose and 0.2 % Bromophenol Blue, the reaction mixtures were electrophoresed for 2 h at the same voltage. The dried gel was autoradiographed at −80 °C. The IRP–IRE complexes were quantified with a GS-700 imaging densitometer (Bio-Rad Laboratories).

Cytosolic aconitase assay

Cytosolic aconitase activity was determined on the liver lysates used for the mobility-shift assay, by monitoring the disappearance of *cis*-aconitate at 240 nm for 10 min [6,7] from a sample containing 0.2 mM *cis*-aconitate and 100 μg of protein in 10 mM Hepes buffer, pH 7.5, in a final volume of 1 ml.

RESULTS

OMA modulates the IRE-binding activities of IRP1 in 3T3-L1 and SH-SY5Y cell lines and in mouse liver

IRP1, a 98-kDa protein, possesses structural identity with 4Fe-4S cytosolic aconitase [16,17], and the mechanism of the switch from aconitase to IRP1 was clarified by experiments of site-directed mutagenesis that revealed the disassembling steps of the [4Fe-4S] cluster of cytosolic aconitase [18,19]. Consequently, it was found that the addition of iron to IRP1 restores aconitase activity, thus preventing RNA-binding, which implies that IRP1/aconitase plays a bifunctional role [20,21]. IRP2 shares 61 % amino acid identity with IRP1, a major difference being a 73-amino acid insertion rich in cysteine residues [22,23], and it has a different pattern of tissue specificity and expression level [24].

Because OMA acts as an efficient competitive inhibitor of liver cytosolic aconitase [6], we predicted that OMA linked to the active site can affect the RNA-binding capacity of IRP1 by preventing the aconitase/IRP1 switch [14]. To verify this hypothesis, we investigated the effect *in vitro* of OMA on lysates obtained from mouse 3T3-L1 fibroblasts, from cells of human neural origin and from mouse livers. We also investigated the effect *in vivo* both by incubating 3T3-L1 fibroblasts with OMA for a short period, and by treating young mice with appropriate doses of OMA precursors (see the Experimental section). Because the addition of high concentrations of reducing agents to cell extracts enhances IRP1 RNA-binding activity, thereby revealing the total 'activatable' amounts of IRP1 [25], we compared the effect of OMA with and without 2 % 2-ME. In all samples tested, OMA greatly decreased the amount of IRP1 available for RNA binding (Figure 1). The experiments in which 2-ME was added after incubation with OMA showed that the recovered 'activatable' RNA-binding activity was always less than that recovered without the inhibitor. This finding indicates that a fraction of the OMA added was tightly bound to the active site of aconitase and consequently less IRP1 was recovered by reduction. Finally, in the presence of OMA, 3T3-L1 cells unexpectedly showed a remarkable decrease in the signal corresponding to the RNA-binding activity of IRP2.

The effect of OMA on IRP1 activity is dose- and time-dependent

Because the results shown in Figure 1 demonstrated that the fraction of aconitase in which OMA is bound to the active site

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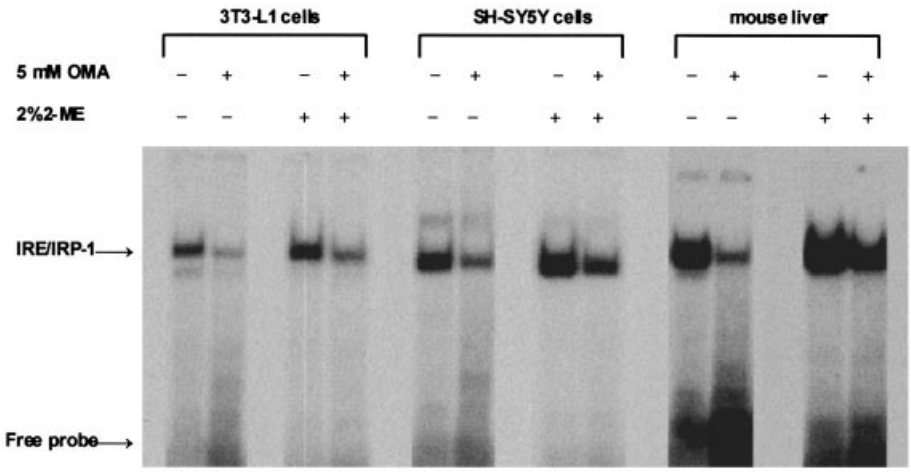

Figure 1 Effect of OMA on the RNA-binding capacity of IRP1 in different experimental systems

Protein extracts (5 μg) were incubated with 5 mM OMA for 10 min, then the RNA–protein binding reaction was started by the addition of 0.2 ng of ^{32}P-labelled IRE probe (6×10^4 c.p.m.) with and without 2% 2-ME, as indicated. The autoradiogram shows the complexes corresponding to IRE-bound IRP1, and the residual unbound RNA probe (free probe). In 3T3-L1 cells a faster-migrating band is present corresponding to IRE-bound IRP2. These data are typical of three experiments.

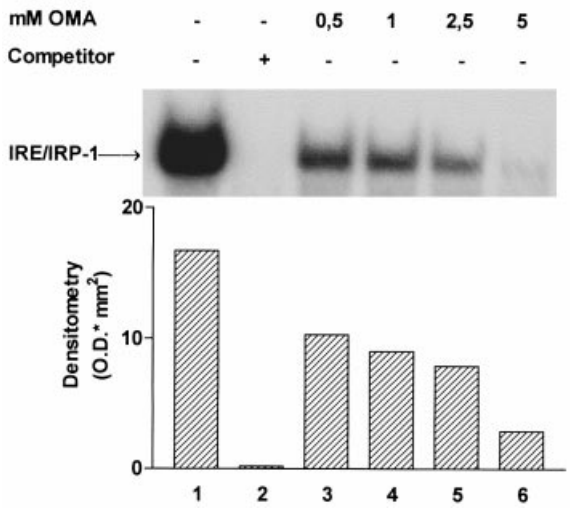

Figure 2 Dose-dependence of the effects of OMA on IRP activity

Cytosolic extracts from mouse liver were treated for 10 min with increasing concentrations of OMA before the addition of ^{32}P-labelled IRE probe. The IRE–IRP1 complexes were determined by electrophoretic mobility-shift assay and quantified with an imaging densitometer. The relative activities are plotted as arbitrary units.

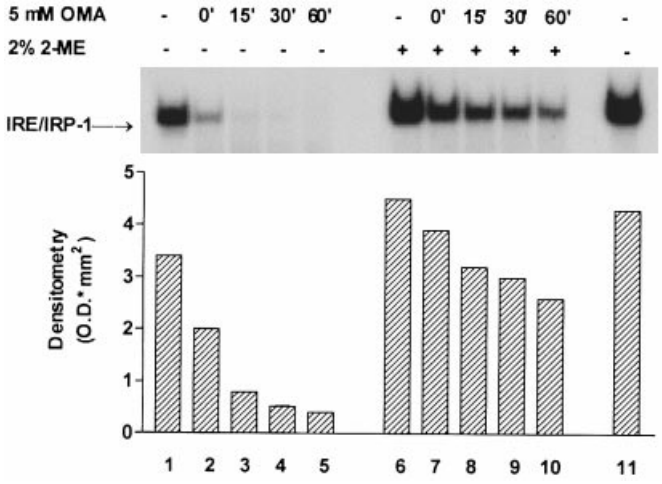

Figure 3 Time dependence of the effects of OMA on IRP activity

Cytosolic extracts from mouse liver were incubated with 5 mM OMA for the times indicated and then treated or not with 2% 2-ME before the addition of ^{32}P-labelled IRE probe. Lane 11 shows the IRE–IRP1 binding activity of the same cytosolic extract kept at room temperature for 60 min before addition of the probe. The IRE–IRP1 complexes were quantified by densitometry. The relative activities are plotted as arbitrary units.

cannot be recovered by 2-ME, we investigated the mechanism by which OMA might modify the residual '2-ME-activatable' fraction of IRP1. We first determined the dose- and time-dependence of the effects exerted by OMA on the cytosolic extract from the liver of untreated mice. As shown in Figure 2, 0.5 mM OMA significantly decreased the RNA-binding activity of IRP1 and the effect increased with concentrations up to 5 mM, at which point RNA-binding activity was negligible. Therefore, we used a 5 mM concentration of OMA in all the experiments described herein.

We next evaluated the time-dependence of the effect of OMA by incubating extracts at room temperature, with and without OMA, and by adding 2% 2-ME at the end of incubation with OMA (Figure 3). IRP1 RNA-binding activity decreased very rapidly and practically disappeared between 15 and 60 min of OMA exposure. This effect was not due to protein degradation because after 60 min (Figure 3, lane 11) the IRP1 binding capacity was not modified in the untreated sample. Moreover, the recovery of the binding activity of IRP1 by 2-ME never reached the value of the control and decreased as the incubation

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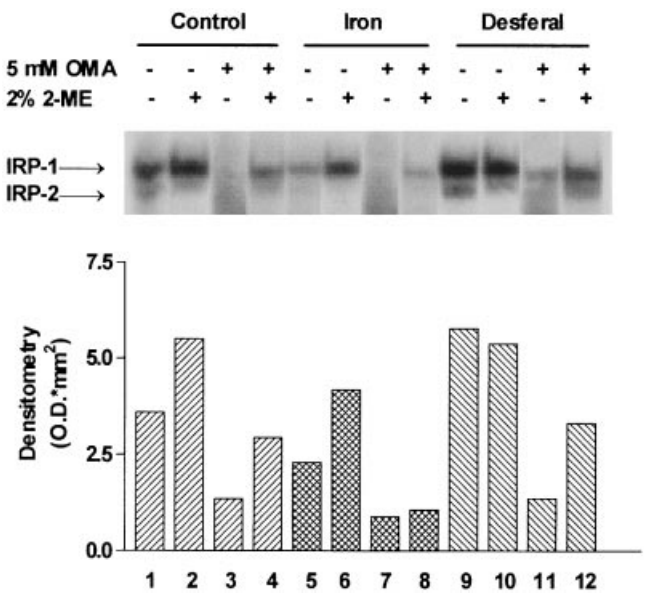

Figure 4 Effects *in vitro* of OMA on IRP from iron-repleted and -depleted cells

3T3-L1 cells were treated for 18 h with 20 μg/ml ferric ammonium citrate (lanes 5–8) or with 100 μM Desferal (lanes 9–12) in comparison with untreated cells (lanes 1–4). The obtained cytosolic extracts were incubated with and without 5 mM OMA for 30 min at room temperature. Where indicated, 2% 2-ME was added before the addition of ^{32}P-labelled IRE probe. The IRP–IRE complexes were resolved by electrophoretic mobility-shift assay and plotted as arbitrary units.

time increased. This finding shows that the formation of the inhibitor–aconitase complex was time-dependent.

OMA *in vitro* modulates the RNA-binding activity of IRP1 and of IRP2 from iron-repleted as well as from iron-depleted cells

The RNA-binding activity of IRP1 depends on the amount of iron available to the cell [8,9,21]. We analysed lysates of 3T3-L1 cells maintained in an iron-rich medium containing ferric ammonium citrate, and in an iron-depleted medium containing Desferal, a potent iron chelator. Also in the 3T3-L1 cells, iron repletion and depletion provoked a decrease and an increase, respectively, in the RNA-binding capacity of IRP1 and IRP2 (Figure 4). As expected, the effect of OMA was much more evident in the iron-repleted than in the iron-depleted cells. It is noteworthy that, in the presence of an excess of iron, 2-ME was unable to restore IRP1, a finding that reinforces the concept of stability of the association between the inhibitor and the [4Fe-4S] cluster. IRP2 was absent from lysates of iron-repleted cells because iron accelerates the turnover of the protein [26,27], but, when present as in the control and in iron-depleted cells, it decreased upon the addition of OMA. Interestingly, 2-ME restored the IRP2 activity in iron-depleted cells. Since IRP2 does not possess an [Fe-S] cluster, but has five cysteine residues in its specific 73-amino acid insertion [22,23], it is not unreasonable to predict that OMA might interfere also with IRP2 cysteine residues.

OMA synthesized *in vivo* inhibits cytosolic aconitase activity and modulates the RNA-binding activity of IRP1

Early experiments demonstrated that OMA synthesis occurs *in vivo* by the condensation of two cellular metabolites, namely oxaloacetate and glyoxylate [1,3,7]. In light of this finding, we investigated the effects of OMA *in vivo* both in the whole animal

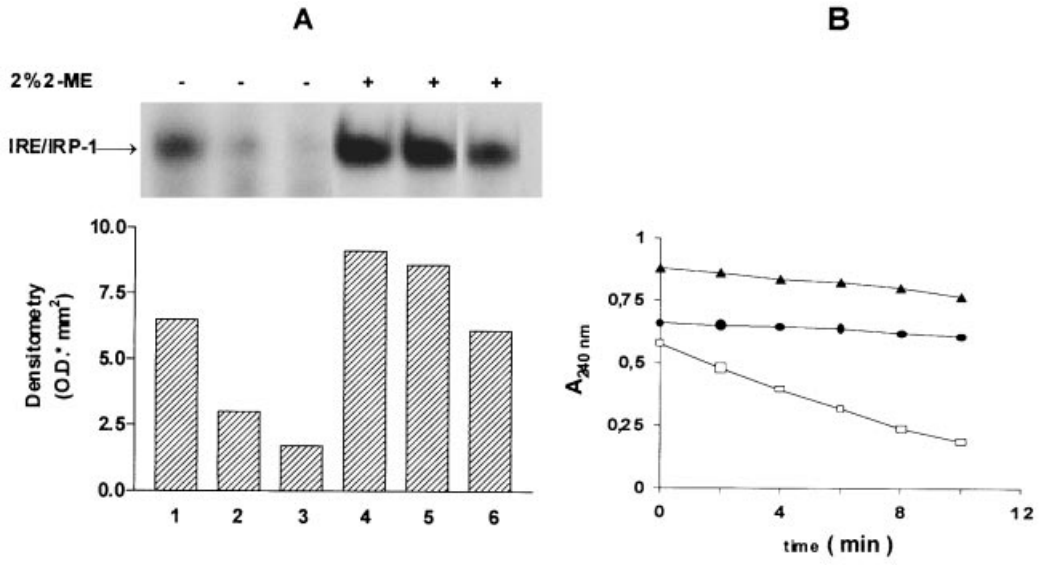

Figure 5 Effects *in vivo* of OMA

(**A**) Cytosolic extracts were prepared either from the livers of mice killed 1 h after injection of equimolar amounts of oxaloacetate plus glyoxylate (corresponding to $\approx$ 2.5 mM OMA in lanes 2 and 5, and $\approx$ 5 mM OMA in lanes 3 and 6), or from livers of mice injected with physiological solution (lanes 1 and 4). As indicated, 2% 2-ME was added before the reaction was started with the ^{32}P-labelled IRE probe. The IRE–IRP1 complexes were quantified by densitometry and reported in arbitrary units. (**B**) The aconitase assay was performed on 100 μg/ml cytosolic extract used for RNA mobility-shift assay. Aconitase activity was evaluated by monitoring the disappearance of *cis*-aconitate at 240 nm: □, control; ▲, lower dose of OMA precursors (2.5 mM); ●, higher dose of OMA precursors (5 mM).

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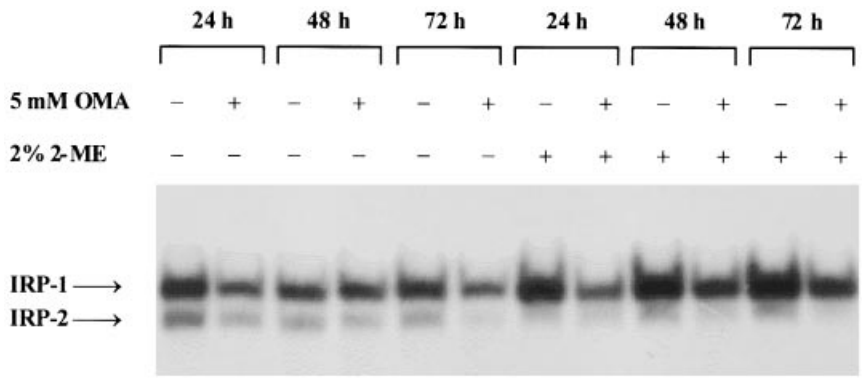

Figure 6 Effects of OMA treatment on IRP1 and IRP2 RNA-binding activity in 3T3-L1 cells

Confluent cultures of 3T3-L1 cells were grown with and without 5 mM OMA for the times indicated. Cytosolic extracts were incubated at room temperature with and without 2% 2-ME before the addition of ^{32}P-labelled IRE probe. Arrows indicate the positions of complexes IRE–IRP1 and IRE–IRP2.

and on cultured 3T3-L1 cells. In the first experiment we injected subcutaneously into young mice amounts of glyoxylate and oxaloacetate that approximately reproduced, in the whole body, the concentrations of 2.5 and 5 mM OMA used *in vitro*. Other mice injected with physiological solution served as controls. All animals were killed 1 h after the injection. Cytosolic extracts were prepared as described in the Experimental section. Aliquots of the same extracts were used to assay aconitase activity and the RNA-binding capacity of IRP1. The effect exerted by the newly synthesized OMA on IRP1 RNA binding resulted in the disappearance of IRP1 (Figure 5A). The addition of 2-ME confirmed the difference in the recovery of IRP1 activity between the control and treated mice (Figure 5A). As expected, *cis*-aconitate absorbance decreased in the liver of controls while it remained unchanged in the treated mice even in those given the lower concentration of OMA precursor (Figure 5B). This finding confirms that aconitase was completely inhibited.

In the second experiment, confluent cultures of 3T3-L1 were treated with 5 mM OMA for the times indicated in Figure 6. In mobility-shift analyses of lysates, the effect of OMA became evident after 24 h and remained almost constant up to 72 h. The 2-ME-induced recovery of IRP1 was always lower versus controls, which confirms yet again that the OMA linked to the active site cannot be removed. IRP2 binding decreased up to 72 h, more remarkably in OMA-treated cells. The addition of 2-ME did not restore IRP2 activity. These results demonstrate that OMA synthesized *in vivo*, or added to long-term cell cultures, possesses the same capacity to inhibit formation of the RNA–IRP complex as observed *in vitro*.

DISCUSSION

The results of our experiments show that the addition of OMA *in vitro* and *in vivo* to lysates from various sources leads to a constant decrease in the RNA-binding activity of IRP1 and, surprisingly, also of IRP2, when present. Experiments with the reducing agent 2-ME, which in the presence of OMA only partially restored the RNA-binding capacity of IRP, confirm that a fraction of OMA is tightly fixed to the active site of aconitase. These findings agree with previous observations that aconitase substrates prevent full recovery of the binding activity promoted by 2-ME in different cell lines [19,21]. OMA decreased the binding of IRP to practically the same extent in iron-repleted cells, which are rich in aconitase, and in iron-depleted cells, rich in IRP1 and IRP2. Interestingly, 2-ME only minimally restored IRP binding in iron-enriched cells compared with iron-depleted cells. This finding indicates that iron also increases the stability of the OMA complex with the [Fe-S] cluster. In all our experiments, OMA decreased the binding activity of IRP2; this suggests reactivity with a protein that lacks an [Fe-S] cluster. Therefore, OMA may also interact with IRP1, in which the [Fe-S] cluster of aconitase is disassembled. Consequently, OMA may prevent binding of IRP to IRE regions in two ways. One entails direct contact with the cluster; in this case OMA mimics the substrate with which it shares close structural analogy. The other is by decreasing the efficiency of the active components of IRP, i.e. the amino acid residues cysteine, arginine and/or lysine [19,28], which are involved in the interaction with the mRNA IRE regions that control iron metabolism.

We believe that, besides reacting with the [4Fe-4S] cluster, the highly negative-charged OMA might well interfere with the amino acid residues involved in IRE binding by impeding the post-transcriptional control exerted by IRP. This hypothesis is supported by the finding that the newly synthesized OMA in mouse livers, which inhibits aconitase activity *in vivo*, promotes the decrease of RNA-binding capacity of IRP1. Moreover, in 3T3-L1 cells cultured with OMA, IRP2 also had a modulating effect on RNA-binding capacity, similar to IRP1, and a response to 2-ME at all times, analysed similarly to that observed *in vitro*. These results obtained *in vivo* strongly support the earlier observation [14], obtained *in vitro*, that OMA, by modulating the activity of IRP1 and IRP2, directly participates in the complex system of iron-metabolism regulation. In addition, the results *in vivo* also support the utility of OMA synthesis in living cells because, besides regulating the flux of the citric acid cycle, OMA appears to exert another important function in the regulation of mRNA expression in iron metabolism.

In conclusion, the data presented shed new light on early reports of OMA synthesis in mammalian tissues [1,3,7]. The specific effect of OMA on the cytosolic and mitochondrial aconitase give a meaning to the presence of the cytosolic form of this enzyme, whose function in the cytoplasm is unknown. Moreover, our data suggest that OMA plays a role in the complex mechanism of iron-metabolism regulation. Since OMA precursors also lead to the synthesis of OMA in animal tissues, events occurring in mitochondria, such as interruption of the citric acid cycle, may be correlated with cytosolic events, such as translational control of specific mRNA.

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