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Amino Acid Deprivation Decreases Intracellular Levels of Reactive Oxygen Species in Hepatic Stellate Cells

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Key Words
Hepatic stellate cells • Histidine deprivation • ROS • GCN2 • SOD

Abstract
In eukaryotic cells amino acid deprivation triggers a response aimed to ensure cell survival in stress conditions. In the present work we analyzed the effects of amino acid deprivation on intracellular levels of reactive oxygen species (ROS) of hepatic stellate cells (HSC), a key cell type in the development of liver fibrosis. Histidine deprivation caused in the human immortalized HSC cell line LX-2 a fast decrease of intracellular ROS levels that was also observed in HSC incubated either with leucine-free or amino acid-free medium, but not with glucose-free medium. Phosphorylation of GCN2 kinase and its substrate eIF2α was induced by histidine deprivation. Reversion studies and activation of GCN2 by tRNA and the proteasome inhibitor MG-132 showed a correlation between GCN2 phosphorylation and diminished ROS levels. However, a lack of correlation between eIF2α phosphorylation and ROS levels was found using salubrinal, an inhibitor of eIF2α phosphorylation, suggesting a role for GCN2 unrelated to its activity as eIF2α kinase. LX-2 cells treated with histidine-free medium presented reduced SOD activity that could account for the decrease on ROS levels. Histidine deprivation as well as activation of GCN2 by treatment with tRNA, caused an increase in LX-2 cell viability, suggesting amino acid restriction to present a protective effect in HSC which is mediated by GCN2 activation.

Introduction

In mammalian cells amino acid availability has been shown to activate signaling molecules and to regulate different cell functions. Unlike other nutrients, free amino acids are not stored in the cell and several of them, the so-called essential amino acids, cannot be synthesized from other metabolic intermediates and need to be provided in the diet. Therefore, changes in intracellular amino acid levels act as nutritional signals regulating molecular mechanisms that are different for amino acid abundance or deficiency. Amino acid abundance leads to the activation of the soluble kinase mTOR, which induces S6K phosphorylation, increased translation of mRNAs

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encoding for ribosomal proteins and a higher protein synthesis rate [1, 2]. These effects can be elicited by leucine, an essential amino acid with specific signaling properties [3, 4]. In contrast, the response to amino acid deficiency is activated by the deprivation of any single essential amino acid or by blocking the synthesis of an otherwise nonessential one [5]. A key mediator of amino acid deficiency is GCN2 (general control nonderepresible 2) kinase, an enzyme that undergoes activation by direct binding of uncharged tRNA through a histidine-tRNA synthetase homologous domain [5, 6]. Regulation of other enzymes such as ERK, JNK, p38 MAPK, Akt and mTOR [7-10] has also been described in different cell lines cultured in the absence of an essential amino acid.

The effects of amino acid deprivation are aimed to ensure cell survival in stress conditions. Global protein synthesis is decreased due to GCN2-dependent phosphorylation of translation initiation factor eIF2α and the subsequent inhibition of eIF2B activity, which is required to recycle eIF2 and maintain high rates of translation initiation. However, a specific set of proteins like transcription factor ATF-4 [11] or CDK inhibitors p21 and p27 [7] escape from this inhibitory effect and present higher expression levels in response to amino acid deprivation. On the whole, these alterations result in a reconfiguration of the gene expression pattern, affecting proteins involved in cell cycle control and amino acid transport and metabolism [5]. Interestingly, recent data suggest that amino acid deprivation can also regulate other pathways unrelated to protein biosynthesis such as hepatic lipid metabolism [12-14].

Moderate production of ROS has been shown to regulate gene expression and to mediate the action of cytokines and growth factors through the activation of different signaling pathways [15, 16]. The effects of amino acid deficiency on ROS production are controversial. Studies performed in animal models for methionine restriction, describe a decrease in mitochondrial ROS levels and oxidative damage to mitochondrial DNA that could be responsible for the well-documented beneficial effects of caloric or protein restriction in the aging process [17, 18]. Although these results suggest a protective antioxidant action for amino acid restriction, there are also data both in yeast [19] and mammalian cells [9, 10] indicating that amino acid deprivation can also cause a pro-oxidant effect.

Hepatic stellate cells (HSC) are the cell type mainly responsible for the accumulation of extracellular matrix characteristic of liver fibrosis. Activation of HSC caused by liver damage leads to an increased proliferation and fibrogenic activity of these cells, and to a scar-formation process that can become irreversible. Several reports indicate that oxidative stress regulates the fibrogenic activity of HSC [20]. ROS produced by HSC or generated by other cell types have been demonstrated to induce the production of collagen type I and other extracellular matrix proteins and to mediate the effects of the profibrogenic cytokine TGF-β [21, 22]. We have previously shown that HSC respond to nutritional signals such as increased leucine levels with an enhanced production of ROS and a specific up-regulation of collagen translation [23, 24]. In the present work we analyzed the effect of amino acid deprivation on ROS generation by HSC, as well as the molecular mechanisms involved.

Materials and Methods

Reagents

Earle's Balanced Salt Solution (EBSS), MEM without histidine, without leucine, or without glucose, trypan blue and tRNA were obtained from Sigma-Aldrich (St. Louis, MO). Cell culture reagents were obtained from Gibco BRL (Grand Island, NY). Chloromethyl-2',7'-dichlorofluorescein diacetate (CM-H_2DCFDA) was from Molecular Probes (Eugene, OR). Salubrinal and Superoxide Dismutase Assay kit were purchased from Calbiochem® (Darmstadt, Germany). MG-132 was from Alexis Biochemicals (Lausen, Switzerland).

Cell culture and treatments

Most experiments were carried out using the human HSC line LX-2. This non-tumoral cell line was obtained after immortalization in low media culture of HSC isolated from normal primary human HSC [25] and was kindly provided by Dr S.L. Friedman, Mount Sinai School of Medicine, New York. Cells were cultured in Dulbeccos's modified Eagle's medium (DMEM) supplemented with 1% bovine foetal serum (FBS), penicillin and of streptomycin and non-essential amino acid for 36 h. The medium was replaced for serum-free DMEM for 12 h, after which treatments were carried out.

Primary hepatic stellate cells (HSC) were isolated from Wistar rats by liver pronase and liberase perfusion, followed by a nycodenz gradient centrifugation. Isolated rat HSC were maintained in culture with DMEM-F12 supplemented with 10% FBS, penicillin, streptomycin and fungizone for 6 days. After that the medium was replaced for serum-free DMEM-F12 for 12 h and cells treated as above.

Cells were treated by replacing the media for MEM without histidine, leucine or glucose. Medium of control cells was also replaced by MEM. In some experiments cells were pretreated for 12 hours with salubrinal (75 μM). When indicated, cells were treated for 30 min with 10, 20 or 50 μg/ml of tRNA or with MG-132 (10 μg/ml).

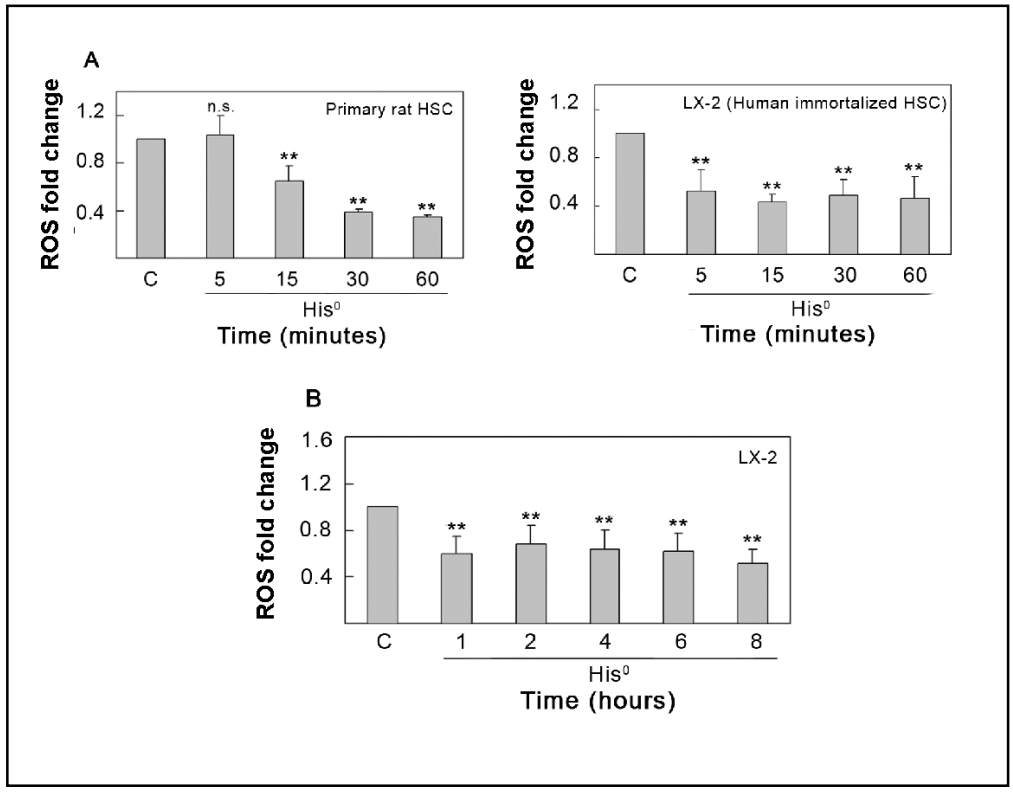

Fig. 1. Time course analysis of ROS production in hepatic stellate cells (HSC) incubated with histidine-deprived medium. Intracellular ROS levels were determined by fluorimetric assay using CM-H_2DCFDA as a probe. (A) Intracellular ROS levels in primary rat HSC and the cell line LX-2 (human immortalized HSC) incubated in medium without histidine for 5 to 60 min. (B) Intracellular ROS levels in LX-2 incubated in medium without histidine for 1 to 8 hours. Control represents non-treated cells. Each bar represents the mean±SD of fluorescence fold change compared to controls of at least triplicate data from four independent experiments (**p<0.01, vs. control, n.s., not significant).

Cell Viability

To evaluate cell viability *in vitro*, trypan blue staining was used for determination of dead cells by dye exclusion. For these assays 1 x 10^6 cells were seeded in 100 mm culture dishes and treated during 24 h either with histidine-free medium or with medium containing the indicated amounts of tRNA. The cells were trypsinized, incubated with trypan blue [0.2% in phosphate-buffered saline (PBS)] and counted within 3 minutes under light microscopy using a hemocytometer. Triplicate samples form two independent experiments were examined for each data point.

Measurement of intracellular ROS levels

Production of ROS was measured using the fluorescent probe CM-H_2DCFDA. CM-H_2DCFDA is freely permeable across cell membranes and is incorporated into hydrophobic lipid regions of the cell. ROS produced by the cell oxidizes H_2DCFDA to 2,7-dichlorofluorescein (DCF), the fluorescence of which is proportional to the ROS produced. The excitation and emission wavelengths for DCFDA were 485 nm and 530 nm. For time-course studies HSC were plated to subconfluence in 60 mm culture dishes and treated without either histidine, leucine or glucose for time periods ranging from 5 min to 8 h. Cells were loaded for 20 min with 10 μM CM-H_2DCFDA at 37 °C in the dark prior the treatment without the amino acid. Long time experiments were performed in a Cytofluor 2350 (Milipore). When indicated, HSC were pretreated with salubrinal or treated with tRNA or MG-132 in the above described conditions. Values are mean±SD of at least triplicate data from four independent experiments.

Western blot

For Western blot analysis 6 x 10^5 cells were seeded on 60 mm culture dishes. After treatment proteins were extracted in Triton ice-cold buffer containing 1% triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.1% SDS, 1mM EDTA, 1mM PMSF, 0.1 mM DTT and 1 μg/ml aprotinin. Protein concentration of the samples was determined by BCA (bicinchoninic acid assay). For immunobloting assay, equal amounts of protein (30 μg) were electrophoresed on polyacrilamide gels and proteins were electrophoretically transferred on nitrocellulose membranes (BioRad, Hercules, CA). Membranes were incubated with blocking solution at room temperature and with specific antibodies at 4 °C overnight. Antibodies against total GCN2, phospho-specific GCN2, total eIF2α, and phospho-specific eIF2α were purchased from Cell Signalling Technology (Beverly, MA). Anti β-actin antibody was from Sigma. After incubation with primary antibodies, membranes were washed and incubated with a secondary polyclonal (BioRad, Hercules, CA) or monoclonal (Amersham Life Science, Arlington Heights, IL) antibody, conjugated to horseradish peroxidase. Immunoreactive proteins were detected by enhanced chemiluminescence system (ECL; Roche Molecular Biochemicals, Lewes, United Kingdom). Figures are representative of at least three independent experiments.

Measurement of SOD activity in cell lysates

SOD activity was analyzed using the Superoxide Dismutase Assay Kit, which detects the activities of the three types of SOD (Cu/Zn-, Mn-, and Fe-SOD). This kit uses a tetrazolium salt for detection of superoxide radicals generated

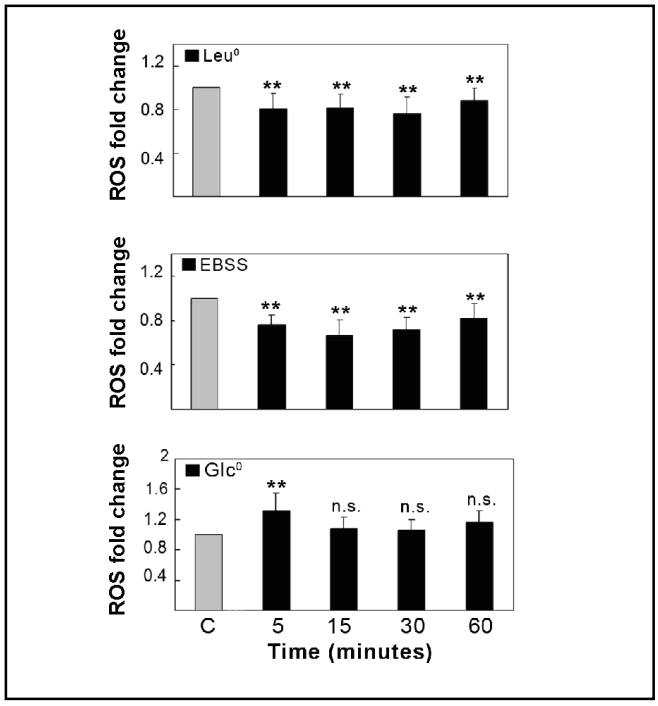

Fig. 2. Time course analysis of ROS levels in LX-2 incubated either with leucine-deprived medium, Earle's balanced salt solution (EBSS) or glucose-deprived medium. Intracellular ROS levels were determined using CM-H_2DCFDA as a probe. Control represents non-treated cells. Each bar represents the mean±SD of fluorescence fold change compared to controls of at least triplicate data from four independent experiments (**p<0.01, vs. control, n.s., not significant).

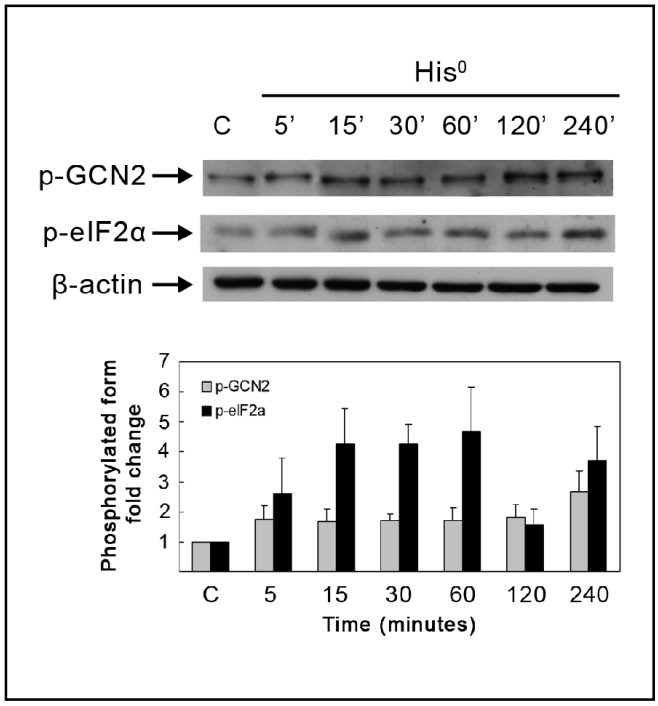

Fig. 3. GCN2 activation in LX-2 cells deprived of histidine. LX-2 cells were incubated with medium without histidine for the indicated times. GCN2 and eIF2α phosphorylation levels were determined by Western blot using specific antibodies. A representative immunoblot and data of GCN2 and eIF2-α phosphorylation fold change are included.

by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The absorbance was measured at 450 nm. For time-course studies HSC were plated to subconfluence in 60 mm culture dishes and treated either without histidine or with tRNA (20 μg/ml), for time periods ranging from 5 to 30 min. Cells were scraped and collected by centrifugation. Cell pellets were sonicated in cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose. The supernatants were stored for the assay. Measurement of SOD activity in cell lysates was carried out according to the manufacturer protocol.

Statistical analysis

Data were analyzed using the Kruskal-Wallis test to determine differences between all independent groups. When significant differences were obtained ($p<0.05$), differences between two groups were tested using the Mann-Whitney U test. In the neutral red assay in which the normality test was also significant, the Wilcoxon Test was used in order to test differences between the values obtained in the two groups.

Results

Histidine deprivation decreases intracellular ROS levels in HSC

The effects caused by amino acid deprivation in hepatic stellate cells (HSC) were studied by incubation of the cells in a medium lacking the essential amino acid histidine. Determination of reactive oxygen species (ROS) levels in amino acid-deprived HSC was carried out by fluorimetry using the probe CM-H_2DCFDA, which reacts mainly with peroxides and to a lesser extent with other ROS. Fluorimetric assays carried out at short incubation time periods in LX-2 cells and primary rat HSC showed a rapid and significant decrease on ROS levels caused by histidine-free medium (Fig. 1A). In LX-2 cells ROS levels diminished after 5 min without histidine, while in primary rat HSC the effect was observed after 15 min of incubation,

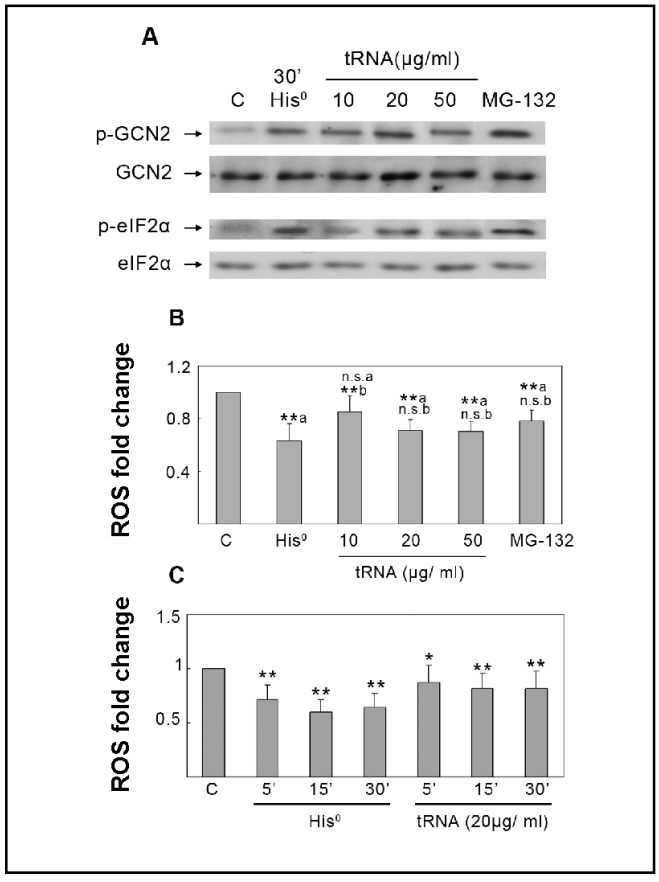

Fig. 4. Effect of tRNA and MG-132 (proteasome inhibitor) on GCN2 and eIF2α phosphorylation and intracellular H_2O_2 levels. (A) Effect of histidine deprivation, tRNA and MG-132 addition on phosphorylation levels of GCN2 and eIF2α. LX-2 cells were incubated for 30 min either with medium without histidine (His^0) or MG-132 (10 µg/ml) or with increasing amounts of tRNA (10 to 50 µg/ml). GCN2 and eIF2α phosphorylation levels were determined by Western blot using specific antibodies for the proteins and their phosphorylated forms. A representative immunoblot is shown. (B) Intracellular ROS levels in the cell line LX-2 (human immortalized HSC) treated with medium without histidine or MG-132 (10 µg/ml) with increasing amounts of tRNA (10 to 50 µg/ml). (C) Intracellular ROS levels after incubation for 5 to 30 min with medium without histidine or with 20 µg/ml tRNA. Intracellular ROS levels were determined using CM-H_2DCFDA as a probe. Control represents non-treated HSC. Each bar represents the mean±SD of fluorescence fold change compared to controls of at least triplicate data from four independent experiments (**p<0.01, a, vs. control, b vs. 30 min. without histidine treated cells, n.s., not significant).

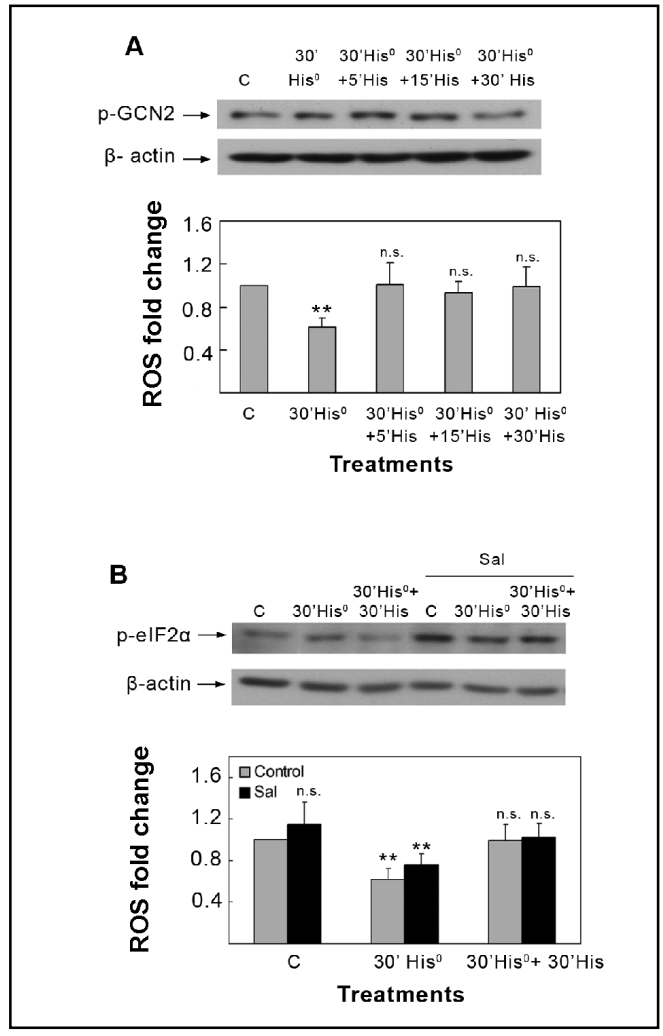

Fig. 5. Effect of histidine restoration on GCN2 and eIF2α phosphorylation and intracellular ROS levels in absence or presence of salubrinal. LX-2 cells were treated with medium without histidine for 30 min and after 5, 15 or 30 min the medium was replaced for complete medium containing histidine 270 µM. Phosphorylation levels of GCN2 were determined by Western blot using specific antibodies. Representative immunoblot is shown. Intracellular ROS levels were determined using CM-H_2 DCFDA as a probe. Control represents non-treated HSC. Each bar represents the mean±SD of fluorescence fold change compared to controls of at least triplicate data from four independent experiments (**p<0.01, n.s., not significant). (A) Reversion assay in the absence of salubrinal. (B) LX-2 cells were treated for 12 hours with 75 µM salubrinal, prior incubation for 30 min with medium without histidine.

reaching a maximum effect at 30 min. Similar experiments were performed in rat hepatocytes and mouse hepatoma cells HepaC1, and a moderate depletion of ROS levels was also observed in these cell types (data not shown).

ROS levels were also analyzed after incubation of LX-2 cells in the absence of histidine for longer time periods. As shown in Fig. 1B, the decrease on ROS levels caused by histidine-free culture medium was maintained for at least 8 hours.

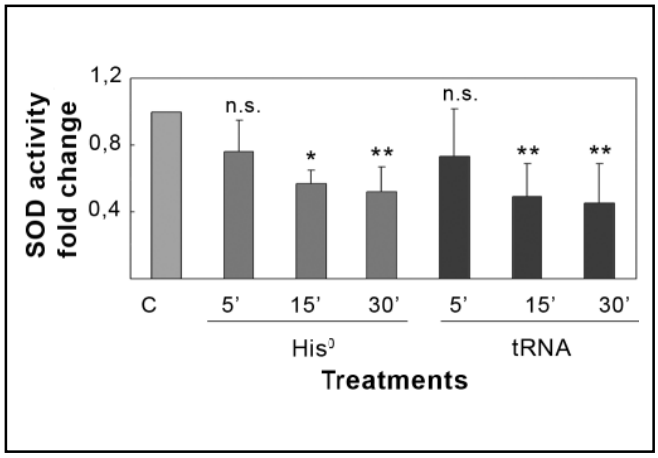

Fig. 6. Analysis of superoxide dismutase (SOD) activity in LX-2 cells treated with histidine-free medium or with tRNA. LX-2 cells were incubated for 5 to 30 minutes either with medium without histidine or with 20 μg/ml tRNA. SOD activity was measured using the Superoxide Dismutase Assay kit, as described in Materials and Methods. Each bar represents the mean±SD of SOD activity fold change compared to controls of at least triplicate data from four independent experiments (**p<0.01, *p<0.05, n.s., not significant).

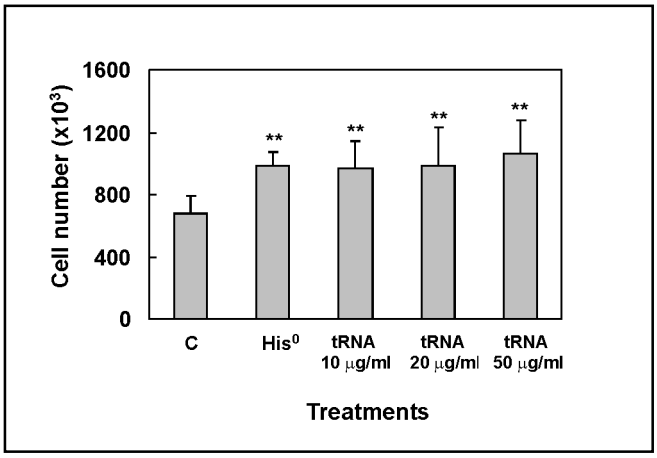

Fig. 7. Analysis of cell viability in LX- 2 incubated with histidine-free medium or tRNA for 24 h. Cell viability was determined by trypan blue exclusion assay. Control represents non-treated cells. Each bar represents the mean±SD of cell number obtained from triplicate values from two independent experiments (**p<0.01, vs. control).

Characterization of the response of HSC to amino acid deprivation

Once the effect of histidine deprivation on ROS levels was detected, we evaluated whether the absence of other amino acids exerted the same response. Deprivation of leucine, an essential amino acid, was therefore tested in LX-2 cells. Cells were treated for 5 to 60 min with leucine-free medium and intracellular ROS levels determined using the fluorescent probe CM-H_2DCFDA. As shown in Fig. 2, leucine deprivation also decreased ROS levels in LX-2 cells, although to a lesser extent than histidine deprivation. Similar results were obtained by incubation of LX-2 cells with Earle's Balanced Salt Solution, a medium lacking all the amino acids: after 5 min of treatment ROS levels were 25% lower than those of control cells.

Some of the effects of amino acid deprivation have also been observed in glucose starvation models, and are supposed to be a consequence of diminished nutrient availability [26, 27]. To determine if the effect of amino acid deprivation on ROS levels was caused by caloric limitation, LX-2 cells were incubated in the absence of glucose for 5 to 60 min and intracellular ROS levels determined as above. Glucose deprivation did not diminish ROS levels on LX-2 cells; on the contrary, a slight increase was detected at 5 min, decreasing to baseline values by 15 min (Fig. 2).

Role of GCN2 in the effects of histidine deprivation on ROS levels

Amino acid restriction causes the activation of GCN2 kinase through the binding of uncharged tRNA, which leads to the autophosphorylation and activation of the enzyme. The role played by GCN2 in the response of LX-2 to histidine deprivation was studied. First, we determined whether phosphorylation levels of GCN2 as well as its substrate eIF2α were altered by histidine deprivation. LX-2 cells were treated without histidine for 5 to 240 min, and the total and phosphorylated forms of GCN2 and eIF2α analyzed by Western blot. As shown in Fig. 3, histidine deprivation activated GCN2 at short time periods, and this effect correlated with increased phosphorylation levels of its substrate eIF2α. Activation of other signaling enzymes such as ERK, JNK, p38 MAPK, Akt and mTOR was also detected in LX-2 cells treated without histidine but pre-treatment of HSC with their inhibitors did not prevent the effect of histidine deprivation on intracellular ROS levels, suggesting that these enzymes were activated either independently or downstream of ROS production (data not shown).

To establish the relationship between GCN2 activation and the decrease of ROS levels caused by histidine deprivation we activated GCN2 independently of amino acid starvation. LX-2 cells were treated for 20

min with increased amounts of tRNA (10 to 50 μg/ml) or with the proteasome inhibitor MG-132, an agent that has been described to activate GCN2 [28]. Cells incubated for 30 min without histidine were used as a positive control. Analysis by Western blot of the phosphorylated and total protein levels of GCN2 and eIF2α showed that addition of free tRNAs or MG-132 (10 μg/ml) activated GCN2 and caused the phosphorylation of its substrate eIF2α (Fig. 4A). Once GCN2 activation was established, we studied the effect on ROS levels in the same conditions, using both primary rat HSC and LX-2 cells. Cells were treated as above and ROS levels were analyzed 30 minutes later. As shown in Fig. 4B, ROS levels significantly decreased both in primary rat HSC and in the cell line LX-2 in response to 20 and 50 μg/ml tRNA and MG-132, although the effect was higher in histidine-deprived cells (Fig. 4B). The effect on ROS levels induced by tRNA was also an early event, since 5 min after treatment a significant decrease was observed (Fig. 4C).

Reversion of the effects of histidine deprivation

Once we had demonstrated the correlation between GCN2 activation and ROS decrease caused by histidine deprivation, the effects of restoring histidine levels was studied. LX-2 cells were treated without histidine for 30 min and the medium was replaced for complete medium (containing histidine 270 μM) after 5, 15 or 30 min. The phosphorylated and total protein levels of GCN2 were analyzed by Western blot. Phosphorylation of GCN2 was restored to basal levels after adding complete medium for 15 min (Fig. 5A, upper panel). To determine whether restoration of GCN2 phosphorylation levels correlated with changes in intracellular ROS levels, LX-2 cells were treated as above and ROS levels analyzed by fluorimetry. ROS intracellular levels diminished by histidine deprivation reversed to baseline values after adding complete medium for 5 min (Fig. 5A, lower panel).

Salubrinal is a compound that inhibits eIF2α dephosphorylation [29]. Reversion studies in the presence of salubrinal were carried out to evaluate the role played by eIF2α phosphorylation in the effect caused by histidine deprivation on ROS levels. Western blot analysis showed that the reversion of eIF2α phosphorylation caused by incubation with complete medium after histidine deprivation did not take place when salubrinal was present in the medium (Fig. 5B, upper panel). However, ROS levels that were diminished by histidine deprivation were restored to normal values by the addition of complete medium also in the presence of salubrinal (Fig. 5B, lower panel). These results indicate that eIF2α phosphorylation is not required for the decrease of ROS levels caused by histidine deprivation.

Role of superoxide dismutase in H_2O_2 depletion caused by histidine deprivation

The enzymes superoxide dismutases (SOD) are the main responsible for hydrogen peroxide generation in the cell, through dismutation of the superoxide radical. The effect of histidine deprivation on SOD activity was analyzed by an assay that detects the activities of both the cytosolic Cu/Zn-dependent and the mitochondrial Mn-dependent SOD in cell lysates. Incubation of LX-2 cells with histidine-free medium caused a time-dependent decrease in SOD activity, with values 50% lower than those of control cells after 30 minutes of treatment. Cells lysates obtained from LX-2 cells incubated with tRNA also showed diminished SOD activities (Fig. 6). These results suggest that diminished SOD activity could contribute to ROS decrease induced by histidine deprivation.

Histidine deprivation and GCN2 activation enhance cell survival in HSC

Finally, we evaluated whether histidine deprivation affected cell survival, since data in the literature have described both a protective or an apoptotic effect for amino acid deprivation, depending on the cell type analyzed. LX-2 cells were incubated for 24 hours with histidine-free medium in the absence of FBS, and cell viability was determined by trypan blue exclusion assay, as described in Materials and Methods. The effect of GCN2 activation on cell survival was also analyzed by incubation of LX-2 cells with tRNA. As shown in Fig. 7, histidine deprivation as well as incubation of LX-2 with tRNA enhanced cell viability, suggesting amino acid restriction to exert a protective action in HSC which is mediated by GCN2 activation.

Discussion

Oxygen-based metabolism leads to the constant generation of intracellular reactive oxygen species (ROS) and is responsible for basal ROS production. Different physiological and pathological processes, including changes in nutrient availability can alter basal ROS levels. The relationship between amino acid deprivation and oxidative stress caused by ROS accumulation has been previously studied in animal models in the context of the free radical theory of aging. It has been demonstrated that protein restriction leads to a diminished expression

of mitochondrial complex I and a decreased rate of mitochondrial ROS production [16, 17]. This effect is caused mainly by the absence of the essential amino acid methionine, a precursor of metabolites involved in the antioxidant defense like homocysteine and S-Adenosyl-methionine [17]. Another example of a potential role for amino acid deprivation in the regulation of intracellular oxidative status is the recently reported induction of Mn-SOD in histidine-deprived human hepatoma cells, an effect that requires MEK/ERK and mTOR activities and the presence of the amino acid glutamine [30].

In the present work we studied the early effects of amino acid deprivation in endogenous intracellular ROS levels on hepatic stellate cells (HSC). We found histidine deprivation to cause a rapid and significant decrease in ROS levels that had not been previously described. Unlike the effects above mentioned that are either specific for an amino acid or cannot be triggered by amino acid-free medium, the decrease in ROS levels was also induced by leucine-free medium or by incubating the cells in the absence of all amino acids. These results suggest the action of amino acid deprivation on ROS levels to be a general mechanism by which the cell reacts to a nutrient-deprived medium. Several reports indicate that ROS can elicit cellular responses leading to the regulation of cell growth, gene expression and apoptosis [31]. We found the decrease in ROS levels induced by histidine deprivation to correlate with enhanced cell viability. This fact suggests that changes in basal ROS levels could mediate the reprogramming of gene expression that takes place in response to amino acid deprivation in order to ensure cell survival.

One of the questions raised by these findings was the precise molecular mechanism by which the absence of histidine leads to diminished ROS levels. Amino acid limitation has been shown to regulate different signaling pathways, although few concordances among data can be found. In Hela cells amino acid deprivation induces JNK phosphorylation, while ERK and p38 activities are decreased [9]; histidine depletion causes on human hepatoma cells an enhancement of ERK phosphorylation [7, 8]; and amino acid restriction blocks Akt activation in human tumoral prostate cells [10], but not in hepatoma [8]. These discrepancies may reflect differences in experimental approaches or the importance of cell phenotype for the specificity of the responses. In our system, histidine deprivation caused in HSC an early activation of ERK, JNK, mTOR and Akt, and to a lesser extent of p38. However, none of the corresponding signaling pathways seemed to be responsible for ROS depletion in response to histidine deprivation, since inhibitors for these enzymes did not prevent the effect. Our results suggest that the decrease on intracellular ROS levels is mediated, at least in part, by GCN2 activation. The following data support this: 1. glucose starvation does not alter intracellular ROS levels, indicating that the effect of amino acid deprivation is not due to caloric restriction; 2. ROS decrease takes place either by the absence of an essential amino acid like histidine or leucine or by total amino acid deprivation, suggesting the involvement of uncharged tRNAs accumulation in the response; 3. addition of free tRNAs, which are known to bind and activate GCN2, and MG-132, a GCN2 activator unrelated to amino acid deprivation, also diminish intracellular ROS levels. To date the effects described for GCN2 are mainly mediated by phosphorylation of eIF2α, and typically affect protein synthesis. However, we found ROS depletion to take place after exposing the cells to histidine deprivation for very short times (5-15 min), and this fact seems to rule out an effect mediated by regulation of protein synthesis. Moreover, the lack of correlation found between ROS levels and eIF2α phosphorylation in salubrinal-treated cells suggests a role for GCN2 unrelated to its characteristic action as eIF2α kinase. ROS decrease in response to histidine-free medium was found to correlate with diminished superoxide dismutase (SOD) activity, pointing to a role for these enzymes in the effect caused by histidine deprivation. Further research would be necessary to explore the possibility of GCN2 exerting a regulatory effect on any of the SOD variants.

Interestingly, the effect of histidine deprivation was especially relevant in hepatic stellate cells (HSC), a cell type that has been shown to be both a source and a target of ROS in the fibrotic liver and to regulate collagen expression in response to ROS [32]. Moreover, endogenous ROS production seems to be a key mediator for the action on HSC of different agents and cytokines like leucine [24], TGF-β [32] or acetaldehyde [33]. In all cases, increased ROS levels are related to an enhanced profibrogenic activity of HSC. Therefore, finding novel molecular mechanisms involved in the control of ROS production in HSC, could contribute to a better understanding of the biology of this cell type and to the identification of potential anti-fibrotic strategies.

Abbreviations

HSC (hepatic stellate cells); ROS (reactive oxygen species); GCN2 (general control nonderepresible 2 kinase); eIF2α (eukaryotic initiation factor 2 alpha); SOD (superoxide dismutase).

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