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Reactive oxygen species (ROS) mediate the effects of leucine on translation regulation and type I collagen production in hepatic stellate cells

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Abstract

The amino acid leucine causes an increase of collagen α1(I) synthesis in hepatic stellate cells through the activation of translational regulatory mechanisms and PI3K/Akt/mTOR and ERK signaling pathways. The aim of the present study was to evaluate the role played by reactive oxygen species on these effects. Intracellular reactive oxygen species levels were increased in hepatic stellate cells incubated with leucine 5 mM at early time points, and this effect was abolished by pretreatment with the antioxidant glutathione. Preincubation with glutathione also prevented 4E-BP1, eIF4E and Mnk-1 phosphorylation induced by leucine, as well as enhancement of procollagen α1(I) protein levels. Inhibitors for MEK-1 (PD98059), PI3K (wortmannin) or mTOR (rapamycin) did not affect leucine-induced reactive oxygen species production. However, preincubation with glutathione prevented ERK, Akt and mTOR phosphorylation caused by treatment with leucine. The mitochondrial electron chain inhibitor rotenone and the NADPH oxidase inhibitor apocynin prevented reactive oxygen species production caused by leucine. Leucine also induced an increased phosphorylation of IR/IGF-R that was abolished by pretreatment with either rotenone or apocynin. Therefore, leucine exerts on hepatic stellate cells a prooxidant action through NADPH oxidase and mitochondrial Reactive oxygen species production and these effects mediate the activation of IR/IGF-IR and signaling pathways, finally leading to changes in translational regulation of collagen synthesis.

Keywords: Leucine; Hepatic stellate cells; Type I collagen; Translational regulation; Oxidative stress

1. Introduction

Reactive oxygen species (ROS) generated in the cell as a consequence of oxygen-based metabolism were initially considered as harmful derivatives that in some instances could be used for the defense of the cell. In the last years, however, growing evidence of regulation of different cell functions by moderate levels of ROS have lead to the concept of these molecules acting like secondary messengers with active roles in signaling processes. One of the facts that support this view is the increased ROS production found in response to a wide range of agents, from inflammatory cytokines like TNF-α [1] to hormones or growth factors like insulin [2], EGF [3], PDGF [4] or TGF-β [5]. Moreover, inhibition of oxidative stress generation by the addition of antioxidants usually prevents the effects of the agonist, indicating that ROS are indeed key mediators of specific signaling events. Among other effects, ROS have been shown to modulate signal transduction through the inhibition of protein phosphatases, a family of redox-sensitive enzymes [6].

The precise mechanism(s) through which intracellular ROS are generated in response to extracellular signals is in most cases not fully established and seems to be of different nature depending on the agent and the cell type involved. In general terms, oxidative stress can be caused either by the inhibition of

Abbreviations: HSC, hepatic stellate cells; ROS, reactive oxygen species; eIF4E, eukaryotic initiation factor 4E; mTOR, mammalian target of rapamycin; PI3K, phosphatidyl inositol 3 kinase; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; GSH, glutathione (reduced form); NAC, N-acetylcysteine

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antioxidant systems, for example by downregulating the expression of antioxidant enzymes, or by direct activation of ROS production. The main sources of ROS are the mitochondria, that generates superoxide radical ($O_2^{\bullet -}$) as a result of defective electron transfer, and the NADPH oxidase complex that was initially described in phagocytic cells but has been recently characterized in many cell types as an non-phagocytic complex that constitutively produces basal ROS levels [7].

Liver fibrosis is a pathophysiological condition that has been extensively related to oxidative stress. Many experimental data demonstrate that ROS accumulate in the injured liver and mediate cellular and molecular events that contribute to the development of liver fibrosis. Most importantly, ROS participate in the activation and fibrogenic effects of hepatic stellate cells (HSC), the main source of extracellular matrix proteins in the fibrotic liver [8]. Studies carried out in cell culture models have shown that the responses elicited by ROS in HSC range from necrotic or apoptotic cell death to up-regulation of collagen production, depending on the prooxidant agent employed and the localization and extent of oxidative-stress [9]. Moreover, oxidative stress caused either by prooxidant agents or by profibrogenic cytokines like TGF-β has also been shown to modulate intracellular signaling pathways in HSC, including ERK and Akt activation, and to regulate collagen synthesis at translational and post-translational levels [10–13].

We have previously shown that leucine, an essential amino acid that is also a nutritional signal, exerts profibrogenic actions on HSC, inducing collagen I protein synthesis through the activation of signaling pathways and the modulation of translational regulatory steps [14]. The aim of the present paper was to study the possible role played by oxidative stress as a mediator of leucine effects in HSC, and to establish the molecular mechanisms involved.

2. Materials and methods

2.1. Reagents

Leucine, glutathione (GSH), N-acetylcysteine (NAC), menadione, rapamycin, lucigenin, apocynin and rotenone were from Sigma Chemical Company (St. Louis, MO). Cell culture reagents were from Gibco BRL (Grand Island, NY). 2′,7′-dichlorofluorescein diacetate (DCFA-DA) was from Molecular Probes (Eugene, OR). Wortmannin and PD098059 were purchased from Calbiochem® (Germany).

2.2. Cell culture and treatment

The experiments were carried out using the rat HSC line CFSC-2G. This non-tumoral cell line was obtained after spontaneous immortalization in culture of HSC isolated from a CCl4-cirrhotic liver [15] and is characterized by low basal levels of expression of type I collagen genes and by the presence of mRNA for nestin and α-SMA [16]. Therefore, it can be considered as a ''transitional'' HSC, in which the activation process is already initiated. Cells were cultured in minimum essential medium (MEM) supplemented with 10% bovine fetal serum and non-essential amino acids for 36 h. The medium was replaced for serum-free MEM for 12 h, after which treatments were carried out. Unless otherwise indicated, HSC were treated with leucine 5 mM. In some experiments either GSH (2 mM), NAC (5 mM), menadione (100 μM), PD098059 (10 μM), wortmannin (200 nM), apocynin (10 μM) or rotenone (20 μM) were added 30 min before treating the cells with leucine. Rapamycin (100 nM) was added 2 h before treatment with leucine.

2.3. Western blot

For Western blot analysis 6×10^5 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, 1 mM EDTA, 1 mM PMSF, 0.1 mM DTT and 1 μg/ml aprotinin. Protein concentration of the samples was determined by BCA (bicinchoninic acid assay). For immunoblotting 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 a blocking solution at room temperature and with specific primary antibodies at 4 °C overnight. Antibodies against total 4E-BP1, phospho-specific eIF4E, phospho-specific and total Akt, phospho-specific and total mTOR, phospho-specific ERK, phospho-specific Mnk-1 and phospho IR/IGF-IR were purchased from Cell Signalling Technology (Beverly, MA). Antibody against total ERK was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti β-actin antibody was from Sigma. Membranes were also incubated with an antibody raised against human collagen type I (Rockland, Inc., Rockland, PA). After incubation with primary antibodies, membranes were washed and incubated with the 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.

2.4. Measurement of ROS

Production of ROS, mainly peroxides, 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. H_2O_2 produced by the cell oxidizes H_2DCF-DA to 2,7-dichlorofluorescein (DCF), the fluorescence of which is proportional to the H_2O_2 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 with 5 mM leucine for time points ranging from 5 min to 4 h. Short-time image analysis was performed by fluorescence microscopy. Cells were loaded for 20 min with 10 μM CM-H_2DCFDA at 37 °C in the dark prior the treatment with the amino acid. Data from long time experiments were performed in a Cytofluor 2350 (Millipore). Dose–response studies were carried out treating HSC for 1 h with different concentrations of leucine (1–7.5 mM). When indicated, HSC were pretreated with inhibitors for signaling pathways or with GSH, rotenone or apocynin in the above described conditions. Values are means ± SD of at least triplicate data from four independent experiments.

2.5. Superoxide detection

Measurement of intracellular superoxide anion ($O_2^{\bullet -}$) production was carried out using lucigenin enhanced chemiluminescence assay. This method is based on the reaction between reduced lucigenin and $O_2^{\bullet -}$, resulting in the emission of photons that can be quantified using a luminometer. HSC were cultured in 60 mm Petri dishes and serum starved for 12 h. Cells were treated with leucine 5 mM for 5 or 40 min and then washed and transferred into a cuvette in 1 ml PBS. Cellular suspensions were loaded into the luminometer and lucigenin at 250 μM was injected. The readings were recorded 5 min later.

2.6. 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.

3. Results

3.1. Leucine induces ROS production in HSC

The effect caused by leucine on intracellular levels of reactive oxygen species (ROS) was studied using the fluorescent probe CM-H_2DCFDA, which reacts mainly with hydrogen peroxide and to a lesser extent with other ROS. Fluorescent microscopy analysis demonstrated that HSC incubated with leucine 5 mM for 5 or 60 min presented an increased fluorescence compared to untreated cells (Fig. 1A). Fluorimetric assays at later incubation time periods showed that ROS reached maximum levels by 1 h of treatment with leucine 5 mM, and decreased to basal levels after 2 h (Fig. 1B). Dose–response experiments were carried out incubating HSC for 1 h with different concentrations of leucine, ranging from 1 to 7.5 mM. As shown in Fig. 1C, ROS levels increased in a dose-dependent fashion (Fig. 1C). ROS accumulation induced by 5 mM leucine was similar to that obtained by incubation of HSC with 100 μM menadione, and was prevented by pretreatment with either 2 mM of the intracellular antioxidant glutathione (reduced form, GSH), or 5 mM of its precursor N-acetylcysteine (Fig. 1D).

3.2. ROS mediate the effects of leucine on procollagen α1(I) levels and translation regulatory steps

Once the prooxidant action of leucine was established, we studied the role played by ROS on the effects caused by this amino acid in HSC. Leucine had been shown to induce procollagen α1(I) production in HSC through the activation of translational regulatory steps: 4E-BP1 and eIF4E phosphorylation. Hyperphosphorylation of 4E-BP1 leads to an induction of protein synthesis through the release of eIF4E, a translation initiation factor that binds to the 5′ end of mRNA and is required to form the eIF4F complex. Phosphorylation of eIF4E can also result in enhanced protein synthesis, since it facilitates the formation of the translation initiation complex. Mnk-1, a soluble kinase which is activated by ERK independently of 4E-BP1 modification, is responsible for eIF4E phosphorylation induced by leucine [14].

To determine whether ROS accumulation mediates these effects, HSC were pretreated with 2 mM GSH before the addition of leucine 5 mM, and the phosphorylated forms for 4E-BP1 and eIF4E, as well as procollagen α1(I) levels were analyzed by Western blot. GSH pretreatment abolished the

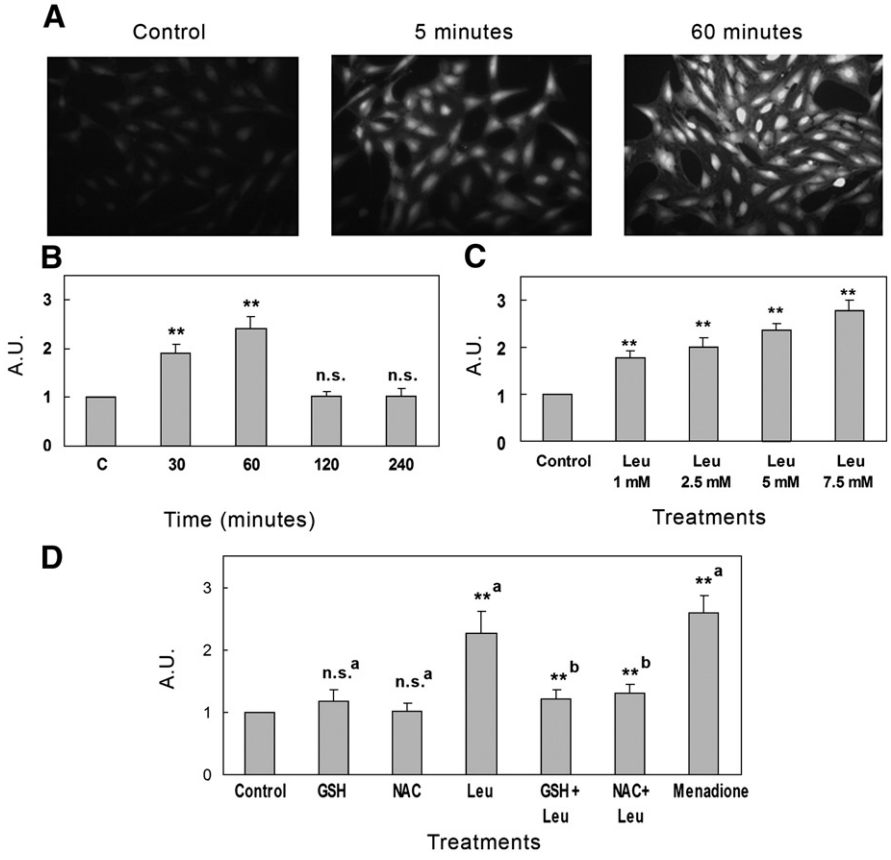

Fig. 1. Measurement of ROS production in HSC treated with leucine. Intracellular ROS levels were determined using CM-H_2DCFDA as a probe. (A) Fluorescent microscopy analysis of ROS production in HSC treated with leucine 5 mM. (B) Time course analysis of ROS levels determined by fluorimetric assay. (C) Intracellular ROS levels in HSC treated for 1 h with different concentrations of leucine. 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). (D) Intracellular ROS levels in HSC pretreated for 30 min with either GSH 2 mM or NAC 5 mM and treated for 1 h with leucine 5 mM. The prooxidant menadione (100 μM, 1 h) was used as a positive control. 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. leucine-treated cells; n.s., not significant).

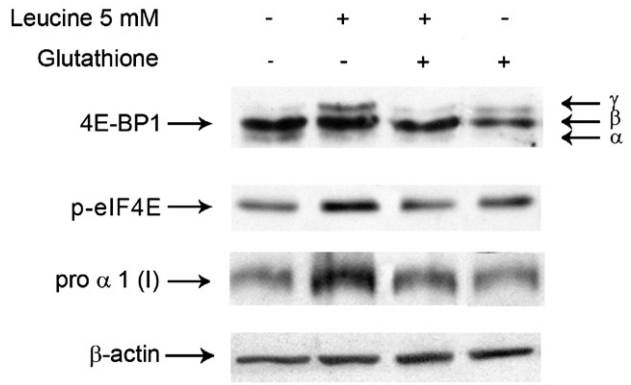

Fig. 2. Effect of GSH on 4E-BP1 and eIF4E phosphorylation and procollagen α1(I) levels induced by leucine. Cells were pretreated with 2 mM GSH and treated with leucine 5 mM either for 2 h (for the detection of 4E-BP1 and eIF4E), or 48 h (for the detection of procollagen α1(I)), and protein levels were analyzed by Western blot. The phosphorylated form of eIF4E was detected using a phospho-specific antibody against the protein. 4E-BP1 resolved into three bands on SDS polyacrylamide gels, with the top band (γ-band) corresponding to the hyperphosphorylated form of the protein.

hyperphosphorylation of the translation inhibitory protein 4E-BP1 and eIF4E phosphorylation induced by leucine in HSC. The effects of GSH on 4E-BP1 and eIF4E correlated with decreased procollagen α1(I) protein levels in HSC pretreated with GSH and treated for 48 h with leucine (Fig. 2).

3.3. The activation of PI3K/Akt/mTOR and ERK in response to leucine is mediated by ROS accumulation

We had previously demonstrated that in HSC leucine activates PI3K/Akt/mTOR and ERK, leading to increased translation of procollagen α1(I). To determine whether these signaling pathways were involved in increased ROS production caused by leucine, pharmacological inhibitors for ERK (MEK-1 inhibitor, PD098059), PI3K (wortmannin) or mTOR (rapamycin) were added prior to treatment with leucine, and ROS levels analyzed as above. Pretreatment of HSC with the inhibitors did not prevent the effect of leucine on intracellular ROS levels, suggesting that these enzymes were activated either independently or downstream of ROS production (Fig. 3).

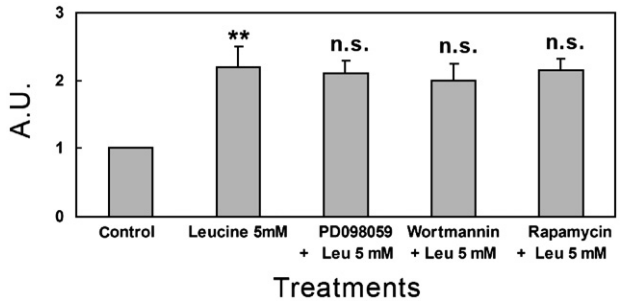

Fig. 3. Effect of signaling pathways inhibitors on intracellular ROS levels induced by leucine. HSC were pretreated for 30 min with 10 μM PD098059 (MEK-1 inhibitor), or 200 nM wortmannin (PI3K inhibitor), or for 2 h with 100 nM rapamycin (mTOR inhibitor) prior incubation with leucine 5 mM for 1 h. ROS levels were analyzed by fluorimetry using CM-H_2DCFDA as a probe. 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).

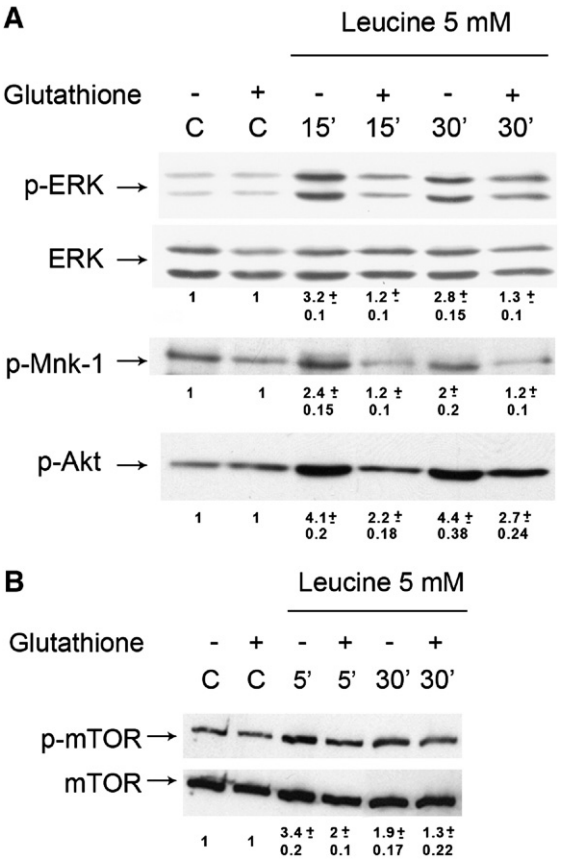

Fig. 4. Role of oxidative stress on signaling pathways activated by leucine in HSC. Cells were incubated for 30 min with GSH 2 mM before adding leucine 5 mM for the indicated times, and protein levels were analyzed by Western blot. (A) Effect of GSH on ERK, Mnk-1 and Akt phosphorylation in HSC treated with leucine for 15 and 30 min. (B) Effect of GSH on mTOR phosphorylation levels in HSC treated with leucine for 5 and 30 min. Data of phosphorylation fold change are included.

The possibility of ROS generation being upstream of signaling pathways activated by leucine was then studied using GSH as an antioxidant and evaluating its effects on leucine-treated HSC. ERK, Mnk-1 and Akt phosphorylation levels were analyzed by Western blot in HSC pretreated with GSH and treated with leucine. GSH prevented the effect of leucine on phosphorylation levels of all the enzymes tested, indicating that ROS contribute to their activation (Fig. 4A).

The effect of GSH on mTOR phosphorylation induced by leucine at different time points was also determined. Phosphorylation of mTOR in response to treatment with leucine presents two peaks depending on the time of incubation with the amino acid. Early activation of mTOR (5 min) is PI3K-independent, while activation at later time points takes place through PI3K-dependent mechanisms, since inhibitors like wortmannin can prevent it [14]. Pretreatment of HSC with GSH abolished mTOR phosphorylation induced by leucine at 5 and 30 min, indicating that ROS accumulation is responsible for both PI3K dependent and independent mTOR activation (Fig. 4B).

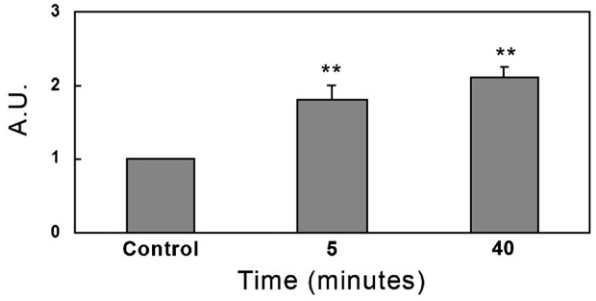

Fig. 5. Superoxide radical production in HSC treated with leucine. Intracellular superoxide radical levels were measured by luminescence, using lucigenin as a probe. HSC were incubated for 5 or 40 min with leucine 5 mM and luminescence determined as described in Materials and methods. Each bar represents the mean ± SD of luminescence fold change compared to control of duplicate data from three independent experiments (**$p<0.01$, vs. control).

3.4. Source of ROS generation in HSC treated with leucine

In order to establish the mechanism(s) of oxidative stress generation induced by leucine on HSC we determined the intracellular levels of superoxide ($O_2^{\bullet -}$), a ROS which is characterized by its high reactivity and short life. In physiological conditions, $O_2^{\bullet -}$ is metabolized to hydrogen peroxide by the antioxidant enzyme superoxide dismutase (SOD). We analyzed $O_2^{\bullet -}$ levels in intact HSC treated with 5 mM leucine for different time periods (5 to 40 min) using lucigenin as a probe. As shown in Fig. 5, $O_2^{\bullet -}$ levels were rapidly increased by leucine as compared to those of untreated control cells.

The main sources of $O_2^{\bullet -}$ in the cell are the constitutive NADPH oxidase complex, recently characterized in HSC [13], and the mitochondria, mainly through the leakage of electrons in electron chain complex III. The possible participation of NADPH oxidase and mitochondria in ROS generation caused by leucine was analyzed using rotenone, a compound that blocks mitochondrial electron transfer in complex I, and apocynin, a NADPH oxidase inhibitor. HSC were pretreated either with rotenone 20 μM or apocynin 10 μM prior to the addition of leucine 5 mM, and ROS levels determined as above at two different time points, 5 and 40 min. Pretreatment with either compound prevented intracellular ROS levels induced by leucine, although apocynin was more effective at 5 min and rotenone at later time periods (Fig. 6A).

To further confirm that ROS generated from the NADPH oxidase system and mitochondria were involved in the signaling events triggered by leucine, we analyzed the effects of apocynin and rotenone on mTOR phosphorylation. Both rotenone and apocynin partially abolished mTOR phosphorylation induced by leucine at 5 and 40 min, as shown in Fig. 6B.

3.5. Role of IR/IGF- IR activation on the effects caused by leucine on HSC

Since some of the signaling proteins activated by leucine in this study are typically involved in the insulin and IGF-I signaling pathways, we finally explored the possibility of IR/IGF-IR being activated by ROS and mediating the effects of leucine on HSC. Cells were treated with leucine 5 mM for 5 min in the presence or absence of either rotenone or apocynin, and

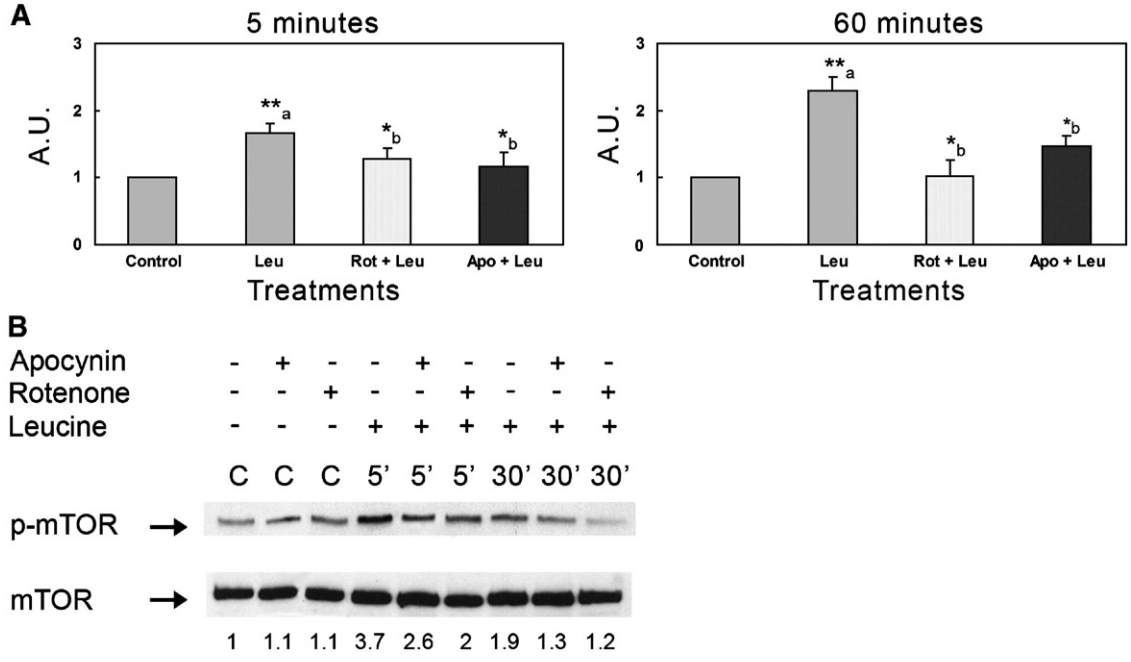

Fig. 6. Effect of rotenone and apocynin on intracellular ROS levels and mTOR phosphorylation induced by leucine. HSC were pretreated for 30 min with either 20 μM rotenone (mitochondrial electron transfer complex I inhibitor) or 10 μM apocynin (NADPH oxidase inhibitor) prior incubation with leucine 5 mM. (A) ROS levels were analyzed by fluorimetry using CM-H_2DCFDA as a probe and treating HSC with leucine 5 mM for either 5 or 60 min. Each bar represents the mean ± SD of fluorescence fold change compared to control of quadruplicate data from three independent experiments (*$p<0.05$, **$p<0.01$; *a*, vs. control; *b*, vs. leucine-treated cells). (B) mTOR phosphorylation levels were analyzed by Western blot at 5 and 30 min of treatment with leucine 5 mM. Data of mTOR phosphorylation fold change are included.

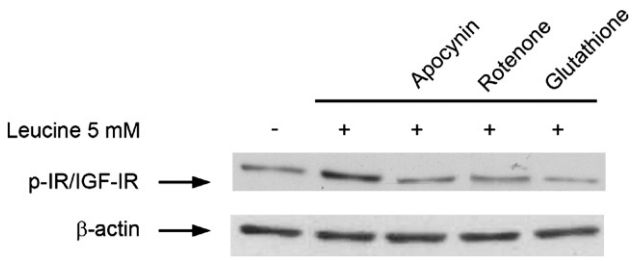

Fig. 7. IR/IGF-IR phosphorylation in HSC treated with leucine and pretreated with rotenone or apocynin. HSC were pretreated for 30 min with 10 μM apocynin (NADPH oxidase inhibitor), 20 μM rotenone (mitochondrial electron transfer I inhibitor) or 2 mM glutathione, prior incubation with leucine 5 mM for 5 min. IR/IGF-IR phosphorylation levels were analyzed by Western blot.

phosphorylated levels of IR/IGF-IR analyzed by Western blot. As shown in Fig. 7, incubation with leucine caused an enhancement of the phosphorylation levels of these receptors. Pretreatment with either rotenone or apocynin prevented IR/IGF-IR phosphorylation, suggesting that ROS produced in response to leucine were responsible for this effect.

4. Discussion

Oxidative stress is involved in the development of liver fibrosis by different mechanisms, one of the most relevant being the fibrogenic activity of hepatic stellate cells (HSC). Recent evidence suggests that HSC are not only a target of reactive oxygen species (ROS) produced by other cell types present in the injured liver such as activated Kupffer cells or infiltrating monocytes, but also contribute to oxidative stress through increased ROS generation in response to cytokines like TGF-β or angiotensin II [5,10]. Since collagen production by HSC can be modulated by ROS at transcriptional and post-transcriptional levels, these data indicate that a positive regulatory loop with ROS acting as mediators or co-activators could contribute to fibrosis progression.

Our results demonstrate that leucine causes in HSC an enhancement of intracellular ROS levels. To date, few reports have described a relationship between leucine and oxidative stress and the data are contradictory, probably because of the different experimental approaches used. At high concentrations (30.5 mM), leucine exerts in pancreatic islets a protective antioxidant action similar to that of D-Glucose, due to its role as a metabolic substrate [17]. However, there is also evidence of the opposite effect: in cerebral cortex leucine causes oxidative stress as a consequence of the inhibition of antioxidant enzymes like catalase and GSH peroxidase and the oxidation of protein thiol groups [18]. To our knowledge, the present study offers the first evidence of ROS generation produced by leucine in a cell culture system. The rapid increase in ROS levels detected in response to leucine (5 min) ruled out transcriptional regulation of antioxidant enzymes as being responsible for oxidative stress generation, and suggested a direct activation of ROS production through other mechanisms. Accordingly, we found that the NADPH oxidase and mitochondrial electron transfer complexes were involved in intracellular ROS levels enhancement by leucine. The nonphagocytic NADPH oxidase has been recently characterized in HSC as a mediator of fibrogenic responses [19]. Unlike the phagocytic form, the nonphagocytic NADPH oxidase produces low basal levels of ROS that can be increased by different stimuli. In cultured HSC reports of NADPH oxidase activation caused by zinc deficiency [20], growth factors like

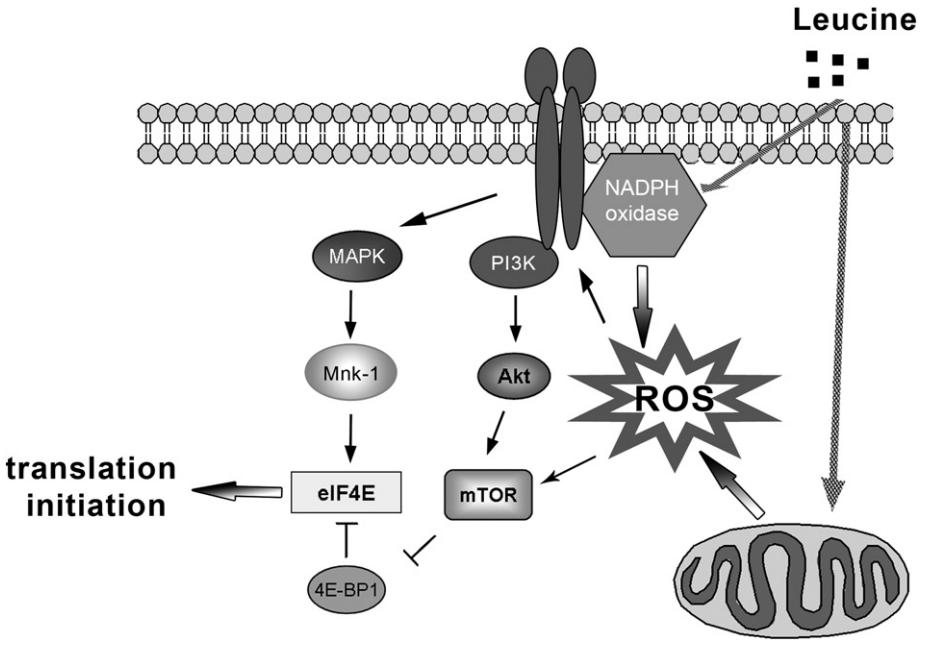

Fig. 8. Schematic representation of leucine effects in hepatic stellate cells. Leucine induces $O_2^{\bullet -}$ production by NADPH oxidase and mitochondria, leading to an increase of intracellular ROS levels. Phosphorylation of receptors for insulin and/or IGF-I as well as activation of PI3K/Akt/mTOR and ERK signaling pathways is induced by ROS. mTOR phosphorylates 4E-BP1 and as a consequence eIF4E is released, allowing the formation of the translation initiation complex. eIF4E phosphorylation by Mnk-1 also contributes to enhanced protein synthesis.

PDGF [21], agonists like angiotensin II [10], or by phagocytosis of apoptotic bodies [22] have been recently published. In all cases, as well as in animal models in which the activity of the enzymatic complex is enhanced [23], NADPH oxidase activation is related to the proliferation and/or increased fibrogenic activity of HSC. Although little is known about mitochondrial ROS production in HSC, a relationship between NADPH oxidase and mitochondrial generation of ROS has been suggested in other cell types and could also take place in our system [24,25].

Increased ROS production caused by leucine mediated the effects this amino acid on 4E-BP1 and eIF4E phosphorylation, two translation regulatory steps that lead to enhanced collagen type I production by HSC. Some reports found in the literature show an inhibitory role for oxidative stress in the translational machinery [26,27], but positive regulation by ROS through the phosphorylation of eIF4E mediated by MAP kinases has also been demonstrated [28]. This mechanism could explain some of our results, since ERK, Mnk-1 and eIF4E phosphorylation caused by incubation with leucine were prevented by GSH pretreatment, pointing to a regulation of these proteins by ROS. GSH also abrogated the phosphorylation of mTOR induced by leucine at all the time points tested, showing that ROS modulate both the PI3K-independent and PI3K-dependent activation of mTOR that takes place at early and late incubation times, respectively. Taking into account that ROS levels induced by leucine were not altered by inhibition of any of the pathways studied and that all the signaling steps analyzed in leucine-treated HSC were ROS-dependent, the possibility of an upstream common mechanism could be suggested.

Early activated HSC express receptors for insulin and IGF-I [29]. We investigated the possibility of IR/IGF-R mediating leucine-induced effects because leucine and insulin/IGF-I activate similar signaling pathways and both the insulin and IGF-I receptors have been shown to be transactivated in response to oxidative stress [2,30,31], probably through the redox-dependent inhibition of protein phosphatase PTP1B [32]. We found that leucine caused an enhanced phosphorylation of IR/IGF-IR at early time points of incubation and that both rotenone and apocynin abolished IR/IGF-IR phosphorylation as well as downstream signaling effects triggered by leucine such as mTOR phosphorylation. These results suggest the following sequence of events (Fig. 8): (1) intracellular ROS levels produced by NADPH oxidase and mitochondrial electron transfer complexes are increased in HSC by treatment with leucine; (2) ROS accumulation induces the phosphorylation of IR/IGF-IR and the activation of PI3K/Akt/mTOR and ERK signaling pathways; (3) translational regulatory mechanisms are modulated by PI3K/Akt/mTOR and ERK, finally leading to the enhancement of collagen synthesis.

The role played by IR/IGF-IR as mediators of leucine-induced responses could also explain a common effect described for leucine and IGF-I on HSC: both agents stimulate the production of hepatocyte growth factor (HGF) the main mitogenic cytokine in hepatocytes and a key modulator for liver function [33,34]. Interestingly, HGF has been shown to be an antifibrogenic factor, due in part to the inhibition of TGF-β expression by activated HSC [35]. In addition, IGF-1 has been recently described to present antifibrogenic effects in animal models for liver fibrosis [36,37] although in cell cultures of HSC exerts a profibrogenic action [29]. These facts reflect the complex network existing among cytokines and growth factors, and suggest that leucine could cause different effects on HSC by potentiating or counteracting the action of several agents involved in fibrosis regulation. Further research would be required to clarify the connections that appear to exist between cytokines and nutritional factors, and their role in the regulation of pathophysiological processes like liver fibrosis.

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