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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.