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Low level of hydrogen peroxide induces lipid synthesis in BRL-3A cells through a CAP-independent SREBP-1a activation

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ABSTRACT

Although H_2O_2 is traditionally known to have cytotoxic effects, recent studies argue about its regulatory role on lipid metabolism. However, the mechanism underlying the induction of lipid biosynthesis by oxidative stress still remains unknown. To shed light on this aspect we investigated the H_2O_2-induced lipogenesis in rat liver BRL-3A cells.

We found that a short-term exposition of cells to 35 μM H_2O_2 didn't cause any significant sign of cell damage measured by following diene formation and lactate dehydrogenase release from cells. However, in this stressful condition, a significant increase of [1-^{14}C]acetate incorporation into fatty acids and cholesterol, associated to an increase in the activity and expression of key enzymes of fatty acid and cholesterol synthesis, were measured. mRNA and protein contents of the transcription factors SREBP-1 and SREBP-2, involved in the activation of lipid synthesis, increased as well. The analysis of molecular mechanism of SREBP-1 activation revealed, in treated compared to control cells, a higher SREBP-1a mRNA translation involving an internal ribosome entry side (IRES), present in the leader region of its mRNA. Longer exposition to the pro-oxidant induced a progressive loss of cell viability together with an increase of cell triacylglycerol content.

1. Introduction

In mammalian, Reactive Oxygen Species (ROS) are unavoidably by-products of mitochondrial metabolic processes (Valko et al., 2007). Furthermore, ROS can be produced in different cellular compartments other than mitochondria (i.e. endoplasmic reticulum, peroxisomes, cytosol, plasma membrane etc.) (Brown and Borutaite, 2012).

Under physiological steady state conditions these molecules are eliminated by complex set of non enzymatic and enzymatic detoxification mechanisms. When these systems are altered the intracellular level of ROS can rapidly raise, generating a condition called "oxidative stress" (Packer and Cadenas, 2007). Because of their high reactivity ROS are harmful for many cellular constituents such as lipids, proteins and nucleic acids (Packer and Cadenas, 2007). A wide range of disorders including atherosclerosis, Parkinson's disease, Alzheimer's disease, and drug-induced liver injury have been attributed to oxidative stress (Packer and Cadenas, 2007).

Among ROS, H_2O_2 is the most weak and stable oxidant, so that it is present with a relatively long half-life in the tissues (Fridovich, 1998). Recent studies have demonstrated that in mammalian cells H_2O_2 may act as intracellular messenger, stimulating biological responses and activating specific biochemical pathways (Gautam et al., 2006; Stone and Yang, 2006).

H_2O_2 has been shown to stimulate hexose monophosphate shunt in insulin-responsive tissue in a manner similar to insulin (Czech et al., 1974; May and de Haën, 1979). In particular, it has been demonstrated H_2O_2 stimulates glucose transport, glucose C-l oxidation and glucose incorporation into glycogen in rat adipocytes (Lawrence and Larner, 1978). In addition, H_2O_2 inhibits theophylline-stimulated lipolysis (Livingston et al., 1977).

Recent finding showed that high H_2O_2 concentrations stimulate triacylglycerols accumulation in the hepatic cancer cells HepG2 (Sekiya et al., 2008). Bettzieche and coll. (Bettzieche et al., 2008) measured higher mRNA levels compared to untreated cells, for several lipogenic enzymes in HepG2 cells treated with the pro-oxidant $CuSO_4$.

Despite the interest in the topic, sparse data are available regarding the molecular mechanism/s of the H_2O_2-induced effects on lipid metabolism. Understanding of the mechanism/s of stress-induced lipogenesis could provide new insight into the metabolic background of correlated pathogenesis. Aim of the present study was to deeply investigate the molecular basis of the induction of lipid synthesis in rat liver BRL-3A cells treated with H_2O_2.

Here, we report that during the early phase of oxidative stress, induced by H_2O_2, a remarkable increase of both cholesterol and

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particularly fatty acid synthesis was observed. In these conditions no evident sign of cell damage was reported. The stimulated lipid synthesis was accompanied by a significant increase in the expression of key enzymes of fatty acid and cholesterol synthesis, i.e. acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) respectively. Both Sterol Regulatory Element-Binding Protein (SREBP)-1 and SREBP-2 mRNA and protein contents increased during this phase of active lipid synthesis. The changes of SREBP-1 protein content, analyzed at molecular level, revealed that the H_2O_2 effect can be ascribed to a higher SREBP-1a mRNA translation, through a CAP-independent mechanism, mediated by an IRES located in its 5′ untraslated region (UTR).

Longer incubation with the peroxide induced cell damage, as evidenced by an increase of membrane diene content and cell lactate dehydrogenase (LDH) leakage, together with a significant increase in cell triacylglycerol content.

2. Materials and methods

2.1. Materials

Rat liver BRL-3A cells were from the American Type Culture Collection (CRL-1442™). DMEM, FBS, penicillin/streptomycin, phosphate buffered saline (PBS) were from Gibco-Invitrogen, Ltd. (Paisley, UK); [1-^{14}C]acetate was from GE Healthcare (Little Chalfont, UK); [1-^{14}C]acetyl-CoA, [3-^{14}C]HMG-CoA were from Perkin-Elmer (Boston, MA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO). Primary antibodies for HMG-CoA reductase, FAS, ACCα, nSREBP-1, SREBP-2, β-actin, and horseradish peroxidase conjugated IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA). All reagents were of analytical grade.

2.2. Plasmid construction

The 5′-UTR of the rat SREBP-1a mRNA (GenBank® accession number XM_213329) was amplified from total RNA by RT-PCR. Primers used in the PCR were 5UTRrS1a for: 5′-TAG CTA AGC TTG AAT TCA GGT GGC TCC GCC CGC GGA A-3′; 5UTRrS1a rev: 5′-GAA TTC CCA TGG CGC CGG CGC CCA CC-3′. The amplimer was digested with EcoRI and NcoI and then inserted into the intercistronic region of pRF (Stoneley et al., 1998), in order to produce the dicistronic constructs pRrS1aF.

2.3. Cell cultures and transient transfection assay

BRL-3A cells were grown in DMEM supplemented with 5% heat inactivated FBS and 1% penicillin/streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. BRL-3A cells were seeded at a density of 5×10^5 cells per 35 mm diameter Petri dishes; 24 h after plating, the medium was changed and H_2O_2 at different concentrations (0–150 μM) and for different times (6, 12 and 24 h) was added to the serum rich (5% FBS) medium. In each experiment and for each determination, control dishes without hydrogen peroxide addition were used.

H_2O_2 solutions were daily prepared from 6.7 M stock solution 5 min before the addition to incubation medium. Hydrogen peroxide which remained in the medium at several time points was measured using a Beckman DU 800 spectrophotometer with the horseradish peroxidase assay as described (Root et al., 1975). Living cells were evaluated by using a MTT test. Viability was calculated as percentage of absorbance relative to control cells.

For transient transfections, 5×10^5 cells were seeded into 12-well Plates 48 h before transfection. Cells were transfected using Metafectene® (Biontex) following the manufacturer's recommendations. After an 8 h transfection period, the medium was changed to fresh DMEM supplemented with 10% (v/v) FBS and cells were incubated for 24 h. After cell lysis, RL (*Renilla* luciferase) and FL (firefly luciferase) activities were measured using the Dual Luciferase Reporter Assay System (Promega). The β-galactosidase activity was determined using a β-galactosidase assay. To study the effect of hydrogen peroxide, cells were maintained in DMEM supplemented with hydrogen peroxide 35 μM and incubated for 6 h.

2.4. Cell membrane damage and lipid peroxidation measurements

Damage to plasma membranes was studied by measuring the release into the medium of LDH from cells. After peroxide addition the collected medium was centrifuged and the supernatant was used for the assay of LDH activity measuring at 340 nm the decrease of absorbance due to the oxidation of NADH to NAD^+. LDH activity in the medium was expressed as percentage of the total cellular LDH activity. The percentage of LDH leakage was calculated by relating the LDH activity present in the medium to the total LDH activity (LDH leakage = LDH in medium/total LDH × 100%).

Conjugated dienes were quantified according to the method described by Buege and Aust (Buege and Aust, 1978). Briefly, after treatment with H_2O_2, cells in the plates were washed with PBS. Cells were scraped off and the cell suspension was centrifuged at 200 × *g* for 5 min. Cell pellet was resuspended in 5 ml of PBS at 0 °C. Cells were lysed and after centrifugation at 14,000 × *g* for 10 min at 4 °C the supernatant was immediately used for the lipid peroxidation assay by measuring conjugated dienes.

2.5. Fatty acid and cholesterol synthesis measurements

Total fatty acid and cholesterol synthesis was assayed following [1-^{14}C]acetate (16 mM, 0.96 mCi/mmol) incorporation into these lipid fractions (Giudetti et al., 2005). Next, a procedure that allows to assay directly in situ the activities of the lipogenic enzymes ACC, FAS and HMG-CoA reductase was set up. To this end, after incubation with H_2O_2, culture medium was removed and cells were permeabilized using 400 μL of assay mixture containing digitonin. Permeabilized hepatocytes allow to rapidly assay intracellular enzyme activities under more or less physiological conditions, avoiding any possible post-homogenizing modifications which can occur in subcellular fractionation (Geelen, 2005). The optimal concentration of digitonin and the time of exposure to digitonin necessary to permeabilize cell plasma membrane, without affecting sub-cellular organelle integrity, were determined. ACC and FAS activity were assayed as described (Bijleveld and Geelen, 1987). The HMG-CoA reductase activity assay was performed as in (Natali et al., 2007).

2.6. Oil red O staining and triacylglycerol assay

BRL-3A cells were treated with 35 μM H_2O_2 for 6, 12 and 24 h. At the end of experimental periods, cells were fixed for at least 1 h with 10% formalin in isotonic phosphate buffer. After washing with PBS cells were treated for 50–60 min, at room temperature, with freshly prepared Oil Red-O solution. After dyeing, cells were washed with sterile water and observed under a epi-fluorescence microscope.

To quantify the lipid accumulation, the cells were scraped off from the plates and lipids were extracted with chloroform/methanol (1:2, v/v). After extraction and evaporation of the chloroform phase, a suitable volume of 0.1% Triton X-100 was added. Chloroform was then evaporated under a nitrogen stream and the residual lipid extract was resuspended in water.