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Superoxide anion radicals activate hepatic stellate cells after entry through chloride channels: A new target in liver fibrosis

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ABSTRACT

It is generally accepted that reactive oxygen species (ROS) play an important role in the pathogenesis of liver fibrosis. ROS, however, constitute a group of species with varying properties making it likely that their contribution to the pathological mechanism varies. LX-2 hepatic stellate cells (HSCs) were exposed to superoxide anion radicals ($O_2^{\bullet -}$) generated by xanthine and xanthine oxidase. To rule out that the activation of HSCs is due to hydrogen peroxide derived from $O_2^{\bullet -}$, control incubations with copper, zinc-superoxide dismutase and tempol were studied as well. Influx of $O_2^{\bullet -}$ activated HSCs, evidenced by the expression of α-smooth muscle actin and the secretion of transforming growth factor β1 and collagen. We further found that blockade of chloride channels with 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) or indanyloxyacetic acid (IAA-94) prevented the increase of intracellular $O_2^{\bullet -}$ levels as well as the activation of HSCs. These findings suggest that $O_2^{\bullet -}$ is involved in the development of liver fibrosis and that entry of $O_2^{\bullet -}$, through chloride channels, in stellate cells is critical for their activation. This study provides new insight into the mechanism by which ROS induce liver fibrosis. Furthermore, our data suggest that chloride channels constitute a potential target for new anti-fibrotic drugs.

1. Introduction

Hepatic fibrosis is a persistent, overly exuberant wound healing in which excessive extracellular matrix proteins accumulate in the liver (Davis et al., 1994; Lieber, 1997; Morimoto et al., 1995; Pietrangelo, 1998). Activated hepatic stellate cells (HSCs) have been identified as the cell type responsible for the production and deposition of collagen (Milani et al., 1990; Friedman, 2000; Tsukada et al., 2006; Wallace et al., 2008). Following liver injury, HSCs are activated by cytokines and reactive oxygen species (ROS) released from Kupffer cells (Casini et al., 1997; Simeonova et al., 2001) to proliferate and secrete extracellular matrix (ECM) proteins and cytokines such as TGF-β1. The phenotype transformation of HSCs to myofibroblast-like cells is characterized by the expression of α smooth muscle actin (α-SMA). Human and animal experimental data have shown that increased ROS formation occurs in injured liver and contributes to the pathogenesis of liver fibrosis by activating HSCs (Svegliati Baroni et al., 1998). Furthermore, the activation of HSCs can be suppressed by antioxidants *in vitro* (Kawada et al., 1998) and *in vivo* (Houglum et al., 1997). However, surprisingly little is known about the mechanism by which ROS activate HSCs. Also rarely addressed is the fact that ROS constitute a heterogeneous group of species with widely varying chemical reactivity and therefore biological properties. The uncharged non-radical hydrogen peroxide is an oxidizing agent capable of passing membranes while the negatively charged superoxide anion radical ($O_2^{\bullet -}$) is a reducing agent that is unable to cross the plasma membrane. The third member of the ROS family, the hydroxyl radical is so reactive that it almost instantly reacts with any molecule in its vicinity. Differences between $O_2^{\bullet -}$ and hydrogen peroxide also become apparent when their breakdown routes are reviewed: dismutase enzymes for $O_2^{\bullet -}$ and catalase/peroxidises for

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hydrogen peroxide. Because ROS appear to play a critical role in the activation of HSCs it is relevant to determine the effects of the individual ROS species. Moreover, which of these ROS constitutes the key mediator of HSC activation remains unclear. The aim of this study was to characterize the effect of $O_2^{\bullet -}$ on HSCs *in vitro*.

Here, we show that $O_2^{\bullet -}$ activated HSCs resulting in the release of TGF-β1 and collagen from HSCs, and enhancing the expression of α-SMA. Activation of HSCs could be prevented by the $O_2^{\bullet -}$ scavengers copper, zinc-superoxide dismutase (SOD1) and tempol, indicating that the activation of HSCs is specifically due to $O_2^{\bullet -}$. We also showed that the non-specific chloride channels blockers, DIDS, NPPB and IAA-94 inhibited $O_2^{\bullet -}$-induced HSCs activation by blocking the cellular influx of $O_2^{\bullet -}$ through chloride channels.

2. Materials and methods

2.1. Chemicals

Xanthine, xanthine oxidase from bovine milk (Grade III), copper, zinc-superoxide dismutase (SOD1), tempol, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) and indanyloxyacetic acid (IAA-94) were all purchased from Sigma (St. Louis, MO, USA). Dihydroethidium (DHE) was obtained from Invitrogen (Carlsbad, CA, USA). α-SMA primers were obtained from BioLegio (Nijmegen, The Netherlands). iScript cDNA synthesis kit, Beacon Designer5 and IQ SYBR Green Supermix were purchased from Bio-Rad (Veenendaal, the Netherlands).

2.2. Cell culture

The human HSC line LX-2 (Xu et al., 2005) was kindly provided by Dr. S.L. Friedman, Mount Sinai School of Medicine, NY. LX-2 HSCs were cultured in 5% fetal calf serum (FCS) D-MEM/F-12 medium. Upon reaching 70–80% confluence, HSCs were washed with PBS and starved for 48 h with 0.1% FCS medium (D-MEM/F-12, 1:1). Next the cells were cultured with 0.1% FCS medium (D-MEM/F-12, 1:1) InVitrogen (Breda, The Netherlands). and exposed to $O_2^{\bullet -}$ generated from 0.5 mM xanthine and 100 mU/ml xanthine oxidase. Where indicated, 1500 U/ml SOD, 5 mM tempol, 200 μM DIDS, 100 μM NPPB or 100 μM IAA-94 were added to the HSCS 15 min before exposition to $O_2^{\bullet -}$.

2.3. TGF-β1 immunoassay

TGF-β1 released by HSCs was quantified using the Quantikine Human TGF-β1 immunoassay kit (R&D Systems, Abingdon, UK). Before quantification, latent TGF-β1 was activated to the immunoreactive form.

2.4. Total soluble collagen

Total soluble collagen (type I–VI) in the supernatants was determined using the Sircol® assay (Biocolor Ltd, Newtownabbey, Northern Ireland) according to the manufacturer's instructions.

2.5. Intracellular $O_2^{\bullet -}$ levels

Intracellular $O_2^{\bullet -}$ levels were determined as described by Peshavariya et al. (2007). Briefly, HSCs were washed with pre-warmed (37 °C) HBSS and suspended in Krebs HEPES buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 11 mM glucose and 20 mM HEPES) containing 25 μM dihydroethidium (DHE). Cells were seeded in a 96-well plate at a concentration of 10^4 cells per well. HSCs were then stimulated with $O_2^{\bullet -}$ with or without SOD1, tempol or chloride channel blockers. After 30 min, HSCs were washed to remove any unreacted extracellular DHE. Fluorescence was quantified with excitation and emission wavelengths of 480 ± 10 and 570 ± 10 nm and recorded from each well every 2 min and averaged over 20 min. Background fluorescence caused by oxidized DHE in the absence of cells was subtracted from the total fluorescence to determine the amount of fluorescence caused by intracellularly oxidized DHE only.

2.6. α-SMA expression

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics, Almere, The Netherlands) and contaminating genomic DNA was removed with the DNAse set also provide in the Pure RNA Isolation Kit. RNA quantity was measured with the ND-1000 spectrophotometer and RNA integrity was evaluated on an Agilent 2100 BioAnalyzer. To detect changes in expression levels of α-SMA real-time quantitative PCR (qPCR) was performed using individual cDNA samples. RNA (500 ng) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Primers were designed across exon boundaries using Beacon Designer5. The qPCR reactions were performed in a volume of 25 μl containing 12.5 ng cDNA, 1x IQ SYBR Green Supermix and 400 nM of gene-specific forward and reverse primers (for α-SMA forward, 5′-CAG GGC TGT TTT CCC ATC CAT-3′; and reverse, 5′-GAA GTA CCC GAT AGA ACA TGG C-3′ (BioLegio (Nijmegen, The Netherlands). cDNA was amplified using a two-step program (40 cycles of 10 s at 95 °C and 45 s at 60 °C) with a MyiQ system (Bio-Rad). Specificity of amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. Gene expression levels were determined using qBase analysis software (Hellemans et al., 2007) using a ΔΔ-Ct relative quantification model with PCR efficiency correction and reference gene normalization. The geometric mean of GAPDH gene was calculated and used as a normalization factor in the qBase analysis software (Hellemans et al., 2007).

2.7. Statistical analysis

Experiments were performed in triplicate and data are presented as means ± standard error of the mean (S.E.M.). All data were analyzed using one-way ANOVA followed by *t*-test when ANOVA indicated significant differences. Differences were considered significant at $P < 0.05$.

3. Results

3.1. TGF-β1 release from HSCs

Incubation with $O_2^{\bullet -}$ more than doubled the TGF-β1 release from LX-2 hepatic stellate cells (HSCs) (Fig. 1). SOD1 and tempol completely inhibited the increased TGF-β1 release. Blockade of chloride channels with DIDS, NPPB or IAA-94 before exposure to $O_2^{\bullet -}$ prevented the increased release of TGF-β1 from HSCs. SOD1, tempol and the chloride channels blockers alone did not show an effect on the release of TGF-β1 (data not shown).

3.2. Determination of total soluble collagen

As shown in Fig. 2, the total soluble collagen released from HSCs was strongly increased by $O_2^{\bullet -}$ generated by xanthine and xanthine oxidase. Co-incubation with $O_2^{\bullet -}$ scavengers SOD1 or tempol completely abolished the increased collagen secretion.