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Superoxide radicals increase transforming growth factor-β1 and collagen release from human lung fibroblasts via cellular influx through chloride channels

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ABSTRACT

Reactive oxygen species (ROS) have been implicated in the pathogenesis of fibrosis. However, it remains unclear which ROS is the major cause. We hypothesize that superoxide elicits specific toxicity to human lung fibroblasts and plays an important role in the development of pulmonary fibrosis. In this study, superoxide generated from xanthine and xanthine oxidase activated lung fibroblasts by increasing the release of TGF-β1 and collagen. This was associated with increased levels of intracellular superoxide. SOD and tempol, by scavenging respectively extracellular and intracellular superoxide, prevented the activation of fibroblasts induced by exposure to exogenous superoxide, whereas catalase did not. Moreover, hydrogen peroxide did not activate fibroblasts. Apparently, superoxide rather than hydrogen peroxide is involved in the regulation of TGF-β1 and collagen release in lung fibroblasts. The chloride channel blocker, DIDS, inhibited the increase of intracellular superoxide levels induced by exogenous superoxide and consequently prevented the activation of fibroblasts. This suggests that the cellular influx of superoxide through chloride channels is essential for superoxide-induced activation of fibroblasts. ERK1/2 and p38 MAPKs are involved in the intracellular pathway leading to superoxide-induced fibroblasts activation.

Superoxide possesses until now undiscovered specific pro-fibrotic properties in human lung fibroblasts. This takes place via the cellular influx of superoxide through chloride channels rather than via the formation of hydrogen peroxide.

Introduction

Reactive oxygen species (ROS) including $O_2^{\cdot -}$, H_2O_2 and $HO^{\cdot}$ are normal byproducts of O_2 metabolism and have important roles in biological processes. However, a massive increase in ROS can induce oxidative stress resulting in cell and tissue damage and thus leading to many diseases. It is known that toxicity of doxorubicin, bleomycin and paraquat is related to the production of ROS, which may result in cardiovascular diseases and fibrosis (Copland et al., 1974; Keizer et al., 1990; Manoury et al., 2005). This can be prevented by means of antioxidants like flavonoids and green tea extracts (Kim et al., 2006; Bast et al., 2007). However, no study, until now, has clarified which of the ROS forms the major cause of fibrosis. H_2O_2 and $HO^{\cdot}$ derived from $O_2^{\cdot -}$ are generally assumed to be more toxic and more relevant ROS than $O_2^{\cdot -}$ in disease development due to their high reactivity and cellular membrane permeability (McCord, 2000). It has however been shown that superoxide dismutase (SOD) protects against toxicity induced by doxorubicin, paraquat or radiotherapy damage on skin (Autor, 1974; Delanian et al., 2001; den Hartog et al., 2004). SOD has been shown to potentially improve fibrosis in liver fibrosis patients (Emerit et al., 2006). It also has been reported that reduction of $NO^{\cdot}$ production leads to an increase of fibrosis, which may be due to increased $O_2^{\cdot -}$ bioavailability (Ferrini et al., 2002). Apparently, $O_2^{\cdot -}$ is not as harmless as has been assumed and is probably more involved in disease development than previously suggested. Bellocq et al. (1999) have shown that H_2O_2 induces the production of TGF-β1, the key mediator of fibrosis, in A549 epithelial cells. However, this does not explain why SOD is protective in fibrosis patients.

Recent studies have provided the evidence that membrane chloride channels may function as a port of entry of $O_2^{\cdot -}$ in the cell (Ikebuchi et al., 1991; Hawkins et al., 2007). Chloride channels are widely found anion pores consisting of approximately 13 members. They are regulated by a variety of signals and play various roles in the cells including regulation of pH, volume homeostasis, organic solute transport (Suzuki et al., 2006). We hypothesized that $O_2^{\cdot -}$ displays specific toxicity to lung fibroblasts and plays an important role in development of pulmonary fibrosis through the mechanisms independently from secondary ROS like H_2O_2.

In this study, we explored the effects of $O_2^{\cdot -}$ radicals in particular on release of fibrotic markers such as TGF-β1 and collagen, cell survival and the intracellular redox state in lung fibroblasts IMR-90

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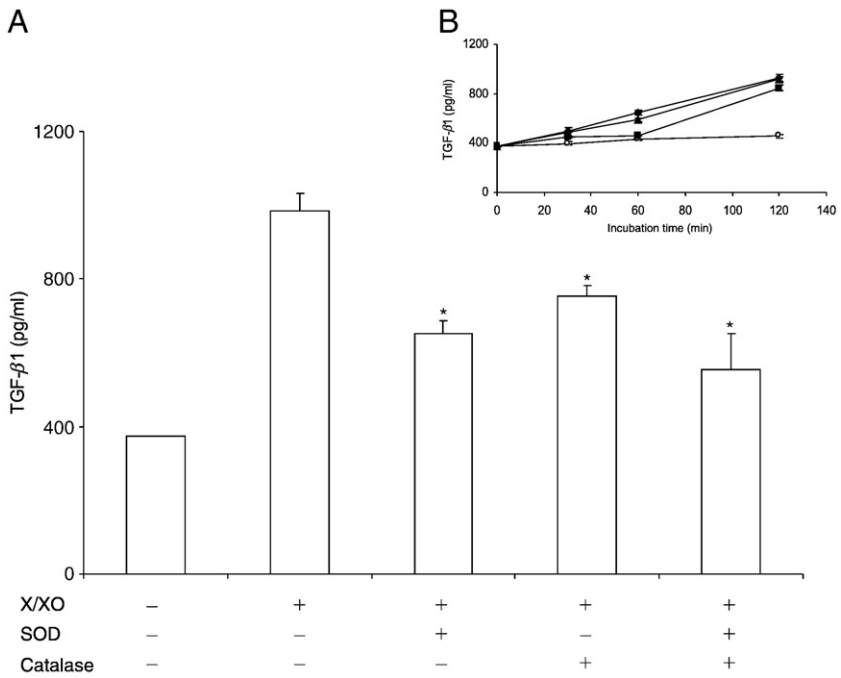

Fig. 1. The release of TGF-β1 in IMR-90 fibroblasts. (A) IMR-90 cells were exposed to $O_2^{\cdot-}$ generated by 0.5 mM xanthine and 100 mU/ml xanthine oxidase for 120 min with (out) 50 U/ml SOD or (and) 1000 U/ml catalase. (B) IMR90 cells were incubated with $O_2^{\cdot-}$ generated by 0.5 mM xanthine and xanthine oxidase (0 mU/ml ○; 50 mU/ml ■; 100 mU/ml ▲; 200 mU/ml ◆) for 30, 60 and 120 min. Results are the means ± SEM of three independent experiments. * Indicates difference from the results in fibroblasts exposed to $O_2^{\cdot-}$ ($P<0.05$).

and HFL1. SOD, tempol and catalase were used to confirm that these effects were due to specific actions of $O_2^{\cdot-}$. To clarify which of the ROS is more important in the activation of fibroblasts, HFL1 fibroblasts were also exposed to H_2O_2 without or with catalase. Furthermore, the possible mechanism of $O_2^{\cdot-}$-induced fibroblast activation was elucidated by assessing the effects of DIDS, a chloride channel blocker, on activation of HFL1 fibroblasts and the effects of $O_2^{\cdot-}$ in particular on MAPKs phosphorylation. Our data provide new insights into the specific cytotoxicity of $O_2^{\cdot-}$ in human lung fibroblasts and the possible underlying mechanism.

Materials and methods

Materials. Xanthine, xanthine oxidase from bovine milk (Grade III), hydrogen peroxide (H_2O_2), NADPH, glutathione reductase, SOD, tempol, catalase, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), dithiobisnitrobenzoic acid (DTNB), trypan blue and nitroblue tetrazolium (NBT) were all purchased from Sigma (St. Louis, MO, USA). The Quantikine Human TGF-β1 immunoassay kit was obtained from R&D systems (Minneapolis, MN, USA). The Caspase-Glo 3/7 Assay kit was from Promega (Madison, USA). Dihydroethidium (DHE) was obtained from Molecular Probes (Eugene, OR, USA). The Sirol assay was purchased from Biocolor (Newtownabbey, Northern Ireland).

Cell culture. Human female lung fibroblasts IMR-90 (CCL-186) and fetus lung fibroblasts HFL1 (CCL-153) purchased from American Type Culture Collection (Manassas, VA, USA) were cultured in Eagle's Minimal Essential medium with Earle's BSS and 2 mM L-glutamine (EMEM; ATCC) supplemented with 10% fetal bovine serum. (FBS, Gibco) and were maintained at 37 °C in a humidified incubator (95% air and 5% CO_2).

Exposure of fibroblasts to $O_2^{\cdot-}$ or H_2O_2. Fibroblasts were washed using Hanks' Balanced Salt Solution (HBSS) and were incubated with xanthine and xanthine oxidase (X/XO). In some cases, HFL1 fibroblasts were incubated with a H_2O_2 solution.

The effects of DIDS on X/XO generating $O_2^{\cdot-}$ were assessed by measuring XO inhibition and $O_2^{\cdot-}$ scavenging abilities of DIDS. XO activity was evaluated by measuring the uric acid formation from xanthine at 293 nm (Noro et al., 1983) and expressed as rate of uric acid formation (μM min^{-1}) ($\varepsilon = 12$ mM^{-1}) (Scheibe et al., 1974). The $O_2^{\cdot-}$ scavenging activity of DIDS was performed by using the nitroblue tetrazolium (NBT) assay (Liu et al., 1997). The transformation of formazan from NBT was determined spectrophotometrically at 560 nm. The rate of formazan formation was calculated using $\varepsilon = 12$ mM^{-1} (Blelski et al., 1980).

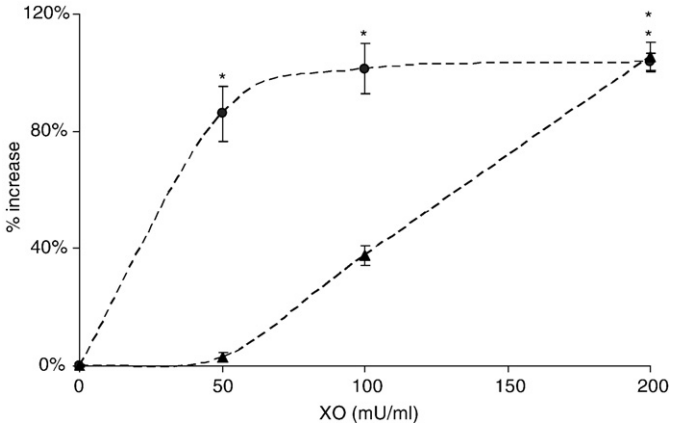

Fig. 2. TGF-β1 release (-●-) and Caspase-3 activity (-▲-) in IMR-90 fibroblasts. Fibroblasts were exposed to increasing concentrations of $O_2^{\cdot-}$ generated by 0.5 mM xanthine and xanthine oxidase (0 mU/ml; 50 mU/ml; 100 mU/ml; 200 mU/ml) for 120 min. Results are the means ± SEM of three independent experiments. * Indicates difference from the results in fibroblasts without treatment ($P<0.05$).