Effects Of Peroxynitrite On Intervertebral Disc Cells

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Introduction: A variety of inflammatory mediators have been implicated in intervertebral disc (IVD) degeneration. Special attention has been drawn recently towards reactive nitrogen species (RNS) for their role in disc abnormalities and sensitization of dorsal root ganglion neurons. RNS include among other species, nitric oxide (NO) and its derivative peroxynitrite (ONOO). Peroxynitrite is formed in vivo from the interaction of superoxide and NO. Peroxynitrite causes generalised oxidative damage, i.e. reacts with DNA, lipids and proteins. Not only nitrosative stress has been detected in human herniated and degenerative IVDs [4-6], but it has also been shown that human nucleus pulposus (NP) cells are able to produce RNS [6-9]. The aim of this study was to determine whether peroxynitrite plays a role in the inflammatory cascade that may lead to disc degeneration and, ultimately, pain.

Materials and Methods: The in vitro continuous flux of peroxynitrite, the peroxynitrite donor 3-morpholinesydnonimine (SIN-1) was used. SIN-1 slowly decomposes to release both superoxide and NO, which react together to produce peroxynitrite [1].

Cultivation media was DMEM/F12 medium with low glucose and low serum, i.e. 2%FCS and 1%ITS (Insulin; Transferrin; Selenium) (Sigma, Switzerland). The concentration of the reagents used to stimulate the cultures were 100μM of 3-morpholinesydnonimine (SIN-1) (Sigma, Switzerland); 10ng/ml of recombinant human TNF-α (R&D Systems, Inc.). 

Disc chondron cultures: Nucleus pulposus cells were isolated from bovine caudal spines with their pericellular matrix (PCM), i.e. chondrons, by overnight digestion with collagenase/dispase (37°C). Bovine chondrons were grown in monolayer and used as second or third passage cells. 

Quantitative PCR:

For the gene expression profile, the cultures were stimulated for 3 and 6 hours with SIN-1. Bovine specific probes and primers for IL-1β, IL-6 and TNF-α (Applied Biosystems) were used for real-time RT-PCR with RG-3000A (Corbett Research). Probes and master mix were purchased from Applied Biosystems. The data is shown as mean of three independent experiments.

Immunoblotting: p65 translocation into the nucleus was monitored. Briefly, cultures were stimulated for 40 minutes with SIN-1 or TNF-α and nuclear extracts were prepared as previously described [2]. The extracts were loaded on an SDS-PAGE gel and p65 (Santa Cruz, Biotech.) was detected.

Results: SIN-1 induced an 8-fold IL-1β expression at 3 hours of stimulation which was statistically significant. The level of IL-β returned to basal level at 6 hours (Fig.1A and B). Furthermore, there was no effect upon stimulation on neither, IL-6 or TNF-α, at any of the time points tested (Fig.1C and F). To determine NF-κB dependency, p65 translocation into the nucleus was examined after 40 minutes of stimulation with SIN-1. Nuclear extracts were prepared for unstimulated (UT), SIN-1 and TNF-α treated cells. UT and TNF-α treated cells were used as negative and positive controls, respectively. p65 was present in both TNF-α and SIN-1 treated cells, which suggest that NF-κB might be responsible for the observed IL-1β induction upon SIN-1 stimulation.

Discussion: Oxidative stress has long been related to IVD degeneration and other inflammatory joint diseases [10-12]. Reactive oxygen species are not the only free radical species involved in degeneration as RNS have also been shown to be present in the degenerated disc. To date, most studies have focused on NO and inducible NO synthase (iNOS). However, there has been little or no research on peroxynitrite and its putative role as an even more reactive, damaging and toxic species in the IVD. Peroxynitrite is one of the major damaging oxidants produced in humans during aging, inflammation and neurodegenerative disorders [13].

Our gene profiling data showed that IL-1β is the major cytokine induced by SIN-1 stimulation in the bovine NP cells. There were no changes in gene expression for IL-6 and TNF-α, thus highlighting the importance of IL-1β during nitrosative stress. Supporting evidence by Le Maitre et al. (2007) showed that in human degenerated IVD IL-1β but not TNF-α is the major detected cytokine.

Nuclear factor kappaB (NF-κB) plays an important role in the transcriptional regulation of genes involved in inflammation. In resting cells, NF-κB is maintained in an inactive state in the cytoplasm. In response to pro-inflammatory stimuli, translocation of the NF-κB subunits (p65/p50) to the nucleus is observed. Transcription is initiated upon binding of NF-κB to the promoter regions of responsive genes. The mechanism by which SIN-1 activates NF-κB or induces IL-1β gene expression is so far unknown. Taken together, our results suggest that in the bovine NP cells SIN-1 activates the NF-κB dependent signal transduction pathway and induces IL-1β upregulation.

Our study suggest that peroxynitrite may play a role in the inflammatory cascade occurring during disc degeneration and eventually leading to discogenic back pain.