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Implication of cytosolic phospholipase A_2 ($cPLA_2$) in the regulation of human synoviocyte NADPH oxidase (Nox2) activity

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Abstract

NADPH oxidase Nox2 is involved in the production of superoxide by rheumatoid synovial cells, constitutively and after pro-inflammatory cytokine treatment. The aims of the study were to evaluate the capacity of these cells to produce the superoxide anion in response to arachidonic acid (AA), and to study the involvement of cytosolic phospholipase A_2 ($cPLA_2$) in the cytokine regulation of Nox2. Superoxide production was quantified in synovial cells obtained from six patients with rheumatoid arthritis (RA) and six with osteoarthritis (OA), stimulated with (i) AA, and (ii) PLA_2 inhibitors prior to IL-1β or TNF-α treatment. Total cellular AA concentrations and PLA_2 activity were measured; effects of cytokines and NADPH oxidase inhibitors on the AA-activatable proton channel opening were also studied. Our results demonstrated that AA enhanced superoxide production in RA and OA cells; this production was significantly inhibited by iodonium diphenyl and apocynin. $cPLA_2$ inhibitors inhibited both IL-1β and TNF-α-induced superoxide production in RA and OA cells. Basal PLA_2 activity was significantly more important in RA cells than in OA cells; PLA_2 activity was increased in IL-1β and TNF-α pre-treated RA cells, and $cPLA_2$ inhibitors inhibited this activity. Opening of the AA-activatable proton channel was amplified when RA cells were pre-treated with both IL-1β and TNF-α, and iodonium diphenyl and apocynin inhibited these cytokine effects. We concluded that AA is an important cofactor for synovial NADPH oxidase activity. Despite their direct effects on p47-*phox* phosphorylation, cytokines can also regulate the Nox2 activity though the AA-activatable associated H^+ channel.

Keywords: Cytosolic phospholipase A2; Interleukin-1β; NADPH oxidase; Osteoarthritis; Rheumatoid arthritis; Superoxide anion; Synovial cells; Tumor necrosis factor-α

Introduction

Synovial cells appear to be involved in both the induction and destructive phases of the multicellular process in rheumatoid arthritis (RA) (Firestein and Zvaifler, 2002). Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are pro-inflammatory cytokines present at high levels in RA synovium, and play an important role in RA pathogenesis (Feldmann et al., 1996). The RA synovium is also exposed to reactive oxygen species (ROS) produced by synovial cells and leukocytes, which are responsible for collagen hydrolysis and metalloproteinase activation, leading to degradation of the extracellular matrix (Henrotin et al., 2003). One of the principal sources of superoxide is NADPH oxidase; this plasma membrane-bound NADPH oxidase complex is composed of two membrane-located subunits — p22-*phox* and Nox2/gp91-*phox* — and a ~250 kDa complex made up of p40-*phox*, p47-*phox* and p67-*phox*, localized in the cytosol (Dröge, 2002). We have recently demonstrated that rheumatoid synovial cells constitutively produce superoxide anion ($O_2^{\cdot-}$), and that this production was upregulated by two major RA-cytokines (IL-1β and TNF-α); NADPH oxidase was involved in this production,

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and both Nox2 (gp91-*phox*) and Nox4 were identified in RA synovial cells, suggesting the existence of two different complexes (Chenevier-Gobeaux et al., 2006).

Arachidonic acid (AA), released by cytosolic phospholipase A_2 ($cPLA_2$), has been shown to be essential to the activation of assembled phagocyte NADPH oxidase (Dana et al., 1998). It has been demonstrated that $cPLA_2$ is required for the PMA activation of proton efflux through gp91-*phox* in PLB cell lines, and that AA itself activates the proton channel (Mankelow et al., 2003). This channel mediates the proton efflux that balances the electronic charge translocated by NADPH oxidase, preventing extreme depolarization that would otherwise abolish NADPH oxidase activity (DeCoursey et al., 2003). $cPLA_2$ plays a critical role in the pathogenesis of collagen-induced arthritis, and pannus formation is reduced in $cPLA_2$-deficient mice (Hegen et al., 2003). It has been shown that $cPLA_2$ is upregulated after exposure to IL-1β and TNF-α in rheumatoid synovial fibroblasts (Hulkower et al., 1994; Bidgood et al., 2000).

Although NADPH oxidase and $cPLA_2$ have been studied separately in RA synovial cells (Chenevier-Gobeaux et al., 2006; Hulkower et al., 1994), the relationship between $cPLA_2$, AA and $O_2^{\cdot-}$ production in this cellular model of interest remains unknown. The aims of this study were thus (i) to evaluate the capacity of synovial cells to produce superoxide anion in response to AA, and (ii) to study the involvement of $cPLA_2$ in the IL-1β and TNF-α regulation of synovial NADPH oxidase. We compared the results obtained in RA cells with those obtained in osteoarthritis (OA) cells, as OA is considered to be a disease where the synovium presents mild to moderate inflammatory changes.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM, 10X), phosphate-buffered saline (PBS), trypsin-buffered solution (0.25%) and Hepes (1 M) solution were obtained from Eurobio (Les Ulis, France). The base medium contained DMEM with 2 mM L-glutamine (Eurobio), 100 U/ml penicillin (Sigma Aldrich, St Louis, MO, USA), 100 μg/ml streptomycin (Sigma) and 0.25 μg/ml amphotericin B (Sigma). The complete medium contained base medium plus 10% fetal calf serum (BioWhittaker, Walkersville, MD, USA). The cell lysis reagent (CelLytic™-M) was obtained from Sigma. Mouse monoclonal agarose conjugate antibody raised against p47-*phox* (anti-p47-*phox* AC) came from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and mouse monoclonal antibody anti-phosphoserine from Calbiochem (Merck Biosciences, Darmstadt, Germany). Secondary peroxidase-conjugated antibody was obtained from Amersham Biosciences (Buckinghamshire, UK). The sample buffer contained 6×10^{-2} M Tris/HCl, pH 6.8, 20% glycerol (v/v), 4% sodium dodecyl sulphate (SDS) (v/v) and 2% 2-mercaptoethanol (v/v) (BioRad, Ivry sur Seine, France). All materials for protein quantification, SDS-PAGE and electroblotting were purchased from BioRad. The chemiluminescence detection system (ECL plus Western blotting detection system) and high performance films (Hyperfilm™ ECL) were obtained from Amersham. Recombinant human cytokines (IL-1β and TNF-α), the protease inhibitor cocktail (containing aprotinin, bestatin, leupeptin, E-64, and pepstatin A), type Ia collagenase from *Clostridium histolyticum*, and chemical effectors such as superoxide dismutase (SOD), hypoxanthine (HX), xanthine oxidase (XO), iodonium diphenyl (DPI), 4-hydroxy-3-methoxyacetophenone (apocynin), arachidonic acid (AA), methyl arachidonyl fluorophosphonate (MAFP), palmityl trifluoromethyl ketone (PTK), thioetheramide-PC (TEPC), cytochrome *c*, valinomycin and nigericin were purchased from Sigma. MAFP was evaporated and prepared as a 27 mM stock solution in ethanol. The fluorescent pH-sensitive dye 2′,7′-bis(2-carboxyethyl)-5-(and -6-)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was obtained from Molecular Probes (Eugene, OR, USA) and prepared as a 1 mM stock solution in dried dimethyl sulfoxide.

Synovial cell isolation and culture

Synovial cells from six patients with RA of the knee, wrist or finger, and from six patients with OA, were isolated from synovium obtained during surgery or replacement of the affected joint. RA and OA were diagnosed according to American College of Rheumatology classification criteria (Arnett et al., 1988; Altman et al., 1991). All patients gave written, informed consent. After dissection, the superficial layer of the synovium was digested in trypsin solution and 5 mg/ml collagenase, and cells were cultured as previously described (Chenevier-Gobeaux et al., 2006). When confluence was attained, cells were treated with trypsin and cultured in 25 cm^2 flasks or in 24-well plates (1×10^5 cells/well), at 37 °C in a 5% CO_2 atmosphere.

Quantification of superoxide production

Superoxide production was measured by the superoxide dismutase (SOD) inhibitable reduction of cytochrome *c* (Miller and Griendling, 2002). Briefly, pre-treated (or not) cells in 24-well plates were washed with PBS, and stimuli (AA from 5 to 100 μM in PBS, expressed as final concentrations) and cytochrome *c* (60 μM in PBS) were added to the wells. Control wells were prepared as above with the addition of 200 U/ml of superoxide dismutase (SOD). Superoxide production was measured at 550 nm on a Uvikon941plus® spectrophotometer after 2 h at 37 °C in a 5% CO_2 atmosphere, and the amount of $O_2^{\cdot-}$ was calculated using a molecular extinction coefficient of 21,200/M/cm. Cells were then washed and 500 μl 0.1 N NaOH was added to each well during 1 h under agitation, in order to obtain protein solutions. Protein concentrations were measured at 750 nm. Superoxide production was finally expressed in nmol/min/mg.

In order to assay the potential scavenging effect of MAFP, PTK and TEPC on superoxide anions, we studied different concentrations of each PLA_2 inhibitor on a Hypoxanthine/Xanthine oxidase system. Serial mixes were prepared, containing hypoxanthine (HX, 60 μM), cytochrome *c* (60 μM), xanthine oxidase (XO, 2 mU/ml), and inhibitor (MAFP 0.7–35 μM, PTK 10 or 30 μM, TEPC 2 or 10 μM), expressed in final