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Inflammation Research

Superoxide production and NADPH oxidase expression in human rheumatoid synovial cells: regulation by interleukin-1β and tumour necrosis factor-α

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Abstract. *Objectives:* to evaluate the rheumatoid synovial cell capacity to produce superoxide anion in response to interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), and to study the NADPH oxidase involvement in this production.
Material and Methods: Synovial cells obtained from 7 rheumatoid arthritis (RA), 5 osteoarthritic (OA) patients, and dermal fibroblasts, were stimulated (i) with IL-1β and TNF-α, or (ii) with specific oxidase activators and inhibitors, before studying superoxide production; we also studied NADPH oxidase mRNAs and protein expression, and p47-*phox* phosphorylation.
Results: Constitutive superoxide production by RA cells was increased in comparison to OA cells and dermal fibroblasts, and was stimulated by PMA and ionomycin. This production was increased after cytokine treatment of RA synovial cells. Cytokine-induced superoxide production by RA cells was inhibited by iodonium diphenyl or apocynin, suggesting the involvement of NADPH oxidase. RT-PCR and western blot analysis revealed the presence of p47-*phox*, gp91-*phox* and Nox4 in RA and OA cells, and in dermal fibroblasts. P47-*phox* phosphorylation was enhanced after cytokine-treatment in RA and OA cells, suggesting a PKC-mediated up-regulation of NADPH oxidase.
Conclusions: NADPH oxidase is involved in the superoxide release by RA synovial cells, constitutively and after cytokine up-regulation. These cells express two different homologues (gp91-*phox* and Nox4).

Key words: NADPH oxidase – Superoxide anion – Rheumatoid arthritis – Synovial cells.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease involving hyperproliferation of the synovial membrane and accumulation of activated T cells and macrophages, leading to progressive joint destruction. Synovial cells appear to be involved in both the induction and the destructive phases of the multicellular process as reviewed by Firestein and Zvaifler [1]. Interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) are proinflammatory cytokines present in large quantities in RA synovium; TNF-α and IL-1β activate NF-κB, which in turn activates several steps of the inflammatory process by affecting gene expression [2, 3].

The RA synovium is also exposed to oxidative stress [4]. Reactive oxygen species (ROS) are responsible for collagen hydrolysis and activation of metalloproteinases, leading to the degradation of the extracellular matrix [5]. They can also facilitate p53 mutations in synovial cells [6]. These ROS include radicals, such as superoxide ions ($O_2^{\bullet -}$) and hydroxyl radicals ($HO^{\bullet}$), and non-radical species, such as hydrogen peroxide (H_2O_2).

One of the major sources of superoxide is the NADPH oxidase of neutrophils and macrophages that infiltrate the synovial membrane [7]; this plasma membrane-bound NADPH oxidase complex is composed by two membrane-located subunits – p22-*phox* and gp91-*phox* – and a ~250 kDa complex composed by p40-*phox*, p47-*phox* and p67-*phox* that is localised in the cytosol of resting cells. Many non-phagocytic cells, including endothelial cells, fibroblasts and chondrocytes, have been reported to express NADPH oxidase components and to produce oxygen free radicals [8, 9]. In chondrocytes, the NADPH oxidase contains an isoform of gp91-*phox* [9]. Recently, four homologues of gp91-*phox* (also termed Nox2, for NADPH oxidase), called Nox1, and Nox3 to Nox5, were identified in non-phagocytic cells [10–14]. These Nox proteins may be involved in the control of endothelial cell

spreading, motility, and cell-cell adhesion [15]. In response to growth factors and cytokines, vascular NADPH oxidases produce superoxide anions which serve as second messengers to activate multiple intracellular signaling pathways. Whereas the vascular NADPH oxidases have been found to be essential in the physiological response of vascular cells, including growth, migration, and modification of the extracellular matrix, they have also been linked to hypertension and to pathological states associated with uncontrolled growth and inflammation, such as atherosclerosis [16].

As synovial cells and ROS appear to be of major importance in the pathogenesis of RA [5, 17], the aims of this study were (i) to evaluate the capacity of synovial cells to produce the superoxide anion in response to IL-1β and TNF-α, and (ii) to study the involvement of NADPH oxidase in this ROS production. We compared the results obtained in RA cells to those obtained with osteoarthritis (OA) cells, as OA is considered to be a disease with a synovium presenting mild to moderate inflammatory changes [18], and dermal fibroblasts were used as controls [19].

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM, 10X), phosphate-buffered saline (PBS), and trypsin-buffered solution (0.25%) were obtained from Eurobio (Les Ulis, France). Base medium contained DMEM with 2 mM L-glutamine (Eurobio), 100 U/ml penicillin (Sigma Aldrich, St Louis, USA), 100 µg/ml streptomycin (Sigma) and 0.25 µg/ml amphotericin B (Sigma). Complete medium contained base medium plus 10% foetal calf serum (BioWhittaker, Walkersville, USA). Cell lysis reagent (CelLytic™-M) was obtained from Sigma. Primary polyclonal antibodies (anti-p47-*phox*, anti-gp91-*phox*, and anti-Nox4) and their blocking peptide were from Santa Cruz Biotechnology. Secondary peroxidase-conjugated antibodies were from Amersham Biosciences (Buckinghamshire, UK). Sample buffer contained 0.5 M Tris/HCl (Bio-Rad, Ivry sur Seine, France), pH 6.8, 20% glycerol (v/v) (Sigma), 5% sodium dodecyl sulphate (SDS) (v/v) (BioRad) and 2% 2-mercaptoethanol (v/v) (BioRad). All materials for protein quantification, SDS-PAGE and electroblotting were purchased from BioRad. Chemiluminescence detection system (ECL plus western blotting detection system) and high performance films (Hyperfilm™ ECL) were from Amersham. Recombinant human cytokines (IL-1β and TNF-α), chemical effectors (superoxide dismutase -SOD, phorbol 12-myristate 13-acetate -PMA, ionomycin, iodonium diphenyl -IDP, 4-hydroxy-3-methoxyacetophenone -apocynin, 4,4'-diaminodiphenyl sulfone -dapsone, rotenone, indomethacin, allopurinol, cytochrome c), type Ia collagenase from *Clostridium histolyticum*, phenylmethylsulfonyl fluoride, aprotinin, and leupeptin were purchased from Sigma.

Synovial Cell and Fibroblast Isolation and Culture

Synovial cells from seven patients with RA of the knee, wrist or finger (4 women and 3 men, mean age 61 ± 12 years) and from 5 patients with OA (3 women and 2 men, mean age 77 ± 5 years) were isolated from synovium obtained during surgery or joint replacement of the affected joint. RA and OA were diagnosed according to the American College of Rheumatology classification criteria [20, 21]. All patients gave written informed consent. After dissection, the superficial layer of the synovium was digested in trypsin solution and 5 mg/ml collagenase in base medium for 1 h at 37 °C with agitation. The cell suspension was then centrifuged at 600 g for 10 min at 20 °C, and the pellet was thereafter resuspended in 10 ml of base medium. After centrifugation, cells were suspended in complete medium (2×10^5 cells/ml) and cultured in 75 cm^2 culture flasks at 37 °C in a 5% CO_2 incubator. After overnight culture, non adherent cells were removed. 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 per well), at 37 °C in a 5% CO_2 atmosphere. Experiments were performed on confluent cultures between the 2nd and the 4th passage.

Dermal fibroblast lines were similarly prepared from enzymatically dispersed human skin biopsies.

Quantification of Superoxide Production

Superoxide production was measured by the superoxide dismutase (SOD) inhibitable reduction of cytochrome c [22]. In a first set of experiments, cells in 24-well plates were treated for 2 or 24 h with base medium supplemented with 1 ng/ml IL-1β, 0.5 ng/ml TNF-α, or neither; they were then washed with PBS, and cytochrome c (60 µM in PBS) was added to the wells; whenever necessary, NADPH oxidase inhibitors such as iodonium diphenyl or IDP (5×10^{-6}-10^{-4} M) and apocynin (5×10^{-5}-10^{-3} M) were added before cytokine treatment in base medium during 30 min. In a second set of experiments, cells in 24-well plates were washed with PBS and stimuli (1 µg/ml PMA, 5 µg/ml ionomycin, expressed as final concentrations) and cytochrome c (60 µM in PBS) were added to the wells; whenever necessary, oxidase inhibitors such as IDP (5×10^{-6}-10^{-4} M), apocynin (5×10^{-5}-10^{-3} M), dapsone (5×10^{-4} M), rotenone (10^{-6} M), indomethacin (10^{-4} M) and allopurinol (10^{-5}-10^{-3} M) were added in association with PMA. Control wells were prepared as above with the addition of 50 U/ml superoxide dismutase (SOD).
After two hours at 37 °C in a 5% CO_2 atmosphere, plates were placed on ice in the dark until measuring supernatant absorbance. Superoxide production was measured at 550 nm on an Uvikon941plus® spectrophotometer, and the amount of $O_2^{\bullet-}$ was calculated by using a molecular extinction coefficient of 21,200 M^{-1} cm^{-1}. Cells were then washed and 500 µl of 0.1 N NaOH added to each well. After 1 h of agitation, protein solutions were obtained, and concentrations were measured at 750 nm. The superoxide production was finally expressed in nmol.min^{-1} mg^{-1}.

Preparation of Total RNA

Synovial cells were cultured in 25 cm^2 flasks and were treated (or not) with IL-1β (1 ng/ml) or TNF-α (0.5 ng/ml) for 24 h. Human peripheral neutrophils, cultured NIH3T3 and HEK293 cells were used for RT-PCR positive controls. Cells were harvested and resuspended in Trizol® (Gibco) at approximately 1×10^6 cells/ml as described in the manufacturer's instructions. Purity of the mRNA was estimated by 260 nm: 280 nm spectroscopy, before determining RNA concentration.

Preparation of cDNA and Polymerase Chain Reaction (PCR)

cDNA was synthesised in a total volume of 50 µl (1 µg of RNA was added to the master mix prepared with the QIAGEN® OneStep RT-PCR kit from Qiagen GmbH, Germany) using an Eppendorf® Mastercycler gradient (Brinkmann Instruments Inc., Westbury, NY). The PCR conditions were as follows: first cycle, 50 °C for 30 min, 95 °C for 15 min; second to 27th–40th cycle, 94 °C for 45 s, 57 °C for 45 s, 72 °C for 1 min. The final cycle consisted of 72 °C for 10 min. PCR products from test (p22-*phox*, p47-*phox*, gp91-*phox,* Nox1, Nox3 and Nox4) and control (β-actin) were separated in 2% (w/v) agarose gels containing 10 µl of ethidium bromide (BioRad) per gel, and photographed under UV light. The PCR primers used are listed in Table 1 [23–25].

Whole Cell Protein Extraction

Synovial cells and fibroblasts were cultured in 25 cm^2 flasks. Cells were treated (or not) with IL-1β (1 ng/ml) or TNF-α (0.5 ng/ml) for

Table 1. Sequences of the oligonucleotide PCR primers.

Gene	Primer sequence (5'-3')	Reference
β-actin	Forward-CCACTGGCATCGTGATGGAC Reverse-GCGGATGTCCACGTCACACT	[23]
p22-*phox*	Forward-GTTTGTGTGCCTGCTGGAGT Reverse-TGGGCGGCTGCTTGATGGT	[24]
p47-*phox*	Forward-ACCCAGCCAGCACTATGTGT Reverse-AGTAGCCTGTGACGTCGTCT	[24]
gp91-*phox*	Forward-TGGGCTGTGAATGAGGGGCT Reverse-TGACTCGGGCATTCACACAC	[24]
Nox1	Forward-CTTCCTCACTGGCTGGGATA Reverse-TGACAGCATTTGCGCAGGCT	[25]
Nox3	Forward-GAGTGGCACCCCTTCACCCT Reverse-CTAGAAGCTCTCCTTGTTGT	[25]
Nox4	Forward-AGTCAAACAGATGGGATA Reverse-TGTCCCATATGAGTTGTT	[25]

24 h, or with PMA (1 μg/ml) during 2 h. After washing, cells were treated with trypsin and centrifuged. The pellet was then treated with 125 μl of CelLytic™-M reagent per 10^6 cells for 30 min at 4 °C, and centrifuged for 10 min at 13,000 g. The protein concentration of the resulting solution was first measured using a BioRad kit based on the Lowry method.

Electrophoresis and Immunoblotting

Protein fractions were denatured in sample buffer for 5 min at 95 °C before subjected to SDS-PAGE on a 4–15 % Tris-HCl polyacrylamide gel for 2 h at 100 V. The proteins were then electroblotted onto a nitrocellulose membrane overnight at 30 V, and probed with (i) antibodies for p47-*phox*, gp91-*phox* and Nox4, diluted 1: 500, or (ii) the same antibodies preincubated 24 h at 4 °C with 5-fold specific blocking peptide. The blots were then incubated with peroxidase-conjugated secondary antibodies (diluted 1: 35000) and used to expose Hyperfilm™ ECL as described in the ECL plus western blotting detection system.

Co-immunoprecipitation

Co-immunoprecipitation was performed as described previously using protein samples (250 μg) diluted in Tris-HCl 0.05 M buffer (pH 7.4) containing 50 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin [23]. Proteins were immunoprecipitated down with 10 μl of anti-phosphoserine-specific monoclonal antibody for 2 h at 4 °C. Protein G-agarose conjugate (20 μl) was added and cap tubes were incubated at 4 °C on a rocker platform to overnight. Normal rabbit IgG-coupled protein G-agarose conjugate was used as negative control. Immunocomplex-bound beads were collected by centrifugation at 2,500 rpm for 30 s at 4 °C, washed three times with PBS and resuspended in 40 μl of 2X sample buffer. Samples were boiled for 5 min, and proteins were separated by 4–15 % SDS-PAGE. Immunoprecipitates were then immunoblotted as described above.

Data Presentation and Statistical Analysis

Quantitative data are presented as means ±standard deviations (S.D.) from representative experiments performed in duplicate. Statistical significance was evaluated by Mann-Withney or Wilcoxon tests, and p values less than 0.05 were considered significant.

Results

Quantification of Superoxide Production by Synovial Cells

Unstimulated RA synovial cells produced more superoxide than OA cells (0.27 ± 0.05 *vs*. 0.20 ± 0.05 nmol.min^{-1}.mg^{-1}, $p < 0.05$). In comparison, unstimulated fibroblasts released only 0.18 ± 0.01 nmol.min^{-1}.mg^{-1} ($p < 0.05$ *vs*. RA cells, N.S. *vs.* OA cells).

We tested the effects of prior treatment with IL-1β (1 ng/ml) or TNF-α (0.5 ng/ml) for 2 and 24 h: RA cells produced significantly more $O_2^{\bullet-}$ when stimulated with IL-1β during 2 and 24 h, or with TNF-α during 24 h (Fig. 1A), while IL-1β and TNF-α had no significant effect on superoxide production in OA cells (Fig. 1B) nor in fibroblasts (Fig. 1C). In RA cells, the 24 h IL-1β and TNF-α-induced production was inhibited by NADPH oxidase inhibitors such as IDP and apocynin (Fig. 2A); in OA cells, these inhibitors had no significant effects on the 24 h IL-1β and TNF-α-induced production (Fig. 2B).

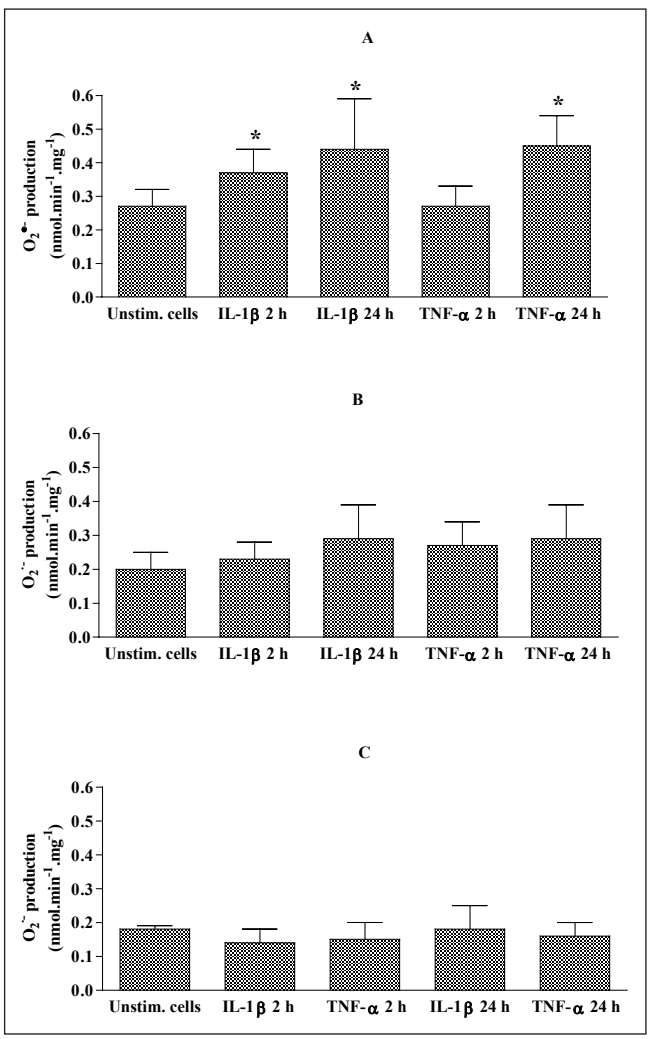

Fig. 1. Effects of IL-1β and TNF-α on superoxide constitutive production by synovial cells. A, RA synovial cells; B, OA synovial cells; C, dermal fibroblasts. ***,** $p < 0.05$ *vs.* unstimulated cells.

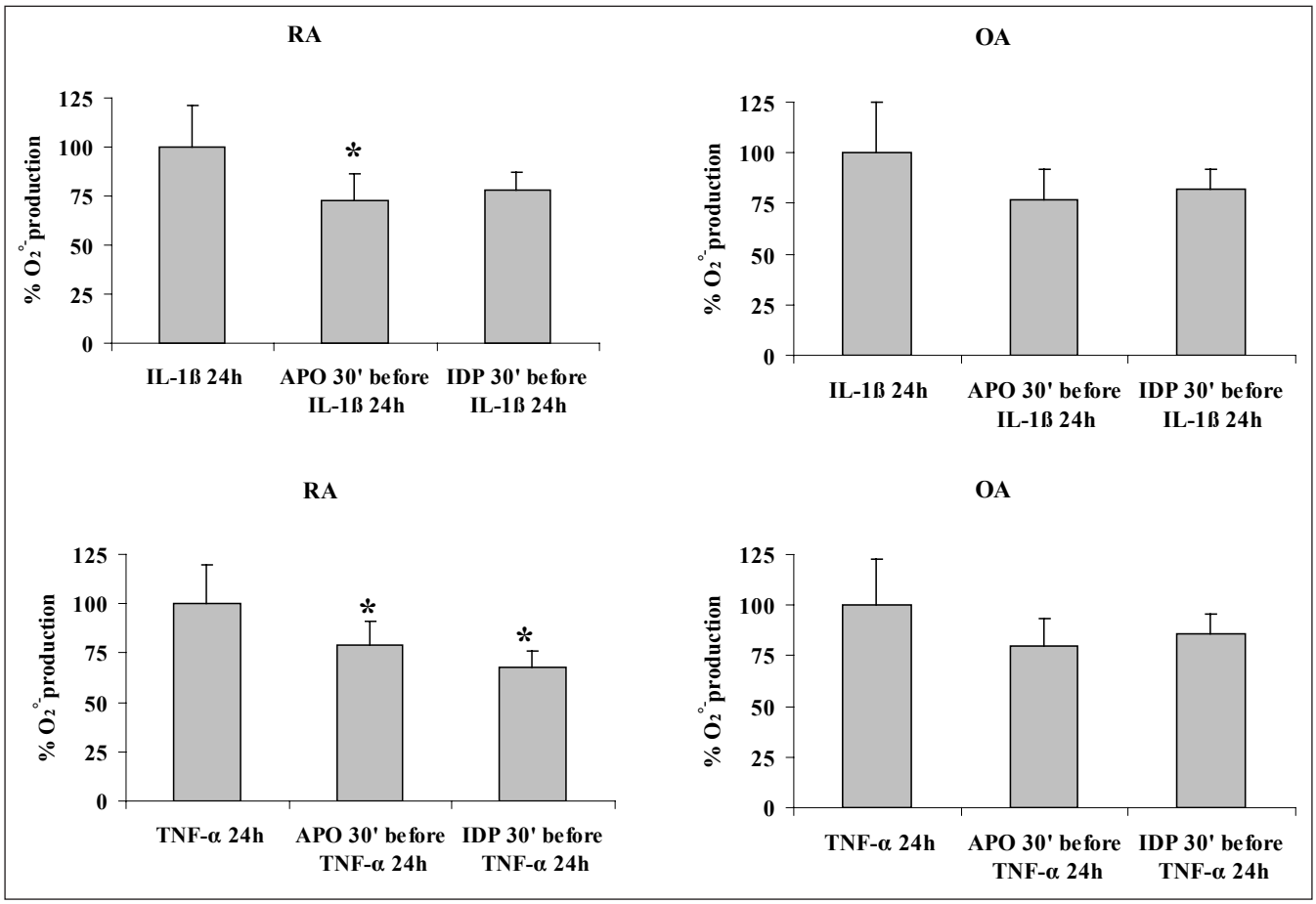

Fig. 2. Effects of apocynin (APO) and IDP on IL-1β and TNF-α-induced superoxide production by synovial cells. RA, RA synovial cells; OA, OA synovial cells. * $p < 0.05$ *vs*. cells treated during 24 h with IL-1β (1 ng/ml) or TNF-α (0.5 ng/ml). Apocynin (2.10^{-4} M) or IDP (2.10^{-5} M) was added during 30 min before cytokine-treatment.

In order to study the involvement of NADPH oxidase in this production, we also performed analysis with specific activators and inhibitors. In RA cells, the basal production was significantly increased by PMA (0.35 ± 0.10 $nmol.min^{-1}.mg^{-1}$, $p < 0.05$) and ionomycin (0.34 ± 0.05 $nmol.min^{-1}.mg^{-1}$, $p < 0.05$); in OA cells, PMA (0.30 ± 0.12 $nmol.min^{-1}.mg^{-1}$, $p < 0.05$) and ionomycin (0.30 ± 0.13 $nmol.min^{-1}.mg^{-1}$, $p < 0.05$) had also significant effects on basal superoxide production. IDP (flavohemoprotein inhibitor) and apocynin (NADPH oxidase inhibitor) inhibited PMA-induced $O_2^{\bullet -}$ production at high concentrations especially in RA cells, whereas the other inhibitors such as dapsone (lipoxygenase inhibitor), rotenone (mitochondrial oxidase inhibitor), indomethacin (cyclooxygenase inhibitor), and allopurinol (xanthine oxidase inhibitor) had no effect (Table 2).

Detection of mRNA for Components of the NADPH Oxidase Complex

In order to identify which NADPH oxidase subunits are implicated in the superoxide production observed, we used RT-PCR to seek the relevant mRNA. cDNA was made from mRNA isolated from IL-1β and TNF-α stimulated (or not) cultured synovial cells. cDNA integrity was checked by using primers designed to amplify the housekeeping gene actin. Primers for known human phagocyte NADPH oxidase components and Nox proteins detected p22-*phox*, p47-*phox*, gp91-*phox* and Nox4 components in RA and OA cells (Fig. 3). However, the IL-1β or TNF-α treatment did not affect the mRNAs production.

Detection of NADPH Oxidase Subunits in Whole Cell Protein Extracts

Synovial cells were treated (or not) with IL-1β (1 ng/ml) or TNF-α (0.5 ng/ml) for 24 h, or with PMA (1 μg/ml) during 2 h. Cells were then lysed and protein extracts immunoblotted. RA and OA stimulated (or not) synovial cells showed a band corresponding to the expected protein for each antibody probed (anti-p47-*phox*, anti-gp91-*phox*, and anti-Nox4); these bands were not detected in presence of specific blocking peptides (Fig. 4). Dermal fibroblasts were examined as controls: we also detected p47-*phox*, gp91-*phox*, and Nox4 in these cells. However, we could not detect any effect of cytokine treatment on protein expression.

Table 2. Superoxide production by synovial cells effects of oxidase inhibitors.

		Superoxide production (nmol. min^{-1}.mg^{-1})	
Addition	**Effector concentration**	**RA cells**	**OA cells**
PMA	1 µg/ml	0.35 ± 0.12	0.30 ± 0.13
PMA plus:			
IDP	5×0^{-6} M	0.32 ± 0.08	0.26 ± 0.06
	10^{-5} M	0.27 ± 0.04	0.29 ± 0.02
	2×0^{-5} M	0.20 ± 0.09 *	0.25 ± 0.13
	10^{-4} M	0.20 ± 0.10 *	0.21 ± 0.05 *
Apocynin	5×10^{-5} M	0.38 ± 0.11	0.36 ± 0.03
	10^{-4} M	0.32 ± 0.08	0.32 ± 0.06
	2×10^{-4} M	0.21 ± 0.06 *	0.23 ± 0.08 *
	10^{-3} M	0.20 ± 0.06 *	0.23 ± 0.07 *
Dapsone	5×10^{-4} M	0.38 ± 0.04	0.38 ± 0.07
Rotenone	10^{-6} M	0.32 ± 0.13	0.34 ± 0.01
Indomethacin	10^{-4} M	0.34 ± 0.0	0.31 ± 0.06
Allopurinol	10^{-5} M	0.25 ± 0.11	0.31 ± 0.08
	10^{-4} M	0.29 ± 0.09	0.27 ± 0.10
	10^{-3} M	0.28 ± 0.07	0.31 ± 0.06

Compounds were added at t = 0 (results are the mean of seven samples).
* $p < 0.05$ vs. PMA-treated cells.

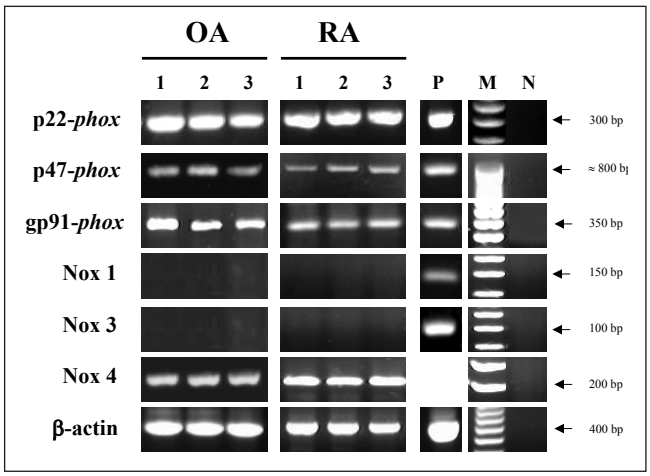

Fig. 3. Representative RT-PCR amplification of RA and OA synovial cell-derived mRNA using primers for NADPH oxidase and Nox proteins. Lanes 1, RT-PCR products obtained without stimulation; lanes 2, products of cells stimulated with IL-1β (1 ng/ml) for 24 h; lanes 3, products of cells stimulated with TNF-α (0.5 ng/ml) for 24 h. M represents 50–1,000 bp DNA marker ladder; N represents negative control, that is, in the absence of cDNA; P represents positive control, that is, the RT-PCR products obtained with control cells (human peripheral neutrophils for p22-phox, p47-phox, and gp91-phox; cultured NIH3T3 for Nox1; HEK293 cells for Nox3).

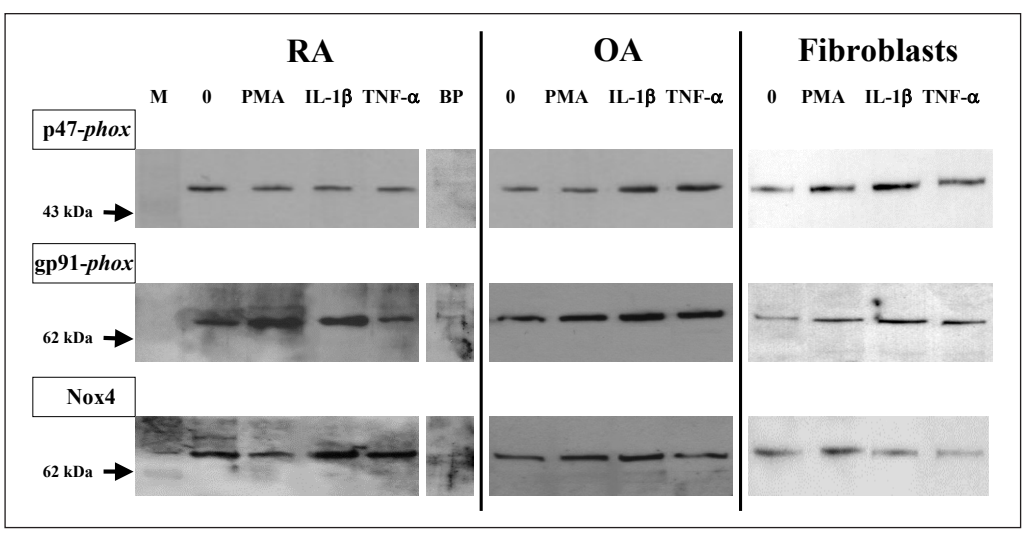

Fig. 4. Representative detection of p47-*phox*, gp91-*phox* and Nox4 in RA and OA synovial cells, and in fibroblasts. Cells were unstimulated (lane 0), or treated with PMA (1 µg/ml) for 2 h, or with IL-1β (1 ng/ml) or TNF-α (0.5 ng/ml) for 24 h. Whole cell protein extract were separated by SDS-PAGE, and blotted on nitrocellulose membranes. Membranes were probed with antibodies specific for p47-*phox*, gp91-*phox* and Nox4, or with the antibody preincubated 24 h at +4 °C in the presence of 5-fold specific blocking peptide (BP lane). M represents protein marker ladder.

Immunoprecipitation for p47-phox

To examine p47-*phox* phosphorylation, we immunoprecipitated serine-phosphorylated proteins and then probed for the existence of p47-*phox* in RA and OA cells, and in fibroblasts (Fig. 5). In usntimulated RA cells, the presence of a phosphorylated subunit before cytokine stimulation was observed, and p47-*phox* phosphorylation was increased after 24 h of IL-1β or TNF-α treatment. In OA cells this activated subunit was only detectable after 2 h of cytokine treatment, while in fibroblasts, p47-*phox* phosphorylation was not enhanced by cytokine treatment. Here again, RA cells appeared more responsive to 24 h of cytokine-treatment than OA cells. However, we did not show a direct correlation between the observed level of phosphorylated p47-*phox* and $O_2^{\bullet-}$ production. Indeed, after 2 h of TNF-α treatment, OA cells appear to generate level of phosphorylated p47-*phox* comparable to those found in RA cells, yet only the RA cells yielded an enhanced $O_2^{\bullet-}$ production (Fig. 1A). Likewise, after 24 h of IL-1β or TNF-α treatment, levels of phosphorylated p47-*phox* appeared much greater than those observed after 2 h of cytokine treatment, yet the increase in $O_2^{\bullet-}$ production was minimal (Fig. 1A).

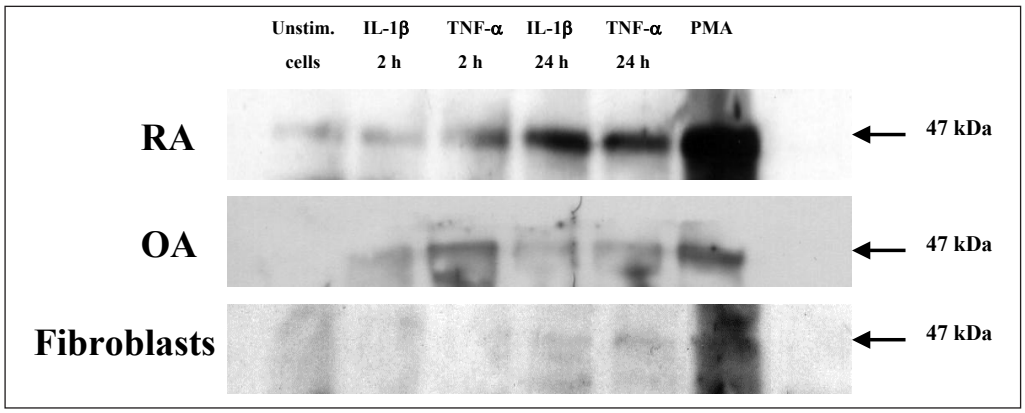

Fig. 5. Representative effects of IL-1β and TNF-α on p47-*phox* phosphorylation in RA and OA cells, and in fibroblasts. Serine phosphorylated proteins were immunoprecipitated and immunoblotted for p47-*phox*. Cells were unstimulated (lane 0), or treated with IL-1β (1 ng/ml) or TNF-α (0.5 ng/ml) for 2 or 24 h, or with PMA (1 μg/ml) for 2 h.

Discussion

We first probed the capacity of synovial cells to produce the superoxide anion: we observed a low level of constitutive superoxide production in RA cells, as described in immortalised chondrocytes and endothelial cells [24, 26]. We estimated that unstimulated RA synovial cells produced ~15 nmol.h^{-1} of superoxide per 10^6 cells, which is lower than for phagocytes (~20–60 nmol.h^{-1} per 10^6 cells, [7]), but higher than for immortalised chondrocytes (~3.2 nmol.h^{-1} per 10^6 cells, [25]). The RA superoxide production was significantly increased in comparison to OA synovial cells and to fibroblasts production.

Moreover, superoxide production was enhanced by the addition of the calcium ionophore, ionomycin, and by PMA, in both RA and OA cells: these results suggested the involvement of a NADPH oxidase regulated by both intracellular calcium concentration and PKC pathway. Indeed, ionomycin increases the cytosolic Ca^{2+} concentration [27], and PMA causes p47-*phox* phosphorylation through the protein kinase C (PKC) [28]. Of note, our results were in accordance with those of Tanabe et al. who detected a PMA-induced oxyradical production in human rheumatoid synovial cells [29]. We also found that IDP and apocynin inhibited the PMA-induced superoxide production in a dose-dependent manner in RA cells, and only at high concentrations in OA cells. IDP is a flavohemoprotein inhibitor [30], whereas apocynin inhibits the assembly of the NADPH oxidase components by interfering with the translocation of p47-*phox* and p67-*phox* [31]. Specific inhibitors of other oxidases (lipoxygenase, mitochondrial oxidase, cyclooxygenase and xanthine oxidase) did not show any effect when used at usual concentrations [32–34].

The existence of a NADPH oxidase in synovial cells was confirmed using RT-PCR and immunoblotting: our results revealed the presence of several NADPH oxidase subunits in unstimulated cells. Even if the expression of p22-*phox* is fairly ubiquitous [35], the expression of the cytosolic component p47-*phox*, associated with the expression of other subunits of NADPH oxidase (gp91-*phox* and Nox4), strongly suggested the presence of NADPH oxidase in these cells, and was in accordance with the observed constitutive production. Dermal fibroblasts were examined as controls in western blot experiments [36, 37].

We then evaluated the capacity of synovial cells to produce superoxide anion in response to IL-1β and TNF-α. When used at concentrations observed in RA [38], IL-1β and TNF-α enhanced superoxide production in RA cells, while they did not have any effect in OA cells or in fibroblasts. Moreover, IL-1β and TNF-α-induced production in RA cells was inhibited by iodonium diphenyl and apocynin, suggesting the involvement of NADPH oxidase.

However, neither IL-1β nor TNF-α showed a regulation of mRNAs or protein expression. Indeed, it has been demonstrated that IL-1β can induce intracellular ROS that act as second messengers [39, 40]; it has also been demonstrated that PKCζ is required for activation of NADPH oxidase in response to TNF-α in endothelial cells [41], and enhance phosphorylation of p47-*phox* in human neutrophils [42]. Then we can hypothesise that these cytokines activate NADPH oxidase through a PKC pathway.

We finally performed a co-immunoprecipitation and demonstrated that in RA cells, p47-*phox* is already phosphorylated before cytokine stimulation, and this phosphorylation is enhanced after 24 h of IL-1β or TNF-α-treatment in RA cells. In comparison, p47-*phox* phosphorylation could be detected only after 2 or 24 h of cytokine treatment in OA cells. These results were in accordance with the hypothesis of a cytokine up-regulation of NADPH oxidase through a PKC-mediated pathway. The presence of the active complex in synovial cells could be the result of the permanent stimulation of these cells by IL-1β and TNF-α in RA. Our results suggest that synovial cells can produce ROS in quantities that are compatible with their aggressive behaviour in RA [17]. In comparison, NADPH oxidase in OA cells did not appear to be preactivated by cytokines, and p47-phox phosphorylation was insufficient to enhance superoxide production. The absence of p47-*phox* phosphorylation by cytokine treatment in normal fibroblasts was in accordance with the superoxide production observed. However, our results concerning the phosphorylation of p47-phox do not provide a complete explanation for the enhanced sensitivity of RA synovial cells, as we did not show a direct correlation between the level of phosphorylated p47-phox and ROS production. Other NADPH oxidase subunits (e. g., p67-*phox*) could be involved in this cytokine upregulated superoxide production.

As we found gp91-*phox* and Nox4 mRNA and protein, synovial cells may express two different NADPH oxidase

systems, as described in vascular smooth muscle cells [43] and in human umbilical vein endothelial cells [15]. Moreover, we can hypothesise that these two NADPH oxidase complexes may have different functions associated with different localisation in the synovial cell, as previously described [44]. In contrast to the activated phagocyte oxidase, which produces large quantities of ROS into a phagosomal compartment in inducible bursts, Nox1 and Nox4 generate low-level, predominantly intracellular ROS constitutively and in response to agonists [16, 45]. In synovial cells, Nox4 could be responsible for a continuous superoxide production, and gp91-*phox* could be responsible be for a cytokine upregulated superoxide production.

In conclusion, our results show that constitutive superoxide anion production by rheumatoid synovial cells is upregulated by the two major RA-cytokines, IL-1β and TNF-α. We also showed that NADPH oxidase is involved in this production. Furthermore, these cells express two different membrane homologues (gp91-*phox* and Nox4), suggesting the existence of two different complexes. As this RA superoxide anion production mediated by NADPH oxidases is upregulated by pro-inflammatory cytokines, therapies such as anti-TNF-α or antagonists to the IL-1 receptor could act by inhibiting these oxidases in RA.

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