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Impairment of thioredoxin reductase activity by oxidative stress in human rheumatoid synoviocytes

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Abstract

The thioredoxin/thioredoxin reductase system is strongly induced in patients with rheumatoid arthritis (RA). We have investigated the impact on TR activity of doses of superoxide anion generated by the hypoxanthine (HX)/xanthine oxidase (XO) system and by hydrogen peroxide, H_2O_2, for various times and compared the findings with synoviocytes obtained from osteoarthritis (OA) patients. At baseline, TR activity in RA cells was significantly higher than in OA cells (2.31 ± 0.65 versus 0.74 ± 0.43 mUnit/mg protein, $p < 0.01$). HX/XO and H_2O_2 in RA cells decreased TR activity, which was found to be unchanged in OA cells. H_2O_2 and superoxide anion caused a time-dependent accumulation of oxidized TR and induced the formation of carbonyl groups in TR protein in RA cells rather than OA cells, and oxidized the selenocysteine of the active site. The oxidation in TR protein was irreversible in RA cells but not in OA cells. In conclusion, we report that the oxidative aggression generates modifications in the redox status of the active site of the TR and induces an alteration of the Trx/TR system, concomitant with those of the other antioxidant systems that could explain the causes of oxidative stress related to RA disease.

Keywords: *Oxidative stress, thioredoxin reductase, synoviocytes, rheumatoid arthritis*

Abbreviations: *BCIP, 5-Bromo-4-Chloro-3-Indolyl Phosphate; BIAM, Biotin-conjugated iodoacetamide; DMEM, Dubelcco modified Eagle milieu; GSH, Glutathione; H_2O_2, Hydrogen peroxide; IAM, Iodoacetamine; NADPH, Reduced nicotinamide adenine dinucleotide phosphate; NBT, Nitro Blue Tetrazolium; $O_2^{\bullet -}$, Superoxide anion; OA, Osteoarthritis; RA, Rheumatoid Arthritis; SOD, Superoxide dismutase; TR, Thioredoxin reductase; Trx, Thioredoxin*

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by progressive and irreversible joint damage, which results in a massive infiltration of immune cells and synovial proliferation [1]. During the inflammatory process, the production of a large array of cell-derived inflammatory mediators such as cytokines, chemokines and growth factors, induce the pathological outgrowth of synovial cells and proteolytic enzymes that affect bone and cartilage structures [2]. Although the pathophysiological basis of this disease is not fully understood, reactive oxygen species (ROS) have been described as an important mechanism underlying destructive proliferative synovitis [3]. Several sources in synovial joints are implicated in the excessive production of ROS during the activation of neutrophils and macrophages, post-ischemic reperfusion injury, vascular changes and the inflammatory process [4,5]. The excessive production of ROS at the site of inflammation contributes to the inflammatory process

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by inducing the expression of adhesion molecules, pro-inflammatory cytokines and chemoattractants [3,6], and T-cell hyporesponsiveness in RA due to effects on proteins and proteosomal degradation [7].

A reduction in antioxidant capacities can also have deleterious effects. Thus, low serum levels of antioxidants have been found to predispose patients to the development of RA [8]. Moreover, several studies report preventive effects of antioxidant supplements in protection against RA [9,10]. By contrast, studies have shown that increased expression of the antioxidant thioredoxin system is correlated with RA disease activity [11–15]. The thioredoxin system, composed of thioredoxin reductase (TR), thioredoxin (Trx) and NADPH, is the most important constituent of the intracellular redox milieu controlling the redox state, the antioxidant defences and the redox regulation of cellular processes [16]. TR exhibits broad substrate specificity, and contains an essential selenocysteine residue that renders TR a promising candidate for an ROS sensor [17]. TR catalyzes the NADPH-dependent reduction of a redox-active disulfide in Trx (a 12-kDa thiol multifunctional protein), which serves a broad range of functions as a reducing agent for protein disulfide, ribonucleotide reductase, peroxiredoxins and transcription factors (e.g. fos, Jun, NF-KB, p53), thus controlling key aspects of cell proliferation and survival [18]. But TR does not only reduce $TrxS_2$, it has the potential to regenerate ascorbate, induce superoxide dismutase, reduce S-nitrosoglutathione and influence the activity of the transcription factors of important genes [18,19]. One of the major consequences of oxidative stress is irreversible protein modifications, such as the generation of carbonyls or a loss of thiol residues [20]. These oxidative modifications alter the biological properties of proteins, and can give rise to enzyme dysfunctions [21]. During this study, we therefore, examined the expression and regulation by ROS of TR in RA synoviocytes, and assessed the possibility that oxidative stress may directly inhibit TR activity through the accumulation of an oxidized form of TR.

Materials and methods

Patients

Human synovial cells were obtained from eight patients with RA (five women and three men, with a mean age of 60.9 ± 7.5 years and a mean disease duration of 13.8 ± 4.3 years). All patients had been diagnosed with RA in accordance with the 1987 definition of the American College of Rheumatology [22]. Patients who had received intra-articular injections of corticosteroids were excluded. The patients were being treated with low-dose glucocorticosteroids (< 5 mg/d) (61%), non-steroidal anti-inflammatory drugs (NSAIDs) (23%), methotrexate (50%) and hydroxychloroquine (12%). Six patients (four women, two men, mean age 71.2 ± 7.7 years, mean disease duration 6.9 ± 4.4 years) with osteoarthritis disease (OA), a low inflammatory syndrome with a weak capacity for proliferation, were also included and considered as controls. Most of these patients were receiving NSAIDs (70%).

Reagents, cytokines and enzymes

Rabbit polyclonal IgG anti-human thioredoxin-reductase 1 (TR), raised against amino acids 71–340 mapping within an internal TR1 region of human origin, was obtained from TEBU (France). DMEM, fetal calf serum (FCS), amphotericin B, netromycin, ceftazidime and vancomycin were obtained from Eurobio (les Ulis, France). Rabbit IgG peroxidase-linked antibodies, HRP-conjugated streptavidin and ECL-Plus western blotting detection reagents were purchased from Amersham Biosciences (Buckinghamshire, UK). Biotin-conjugated iodoacetamide (BIAM) were purchased from Molecular Probe (Invitrogen, France). Recombinant human thioredoxin, TR from rat liver, reduced nicotinamide adenine dinucleotide phosphate (NADPH), 5,5′-dithiobis(2-nitrobenzoic-acid) (DTNB), hydrogen peroxide (H_2O_2), hypoxanthine (HX)/xanthine oxidase (XO) system and all other reagents were purchased from Sigma, St Louis (MO, US).

Cell isolation and culture

Human synovial cells were isolated from fresh synovium collected during synovectomy or joint replacement surgery. The superficial layer of the synovium was dissected out and treated with collagenase and trypsin for 2 h at 37°C, as previously described [23]. The cells obtained were suspended in DMEM supplemented with 10% FCS, amphotericin B, netromycin, ceftazidime and vancomycin, and cultured at a density of $1–3 \times 10^6$ cells per flask in 25 or 75 cm^2 culture flasks at 37°C, in a humidified incubator under 5% CO_2 in air. The culture medium was changed and non-adherent cells removed every 3 days until subconfluency. Adherent cells were harvested by trypsinization and subcultures were maintained in the same medium. All experiments were performed using synoviocytes from between the 2nd and 3rd passages. Fixed concentrations of HX (50 μM) with increasing concentrations of XO (25 and 50 mU/ml), H_2O_2 (15 and 30 μM) were used as oxidants.

Determination of TR activity in cell lysates

The assay of TR activity in biological material was based on the Trx-coupled insulin reduction assay. This determination was an end-point assay measuring

the number of SH groups formed in the added insulin, as described by Arner et al. [24].

After 1, 4 or 24 h of treatment with HX/XO and H_2O_2 in DMEM with 0.1% FCS, the cells were washed twice with cold PBS and lyzed in 0.5 ml ice-cold extraction buffer (350 mM NaCl, 20 mM HEPES-KOH, pH 7.9, 1 mM EDTA, 0.1 mM EGTA, 20% glycerol, 1% NP-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride, 20 mM β-glycerol-P, 0.1 mM Na_3VO_4 and 1 μg ml^{-1} each of aprotinin, pepstatin and leupeptin) at 4°C for 30 min. The cells were harvested and centrifuged at 10,000*g* for 30 min at 4°C. The supernatant was used as a whole cell extract. Briefly, 40 μl of the reaction mixture (composed of 200 μl of 1 M HEPES buffer, pH 7.6, 40 μl of 0.2 M EDTA, 40 μl of 40 mg ml^{-1} NADPH, 500 μl of 10 mg ml^{-1} human insulin) was added to each test tube in an ice bath. Extracts of stimulated cells (20 μg) and 10 μl of rhTrx (60 μM) were included. Water was added to achieve a final volume of 120 μl in all tubes. The TR level was monitored at 412 nm by using a 20-min incubation at 37°C to determine —SH groups formed in insulin by breaking the reaction with 500 μl of 0.4 mg ml^{-1} DTNB-6M guanidine-HCl in 0.2 M Tris–HCl, pH 8.0. A blank, containing everything except Trx, was incubated and treated in the same condition as each sample. Absorbances obtained with enzyme-free controls were subtracted from the absorbance of the samples. TR activity was expressed as mU mg^{-1} protein. As a control for total SH groups, protein from each sample was kept on ice during the incubation, and immediately prior to addition of DTNB-6M guanidine–HCl, Trx, and reaction mixture were added to the cell extract. The absorbance of this control was substracted from the value of the sample determined above.

Total protein per flask was carried out following the manufacturer's instructions (Bio-Rad protein assay kit, Hercules, US).

Quantification of mRNA TR expression using real-time reverse transcription–polymerase chain reaction

Synoviocytes were stimulated with ROS for 12 h. Total RNA was extracted using an RNeasy™ Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA (100 pg) was subjected to RT-PCR using the LightCycler (LC) RNA Amplification Kit SYBR Green I ™ (Roche Diagnostics GmbH) to detect TR mRNA. The oligonucleotide primers were designed according to published cDNA sequence data: TR, forward: 5′-GAAGATCTTCCCAAGTCTA-TGAC-3′, reverse: 5′-ATTTGTTGCCTTAATCCT-GTGAGG-3′ [25]. The primers for β-actin were similar to those described for conventional PCR: forward: 5′-CCACTGGCATCGTGATGGAC-3′, reverse: 5′-GCGGATGTCCACGTCACACT-3′ [26]. The protocol was designed to achieve a final reaction volume of 20 μl containing: 0.4 μl AMV reverse transcriptase, 6 mM $MgCl_2$, 4 μl RT-PCR reaction Mix SYBR Green I, H_2O and an optimum concentration of primers: TR and 0.4 mM for β-actin. The reaction was started after the addition of RNA. Reverse transcription was performed at 55°C for 15 min, followed by 45 cycles of denaturation at 95°C for 5 s, primer annealing at 60°C for 10 s, and extension at 72°C 13 s for TrxR and β-actin. In order to assess the purity of products, a melting curve was produced after each run, allowing discrimination between primer dimers and specific products. The amplification of various RNA dilutions (1000-fold serial dilution) for TR or β-actin produced a standard calibration curve. The expression levels of TR were quantified relative to β-actin using Roche quantification software (version 4.0) that employs the second derivative maximum method to calculate fractional cycle numbers.

Immunoblot for TR protein expression

Subconfluent synoviocytes were treated with oxidants for 1, 4 and 24 h. The cells were washed twice with cold PBS and lyzed in 0.5 ml ice-cold extraction buffer (350 mM NaCl, 20 mM HEPES-KOH, pH 7.9, 1 mM EDTA, 0.1 mM EGTA, 20% glycerol, 1% NP-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride, 20 mM β-glycerol-P, 0.1 mM Na_3VO_4, and 1 μg ml^{-1} each of aprotinin, pepstatin, and leupeptin) at 4°C for 30 min. Samples (20 μg of protein) were loaded onto an SDS-acrylamide gradient gel (4–20%) and subjected to electrophoresis. Gels were electroblotted to polyvinyllidene difluoride (PVDF) membrane and probed for TR using anti-TR1 primary polyclonal antibody and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody followed by chemiluminescence detection with the ECL kit using Hyperfilm MP (Amersham, Buckinghamshire, UK).

Blots were scanned and analyzed for their band surface areas with NIH ImageJ software, using the same sized section of the blot for each scan. The data were expressed as arbitrary densitometric units or as value of change compared to control.

Immunoprecipitation of DNP-TR and biotin-conjugated iodoactamine (BIAM)-labelled TR in synoviocytes

Immunodetection was performed to detect total and TR carbonyl groups formed as a result of protein side-chain oxidation with a specific antibody that recognized the DNP moiety of the proteins. Protein carbonyl groups from lysates were derivatized using 2,4-dinitrophenylhydrazine (DNPH), as described by Reznick et al. [27]. DNP-TR was specifically co-immunoprecipitated with rabbit antibody to TR for 2 h, and then with Protein G-Sephadex overnight

at 4°C. The samples were diluted by adding an equal volume of 2 × reducing sample buffer, loaded directly onto the gel and subjected to electrophoresis. After transfer to nitrocellulose, immunodetection was performed with monoclonal anti-DNP (SPE-7 clone) conjugated with alkaline phosphatase from Sigma (St Louis, MO, US) at 1/15,000, with NBT/BCIP as the substrate. Blots were scanned and analyzed for their band surface, as described above.

The BIAM-labelled TR detection procedure was based on the fact that proteins containing Cys residues were labelled with BIAM, whereas oxidized cysteines were not susceptible to BIAM labelling. BIAM can selectively alkylate proteins by adjusting the pH value; at a high pH (pH 8.5), both —SH and —SeH groups are alkylated, while at a low pH (pH 6.5), only the —SeH group is alkylated. The reduction in BIAM labelling that corresponded to the oxidation of TR was monitored by streptavidin blot analysis [28]. After treatment with oxidants, the cells were rinsed in PBS and then exposed to 1 ml oxygen-free lysis buffer (50 mM Bis–Tris–HCl (pH 6.5), 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, leupeptin (1 μg/ml), aprotinin (1 μg/ml) and 0.1 mM AEBSF) containing 20 μM BIAM. After incubation for 30 min at 37°C in the dark, the labelling reaction was stopped by adding 1 mM iodoacetamine. TR was precipitated with rabbit antibody to TR and Protein G-Sephadex (TEBU, France). TR labelled with BIAM was detected with HRP-conjugated streptavidin and ECL-Plus. In order to assess the reversible process of oxidative modifications to TR, ROS-treated synoviocytes were lysed and incubated with an excess of NADPH (200 μM) or GSH (1 and 2.5 mM) for 10 min at 37°C, and then labelled with BIAM for 30 min at 37°C.

Determination of antioxidant levels in synoviocytes

The SOD activities of RA and OA synoviocytes were evaluated using the nitroblue tetrazolium reduction technique as previously described by Beauchamp and Fridovich [29]. Catalase activity was appreciated according to the technique described by Aebi [30]. Briefly, 100 μl of cell lysates (1:10 in phosphate buffer) were added at the reaction mixture containing 50 mM Tris–HCl (pH 7.3), 0.1 mM EDTA, and 10 mM H_2O_2 in a final volume of 1.0 ml. The decomposition rate of the substrate H_2O_2 was monitored using UV spectrophotometer at 240 nm for 1 min. The activity was expressed as $\mu mol\,min^{-1}$-mg^{-1} of proteins. One unit is equal to 1 μmol of H_2O_2 decomposed by minutes. GSH concentrations were determined using the method described by Baker et al. [31]. Total cellular thiols level (protein SH groups and non-protein thiols) was measured using the Ellman's reagent. DTNB was dissolved to a final concentration of 1 mM in 0.1 M Tris 10 mM EDTA, pH 8.2 (TE). Aliquots of cell lysates (40 μg) were added to 1 ml of 1 mM DTNB in TE. Absorbance of reduced DTNB was measured after 1 min at 412 nm. Antioxidant enzyme activities, GSH and total cellular thiol levels of RA and OA cells were related to the protein levels in each sample.

Statistical analysis

Data were compared using the nonparametric Mann–Whitney (unpaired data) and Wilcoxon (paired data) tests; p values less than 0.05 were considered significant. All quantitative data are expressed as means ± SD.

Results

$O_2^{\bullet-}$ *and* H_2O_2 *inhibited TR activity in RA synoviocytes*

At baseline, TR activity was significantly greater in RA synoviocytes than in OA synoviocytes (2.31 ± 0.65 versus 0.74 ± 0.43 mU/mg protein, $p < 0.01$).

TR activities were monitored for 1, 4 and 24 h after the addition of doses of $O_2^{\bullet-}$ generated by 50 μM HX/25 mU/ml XO and 50 μM HX/50 mU/ml XO, or H_2O_2 at 15 and 30 μM. In RA synoviocytes, TR activity decreased in a time-dependent manner with respect to the HX/XO system (Figure 1A,B), and H_2O_2 (Figure 1C,D). In OA synoviocytes, stimulation with ROS effectors did not reduce TR activity.

mRNA expression of TR by $O_2^{\bullet-}$ *or* H_2O_2

In order to investigate whether ROS could regulate mRNA levels of TR in RA synoviocytes, we assessed the expression of TR mRNA using real-time RT-PCR and compared it with OA synoviocytes. Levels of TR mRNA were quantified relative to β-actin mRNA. As shown in Table I, TR mRNA/β-actin mRNA ratios were higher at baseline in RA synoviocytes than in OA synoviocytes ($p = 0.02$). After treatment for 12 h, $O_2^{\bullet-}$ or H_2O_2 markedly increased TR mRNA levels in RA and OA synoviocytes. However, there was no difference in mRNA levels between RA and OA synoviocytes.

Immunoblot of TR protein expression

To examine whether $O_2^{\bullet-}$ or H_2O_2 treatment modified TR protein levels, Western blot analyses were performed. TR protein expression levels in RA synoviocytes were increased markedly by $O_2^{\bullet-}$ or H_2O_2 treatment when compared with the same treatments in OA cells (Figure 2A). Quantitative densitometry results indicated that TR expression was significantly more elevated at baseline in RA cells than OA cells ($p = 0.01$). ROS treatment during 24 h enhanced this expression in RA synoviocytes to reach significant changes compared with control (Figure 2B).

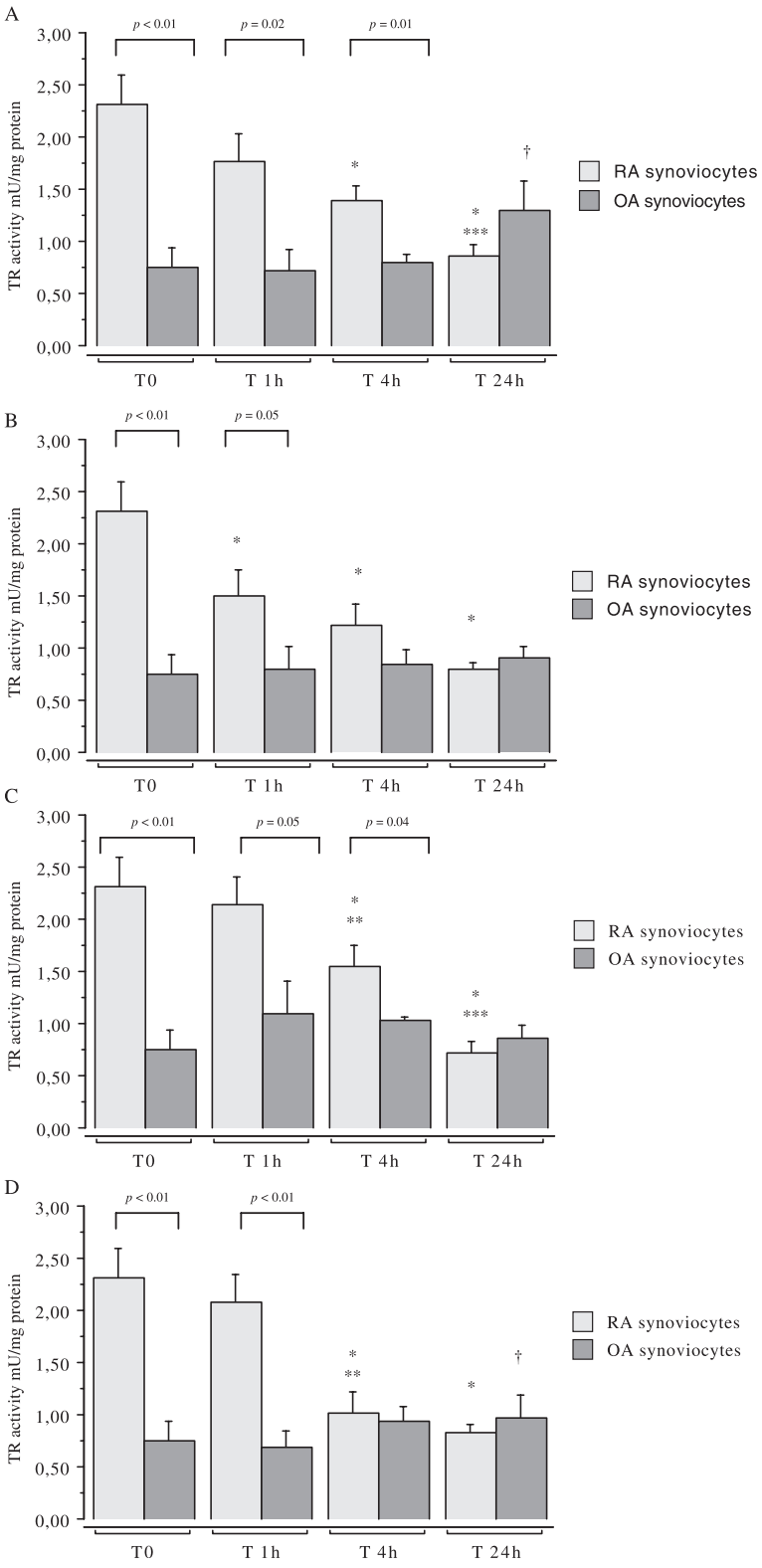

Figure 1. Time profiles of TR activity after treatment with oxidative effectors. Cells were incubated in DMEM at 0.1% SVF containing a fixed concentration of HX (50 μM) with increasing doses of 25 mU/ml (A) and 50 mU/ml (B) XO, or with H_2O_2 at 15 μM (C) and 30 μM (D). TR activity was measured in cell lysates using an insulin reduction assay at 412 nm. Values are expressed as mU/mg of protein. Data are means ± SD from five independent experiments for each of cell type. $\star p < 0.05$ versus RA control cells; $\star\star p < 0.05$ versus RA cells at 1 h; $\star\star\star$ $p < 0.05$ versus RA cells at 4 h; † $p < 0.05$ versus OA control cells.

Table I. TR mRNA/β-actin mRNA ratios after 12 h of HX/XO and H_2O_2 treatments in RA and OA synoviocytes.

	RA cells	OA cells	*p* values RA versus OA
Control	0.91 ± 0.07	0.75 ± 0.06	$p = 0.02$
25 mU/ml XO	2.50 ± 0.78⋆	2.58 ± 0.94†	NS
50 mU/ml XO	3.18 ± 1.59⋆	3.91 ± 1.90†	NS
15 μM H_2O_2	1.91 ± 0.66⋆	1.07 ± 0.22†	NS
30 μM H_2O_2	3.17 ± 0.85⋆	2.68 ± 1.58†	NS

⋆$p < 0.05$ versus RA control; †$p < 0.05$ versus OA control.

In OA cells, only HX/XO at different doses induced statistical changes compared with control.

Oxidative modifications to TR

The formation of carbonyl groups in amino acid side chains, reflecting the degree of oxidative protein modifications, was studied after 24 h of ROS treatment. The immunodetection of carbonyl groups after DNPH derivation of total protein lysates was evaluated by the use of monoclonal anti-DNP. Data showed at baseline that proteins from RA lysates were substantially more oxidized than theses from OA lysates, for the same quantities of proteins lysates (20 μg) (Figure 3A). ROS treatment induced the formation of carbonyl groups, which were more important in RA lysates than in OA lysates. We, therefore, investigated whether ROS caused an accumulation of oxidized TR. The immunoprecipitation of TR after derivatization of the carbonyl groups with DNPH enabled the specific assessment of TR oxidation. As shown in Figure 3B, the level of expression of carbonyl groups after 24 h of HX/XO or H_2O_2 treatment was increased in RA cells when compared with OA cells.

Considering the increase in TR in RA cells after 24 h of oxidation, quantitative densitometry of TR oxidation had been evaluated by the determination of DNP-TR area/TR protein area ratios to estimate amounts derived-TR. These results revealed that the highest concentrations of XO and H_2O_2 induced significant increase of carbonyl groups in RA synoviocytes, whereas there were no changes in OA cells (Table II).

The selective BIAM labelling procedure at pH 6.5 was used as a probe to monitor the oxidation status of selenocysteine at the site of TR catalysis. Blotting results confirmed that the free —SeH group of TR

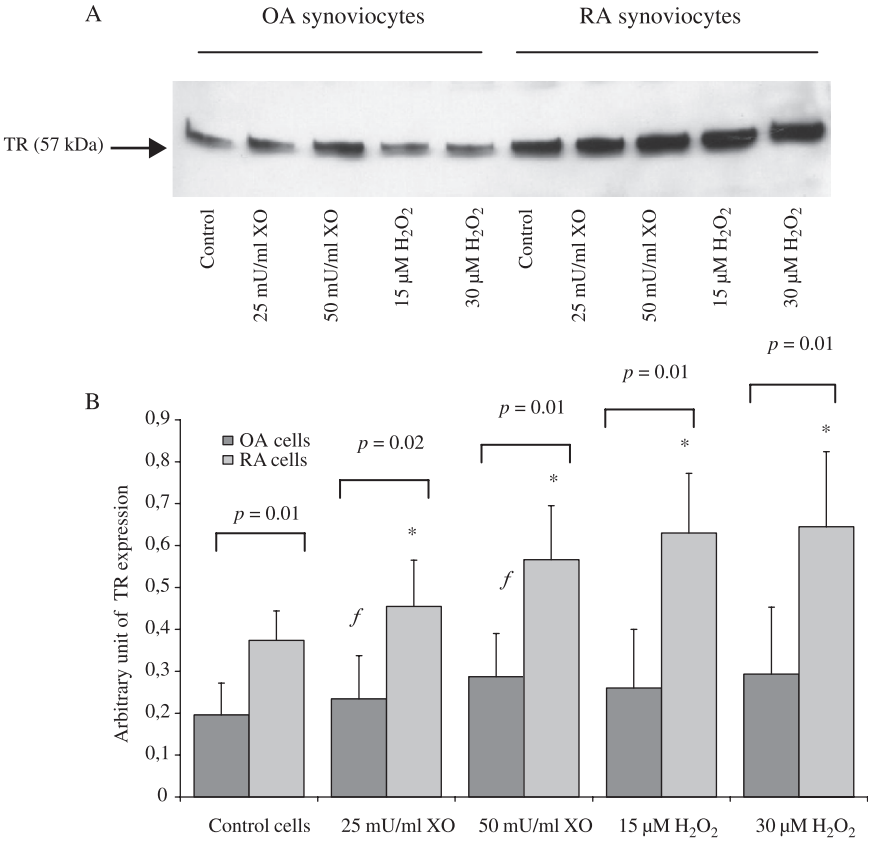

Figure 2. Induction of TR protein by ROS in synoviocytes cells. Cells were incubated for 24 h with a fixed concentration of HX (50 μM) and XO (25 or 50 mU/ml), or with H_2O_2 (15 or 30 μM). Immunoblotting was performed with anti-TR antibody, a representative example from five independent experiments is shown in Figure 2A. Quantitative intracellular TR expression levels were evaluated using NIH ImageJ software and expressed as arbitrary densitometric units (means ± SD) (B). Data were analyzed using the Mann–Whitney test and Wilcoxon test. ⋆$p < 0.05$ versus RA control; †$p < 0.05$ versus OA control.

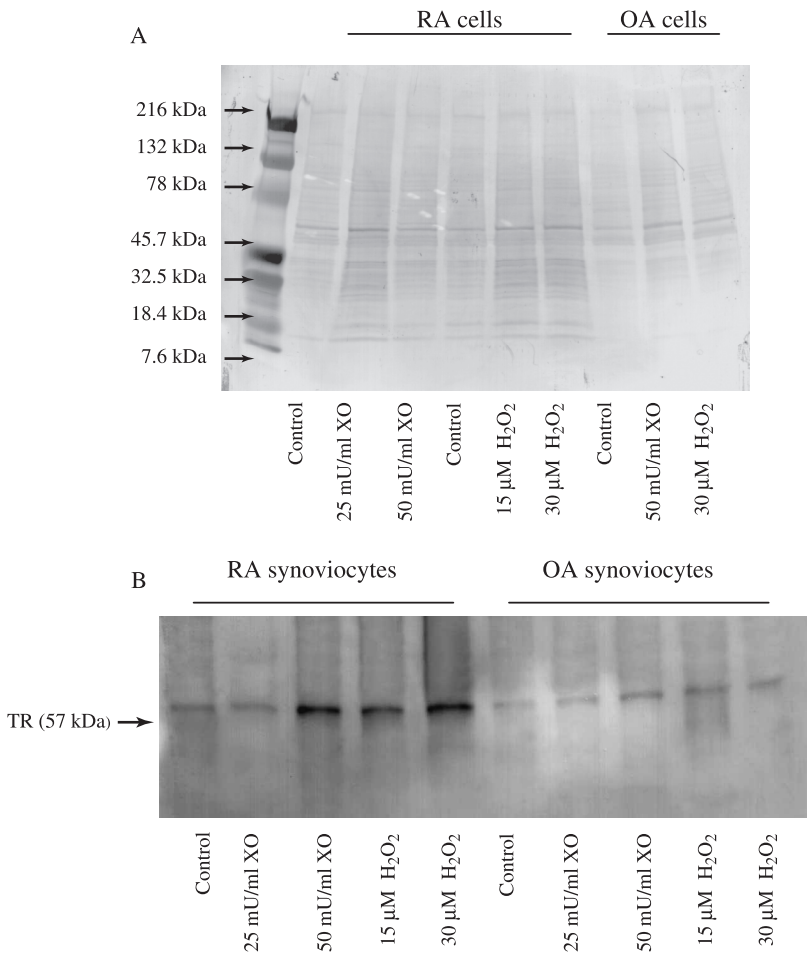

Figure 3. ROS-induced carbonyl groups in TR from RA cells. Synoviocytes were incubated with or without treatment with a fixed concentration of HX (50 µM) and XO (25 or 50 mU/ml), or with H_2O_2 (15 or 30 µM) for 24 h. The cells were lysed and incubated for 30 min at room temperature with one volume of 12% SDS and two volumes of 20 mM DNPH. Total carbonyl groups was derived by DNPH treatment and loaded onto the gel and subjected to electrophoresis. After transfer to PVDF, immunodetection was performed with monoclonal anti-DNP conjugated with alkaline phosphatase and NBT/BCIP as the substrate (Figure 3A). TR was selectively evaluated after DNPH treatment by precipitation with a rabbit antibody to TR for 2 h, then with Protein G-Sephadex overnight at 4°C, and revealed with monoclonal anti-DNP, representative data from four independent experiments is shown (Figure 3B).

enzyme was targeted by HX/XO or by H_2O_2 effectors (Figure 4A). At baseline, the blotting band intensity representing selenol of selenocysteine in 57 kDa was weaker in RA control cells than in OA control cells.

Table II. DNP-TR/TR ratio expressions appreciated by quantitative densitometric analysis after 24 h of HX/XO and H_2O_2 treatments in RA and OA synoviocytes.

	RA cells	OA cells	*p* values RA versus OA
Control	1	1	
25 mU/ml XO	0.92 ± 0.15	0.92 ± 0.28	NS
50 mU/ml XO	1.42 ± 0.37⋆	0.89 ± 0.21	$p = 0.03$
15 µM H_2O_2	1.10 ± 0.1	1.03 ± 0.39	NS
30 µM H_2O_2	1.65 ± 0.38⋆	1.03 ± 0.24	$p = 0.02$

Values were expressed as change compared to control for each cell type (means ± SD) from five independent experiments; ⋆$p < 0.05$ versus RA control.

BIAM-labelled TR levels declined in line with the duration of cell incubation, when compared with controls. This reduction was more marked in RA cells than in OA cells, consistent with the loss of activity.

In order to evaluate the reversibility of TR oxidation state, cells were lysed and incubated with an excess of NADPH (200 µM) for 10 min after ROS treatment, and then treated with BIAM (Figure 4B). TR oxidized by the HX/XO system or H_2O_2 from RA cells for 1 h was labelled with BIAM when reduced by NADPH, and was similar to the RA control, whereas partial biotinylation was detectable in the HX/XO system or H_2O_2-treated RA cells after 4 and 24 h. Moreover, the addition of GSH (1 and 2.5 mM) for 10 min after HX/XO or H_2O_2 treatment partially restored the TR level observed at baseline in RA synoviocytes. These results strongly suggest that selenocysteine could not be fully labelled in RA cells because a proportion of the TR was irreversibly oxidized after prolonged

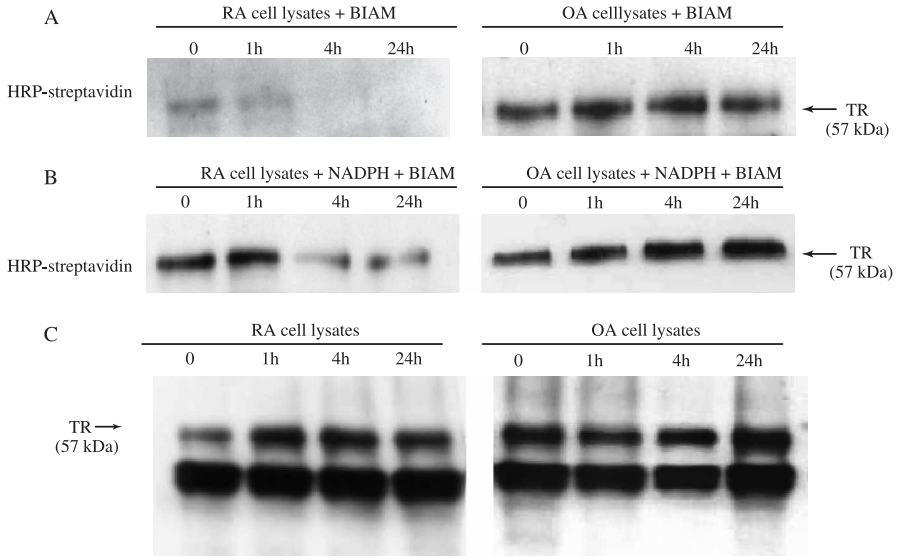

Figure 4. ROS induced oxidation of TR in synoviocytes. Two sets of synoviocytes were incubated with treatment with H_2O_2 (30 μM) for the indicated times. One set of cells (20 μg) were lysed in a buffer containing 20 μM BIAM and incubated for 30 min and then treated with 1 μM IAM (A). The other set of cells (20 μg) was studied for the reversibility of TR oxidation state by the pre-incubation of NADPH in excess in cell lysates after ROS treatment: cells treated with H_2O_2 (30 μM) were lysed in buffer containing 200 μM NAPDH for 10 min. The free selenol was alkylated by 20 μM BIAM for 30 min at pH 6.5 and treated with 1 μM IAM (B). TR was precipitated from cell lysate with polyclonal antibody and subjected to SDS-Page on a 7% gel. The separated proteins were transferred to a PVDF membrane. The membranes were subjected to blot analysis with Biotin-TR detected by streptavidin-conjugated with horseradish peroxidase (upper panel A–B) or with antibody to TR (lower panel) (C). Representative data from three independent experiments are shown.

stimulation by ROS effectors. In contrast, the oxidation of TR was totally reversible by the addition of NADPH in OA cells incubated for long periods.

Deficit of antioxidant status in RA synoviocytes

The oxidative modifications to TR reported above may have resulted from an impairment of the antioxidant capacity of RA synoviocytes. We, therefore, investigated antioxidant status by determining SOD and catalase activities and by GSH protein and total cellular thiol (protein SH groups and non-protein thiols) levels in RA and OA synoviocytes. It was found that SOD and catalase activities were significantly lower in RA than in OA synoviocytes (respectively, 4.9-fold and 1.6-fold lower), that GSH and total cellular thiol levels were, respectively, reduced 2.4-fold and 1.2-fold more in RA cells than in OA cells (Figure 5). Moreover, TR activity was found to be negatively correlated with the reduction in GSH levels in RA synoviocytes (Figure 6), but not with OA cells. This correlation was not observed with SOD, catalase activities or total cellular thiol levels for each cell type.

Discussion

An increased production of ROS may play a role in RA disease [5,32]. Another outcome of increased oxidative stress is the inactivation of enzymes implicated in cellular antioxidant defence systems. In the present study, we found evidence of increased TR activity in RA synovial cells compared to OA synovial cells.

TR maintains the cell redox state through its potential to detoxify hydrogen peroxide and lipid peroxides [33], to regenerate ascorbate [34] and to promote superoxide dismutase activity [35]. A characteristic of TR is its sensitivity to oxidizing conditions, leading to a change in conformation. A conformational change affecting interactions with other molecules could be crucial to triggering cell signalling in response to oxidative stress [16,17]. Moreover, TR was transiently or irreversibly inactivated by H_2O_2-induced TNF-α in HELA cells [36] and by peroxynitrite in HUVECs [37]. Based on the immunoprecipitation of TR after derivatization of the carbonyl groups with DNPH, we show here that TR in RA cells was highly sensitive to oxidation, and that these biochemical modifications could be the cause of TR inactivation. TR exhibits broad substrate specificity, explained by its easily accessible C-terminal active site redox centre, which contains an essential selenocysteine residue. The major role of selenocysteine in the catalytic activity of TR has been demonstrated by replacing it with cysteine or serine [38]. Redox communication between cysteine-selenocysteine at the C-terminus and the active site disulfide at the N-terminus has been proposed as being crucial in the case of mammalian TR [38,39]. Based on several observations [40,41], it has been suggested that the Cys-SH^{496}/$SeCys^{497}$ pair of TR may be the primary site of oxidation by H_2O_2 in the

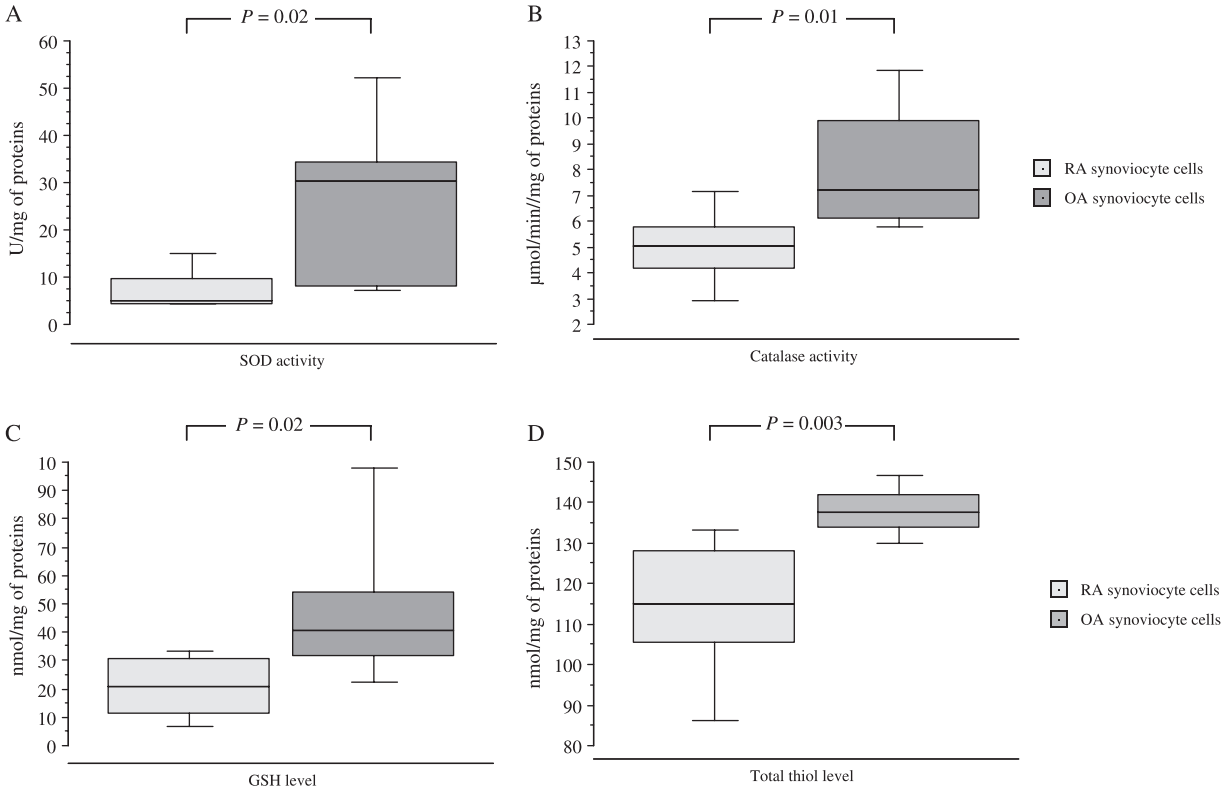

Figure 5. Median antioxidant levels in synoviocytes. Superoxide dismutase (A), catalase (B), glutathione (C) and total cellular thiol (D) levels were measured in each cell type at baseline and compared with the amount of proteins in each sample. Data from at least five independent experiments were pooled and analyzed using the Mann–Whitney test. Boxes denote interquartile ranges, whereas whiskers indicate 5th and 95th percentiles.

cell, which is consistent with a mechanism where the intracellular ROS pathway is regulated through the selenocysteine residue in TR, rather than through effects on Trx or glutathione peroxidase [19]. In order to assess intracellular changes in the redox status of TR, we stimulated synoviocytes with sub-lethal concentrations of ROS effectors under prolonged incubation, and then assessed reversible changes in TR activity. The selective BIAM labelling procedure at pH 6.5 was used as a probe to monitor the oxidation status of SeCys in TR. Our findings show that exogenous H_2O_2 (either authentic H_2O_2 or enzymatically generated) selectively oxidized the —SeH group at the catalytic site of TR protein.

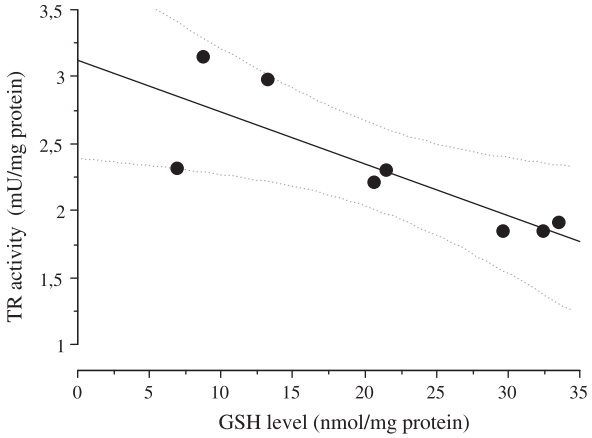

Figure 6. Correlation between TR activity and GSH levels within RA cells at baseline. Values ($n = 8$) of TR activity RA cells (mU/mg protein) and values of RA cells GSH levels (nmol/mg protein) were inversely correlated ($r = -0.82$, $p = 0.02$). Confidence intervals are shown in the figure.

We further showed that this inactivation in RA cells was reversible (by adding an excess of NADPH or GSH) after 1 h, but was less reversible after 4 and 24 h. After these longer periods, we hypothesized that the irreversible oxidation of TR could have been due to an accumulation of lipid peroxidation products such as 4-hydroxy-2-nonenal in the cells after oxidative stress [42].

In contrast, the TR oxidation was totally reversible in OA cells, whatever the duration of incubation. Our results thus revealed a significant reduction in TR activity in RA synoviocytes after 24 h of ROS treatment, whereas TR activity remained unchanged in OA cells, supporting the hypothesis of antioxidant barrier impairment in RA patients [43]. To confirm this, we determined the redox balance in synoviocytes by evaluating SOD, catalase, GSH and total cellular thiol levels. Significantly lower levels of antioxidants were found in RA synoviocytes than in OA synoviocytes, demonstrating the marked activity of the oxidative process in RA. Interestingly, it could be seen at baseline that the drop in GSH levels in RA

synoviocytes was negatively correlated with TR activity, suggesting the existence of a compensatory effect to counteract the intracellular oxidative environment. In this study, we found that the exposure of RA synovial cells to ROS for 12 h increased TR mRNA and TR protein levels. However, this adaptive response to oxidative stress was insufficient to restore TR activity completely in RA cells, if its level was considered after 24 h. One consequence of the inefficiency of TR was that it could affect thioredoxin-dependent cellular components (protein-tyrosine phosphatases, thioredoxin peroxidase), the control of redox-sensitive transcription factors and influence the delicate balance between the promotion of cell viability and the initiation of apoptosis [17,18]. Several studies have reported that oxidized TR may act as a death-inducing factor [44,45]. In particular, oxidized-Trx is able to activate ASK-1 kinase activity, triggering the signalling cascades, which induce the apoptotic process [45].

Increases in the expression of Trx/TR are correlated with oxidative stress markers and RA disease severity [11–15]. In synovial cells from a mouse arthritis model, the administration of recombinant Trx significantly suppresses the development and/or progression of the disease [46], suggesting that Trx has a protective effect against oxidative stress. We hypothesized that the increased expression of Trx in RA synoviocytes was the consequence of TR inactivation, while Trx was maintained in an oxidized form and could not be restored in a reduced form.

In conclusion, inhibition of TR activity by prolonged oxidation with doses of reactive species impairs the antioxidant defences and lead to a chronic dysfunction of the synoviocytes. This may, therefore, constitute an important mechanism to explain the causes of oxidative stress related to RA disease.

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