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Differential regulation of Cu, Zn- and Mn-superoxide dismutases by retinoic acid in normal and psoriatic human fibroblasts

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Abstract

Superoxide dismutases' (SODs) expression is altered in several diseases including Alzheimer, atherosclerosis, cancer and psoriasis. Previously, we reported a marked increase in Mn-SOD and Cu,Zn-SOD functional activity in human dermal psoriatic fibroblasts. As retinoic acid (RA) has been used in the treatment of psoriasis and a mechanism for its beneficial effects is not understood, we investigated the effects of RA on SOD mRNA and protein expression levels in human normal and psoriatic fibroblasts. Prior to RA exposure, Cu,Zn-SOD protein and mRNA levels were similar in normal compared to psoriatic fibroblasts while Mn-SOD protein and mRNA levels were increased in psoriatic cells. However, in contrast to normal fibroblasts, exposure of psoriatic fibroblasts to 1 μM RA down-regulated Mn-SOD mRNA, and also decreased Mn-SOD activity by ~80% with no change in Mn-SOD protein levels. In contrast, Cu,Zn-SOD protein and enzymatic activity were modestly reduced by RA treatment in both normal and psoriatic fibroblasts. Furthermore, RA treatment of psoriatic fibroblasts also caused a decrease in Cu,Zn-SOD steady-state mRNA levels. These results indicate that RA can serve as a regulatory agent to down-regulate the steady-state levels of both Mn-SOD and Cu,Zn-SOD in psoriatic cells. These findings offer a new model for the antiinflammatory activity of RA when used in the treatment of psoriasis.

Keywords: Psoriasis; Superoxide dismutases; Free radicals; Fibroblasts; Retinoic acid

1. Introduction

Studies to define the role of superoxide radicals ($O_2^{\cdot-}$) in both physiological and pathological processes are an area of active investigation. Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide radicals and hydroxyl radicals are generated in cells in response to stimulation by various hormones, growth factors and cytokines [1], such as PDGF [2,3]. The

Abbreviations: CRABPII, Cellular Retinoic Acid Binding Protein II; GAPDH, D-Glyceraldehyde-3-Phosphate Dehydrogenase; PDGF, Platelet Derived Growth Factor; PKA, Protein Kinase A; RA, Retinoic Acid; ROS, Reactive Oxygen Species; SOD, Superoxide Dismutase.

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oxygen radicals generated appear to act as second messengers in transmembrane signaling pathways and modulate cellular functions such as cell proliferation, differentiation, and apoptosis [3]. Oxidative regulation of several key enzymes involved in cellular signal transduction, including protein kinases, has been described [4].

Oxidants also have been implicated in a number of pathological disorders, including Alzheimer disease, psoriasis, aging, atherosclerosis, and cancer [5–8]. The primary cellular defenses against damage that can be caused by $O_2^{\cdot-}$ and by its reactive progeny are the superoxide dismutases (SODs). Mammalian cells have a mitochondrial Mn-SOD, a cytoplasmic Cu,Zn-SOD (also found in peroxysomes) and an extracellular SOD which is a Cu,Zn-SOD that is immunologically distinct from the classical Cu,Zn-SOD [9]. These metalloenzymes act to dismute toxic superoxide radicals to oxygen and H_2O_2[9–11]. Catalase, along with peroxidases such as glutathione peroxide, in turn catalyze the decomposition of H_2O_2 to water and oxygen. Studies have been ongoing for a number of years in our laboratories on the synergism between retinoic acid and cyclic AMP that regulates cell growth and differentiation [12]. In the course of these studies, it was established that cyclic AMP-dependent protein kinase (PKA) activity, as well as 8-azido-[^{32}P] cAMP binding to the RI and RII regulatory subunits, are decreased in cells of psoriatic patients compared to cells of normal subjects [13]. More recent studies determined that exposure of normal human dermal fibroblasts to H_2O_2 and to other oxygen free radical generating systems decreased both PKA activity and cyclic AMP binding to RI and RII, to levels similar to those observed with psoriatic fibroblasts [14]. Treatment of psoriatic fibroblasts with free radical scavenging agents restored the ability of RI and RII to bind cyclic AMP toward normal levels. We also showed that SOD activity, particularly that of the mitochondrial enzyme, Mn-SOD, was elevated in fibroblasts prepared from a psoriatic patient [15]. These results suggested that oxidative modification may serve as a mechanism to alter PKA in human cells, and that an altered oxidative state may be responsible for the decrease in PKA activity and cyclic AMP binding in cells from psoriatic patients.

Retinoic acid derivatives have been used with some success in the therapeutic treatment of psoriasis even though the mechanism of retinoid action responsible for this beneficial effect was not known [16,17]. Previously, we established that the treatment of psoriatic fibroblasts with retinoic acid and with other retinoids, such as 13-*cis*-retinoic acid and arotinoid induced an increase in the oxidatively modified PKA activity back towards normal levels [13]. Again however, the mechanism by which retinoic acid acted to increase PKA activity in psoriatic cells remained to be elucidated. Thus, the present studies were carried out to determine if retinoic acid treatment might differentially regulate antioxidant enzyme activities in normal and psoriatic fibroblasts. To address this question Mn-SOD and Cu,Zn-SOD functional activities, as well as Mn-SOD and Cu,Zn-SOD mRNA levels and protein levels, were determined in cells in the presence and absence of retinoic acid. Retinoid treatment of psoriatic fibroblasts was observed to significantly decrease both Mn-SOD activity and steady-state mRNA levels as well as both Cu,Zn-SOD expression and enzymatic activity.

2. Materials and methods

2.1. Experimental reagents

Retinoic acid (RA) and actinomycin D (ActD) were obtained from Sigma (Saint Louis, MI, USA) and cycloheximide was purchased from Calbiochem (La Jolla, CA, USA). ActD was dissolved in dimethylsulfoxide (Me_2SO). RA was dissolved in 100% ethanol and cycloheximide in double distilled water (ddH_2O). DMEM with L-glutamine, sodium pyruvate, fetal bovine serum and antibiotics were obtained from Invitrogen. The antibody to β-actin was an anti-mouse monoclonal antibody purchased from Sigma. The rabbit anti-human Mn-SOD antibody and the goat anti-human Cu,Zn-SOD antibody were gifts of Dr N. Taniguchi, Osaka, Japan. The horseradish peroxidase-labelled secondary antibody was obtained from Jackson Immunoresearch. Protein assay reagents and polyvinylidene difluoride membranes were purchased from Bio-Rad (Marnes la Coquette, France).

2.2. Cells

Human fibroblasts were isolated from seven normal and seven untreated adult psoriatic patients (*Psoriasis vulgaris* in flare for approximately 3 months) by enzymatic digestion of small pieces (4 mm) of lesional and non-lesional buttock and abdominal skin punch biopsies as previously described [14,18]. These Caucasian subjects were age- and sex-matched. This study was approved by the ethical committee of the Hôpital Cochin (Paris). The patients had received no systemic therapy such as corticosteroids, antimitotic drugs, retinoids for at least a year before the study; no systemic anti-inflammatory drugs for at least two weeks before the biopsy; no local corticosteroids, ultraviolet light, or oral psoralen for at least three months before the assay. The severity of the disease was determined by mean psoriatic area and severity index (PASI)score, as described by Frederiksson and Peterssonn [45]. The PASI score for the 10 patients were 22.54 ± 0.47 (Range 12.8–34.1). Cells were grown as previously described [14] and used

subconfluent (10^6 cells/dish) at the fifth passage. All data were obtained with cells propagated for the same number of passages (fifth passage) under identical culture conditions. For each donor the different assays were performed on the same cell extracts prepared from cells arising from the same cell seeding.

2.3. Preparation of cellular fractions

Fibroblasts were washed three times with ice cold phosphate buffered saline and harvested by scraping into ice cold buffer (0.25 M sucrose, 1 mM $MgCl_2$, 20 mM Tris, pH 7.4) with a cell scraper with anti-proteolytics. The cells were recovered by centrifugation at 1000*g* for 5 min and the cell pellet was then frozen at −80 °C. For antioxidant enzyme activity measurements, the control and psoriatic cell pellets were disrupted by sonication in 500 μl of 10 mM sodium phosphate buffer, pH 7, (whole cell lysates), and the same cellular sample was used to assay each of the indicated activities within the same experiment.

2.4. Superoxide dismutase (SOD) activities

As previously described [15], xanthine–xanthine oxidase was used to generate an $O_2^{\cdot-}$ flux and the reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5 phenyltetrazolium chloride (INT) to red formazan by $O_2^{\cdot-}$ was followed at 505 nm, 30 °C. Each assay tube contained 50 mM (3-[cyclohexylamino]-1-propane-sulfonic acid) (CAPS) buffer, pH 10.2, for determination of Cu,Zn-SOD activity [19], or 50 mM phosphate buffer, pH 7.8, for determination of total SOD activity [20], along with 1 mM EDTA, 25 mM INT, 50 mM xanthine, 1 U/ml catalase, 0.05 mM bathocuproin disulfonate disodium salt (BCS), 0.13 mg/ml bovine serum albumin (BSA), and enough xanthine oxidase to achieve the required 100% of non-inhibition. BCS and BSA were added to inhibit non-enzymatic scavenging of $O_2^{\cdot-}$. Mn-SOD activity was determined by adding cyanide (2 mM) to the assay mixture at pH 7.8 to inhibit Cu,Zn-SOD activity, or was determined by subtracting the Cu,Zn-SOD activity determined at pH 10.2 from the total SOD activity measured at pH 7.8. The two methods gave similar values (data not shown). All data are expressed in units of SOD activity per mg of protein.

2.5. Western blot analysis

Cell pellets were prepared as described above. Then cell lysates were prepared, protein levels were determined and the lysates were then heated for 5 min at 95 °C in Laemmli sample buffer and analysed by electrophoresis on 15% polyacrylamide gels (10 μg/sample). Proteins were electrotransferred to nitrocellulose and Western blots were performed as described in the Supersignal kit (Pierce, Bezons, France). The SOD proteins were detected with a rabbit anti-human Mn-SOD antibody and a goat anti-human Cu,Zn-SOD antibody, both diluted 1000-fold (gift of Dr Taniguchi N., Osaka, Japan).

2.6. Actinomycin D treatment

To determine the stability of Cu,Zn- and Mn-SOD mRNA, with or without retinoic acid (1 μM), the three different types (normal, non-lesional and lesional psoriatic) of human fibroblast primary cultures were untreated or treated for 48 h with 1 μM RA, and then 10 μg/ml ActD was added. Cells were collected, and total RNA was isolated at the indicated time points.

2.7. Cycloheximide treatment

To inhibit protein synthesis, cells were incubated with 10 μg/ml cycloheximide, with or without retinoic acid treatment (1 μM) for 48 h. To determine the turn over of Cu,Zn- and Mn-SOD protein, cells were collected from the three different types of human fibroblasts primary cultures at the indicated time for western blot analysis.

2.8. RNA isolation and real time RT-PCR

Total RNA was extracted with RNA-Plus (Qbiogene, Illkirch, France) from normal (N, Normal Skin), lesional (LP, Lesional Psoriatic Skin) and non-lesional (nLP, non-Lesional Psoriatic Skin) cultured fibroblasts, and 1 μg of each sample was reverse-transcribed into cDNA using a first-strand cDNA-synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Real-time quantitative PCR was conducted with the TaqMan system (Perkin-Elmer Applied Biosystems) for the Cu,Zn-SOD and Mn-SOD steady state mRNA levels with or without RA treatment or the LightCycler system for the Cu,Zn-SOD and Mn-SOD mRNA stability (Roche Diagnostics, Mannheim, Germany) using the DNA-binding dye SYBR Green for the detection of PCR products according to the manufacturer's instructions. Quantitative PCR was performed in a total volume of 20 μl in the presence of 2 μl of cDNA, 2 μl of 10× LightCycler DNA Master SYBR Green I, 1.25 mM $MgCl_2$, 25 pmol of both primers. After denaturation at 95 °C for 10 min, the samples underwent 40 cycles of amplification (15 s at 95 °C; 5 s at 60 °C and 8 s at 72 °C). Sense and antisense primers were respectively: 5′-GGC GAC CTG GAA GTC CAA CT-3′ and 5′-CCA TCA GCA CCA CAG CCT TC-3′ for PO; 5′-CTG AAG GCC TGC ATG GAT TC-3′ and 5′-CCA AGT CTC CAA CAT GCC TCT C-3′ for Cu,Zn-SOD; 5′-CGT GAC TTT GGT TCC TTT GAC A-3′ and 5′-AAG TGT CCC CGT TCC TTA TTG A-3′ for Mn-SOD. Experiments were performed with duplicates

for each point, and all results were normalized to P0 (RPLP0, human acidic ribosomal phosphoprotein) cDNA detected in the same sample.

2.9. Analysis of statistical significance

Data were analysed by the ANOVA test.

3. Results

3.1. Retinoic acid treatment of psoriatic fibroblasts decreased SOD enzymatic activities

In agreement with our previous findings [15], both non-lesional (nLP) and lesional (LP) psoriatic fibroblasts exhibit significantly higher levels of Mn-SOD and Cu,Zn-SOD activities compared to normal (N) fibroblasts (Table 1). Treatment with 1 μM RA for 4 days decreased the Cu,Zn-SOD activity determined in all three cell types by 18% ($p < 0.005$), 26% ($p < 0.005$), and 23% ($p < 0.025$) for normal, nLP, and LP fibroblasts, respectively. Interestingly, the Mn-SOD activity was dramatically decreased by 1 μM RA treatment for 4 days in psoriatic fibroblasts and returned to levels equivalent to that in normal firbroblasts (83% and 78% decrease in nLP and LP psoriatic fibroblasts, respectively, $p < 0.005$), whereas RA had no effect on Mn-SOD activity in normal fibroblasts (Table 1).

3.2. Time dependency of decreased SOD enzymatic activities upon retinoic acid treatment

The effects of treating cells with RA for increasing periods of time on Cu,Zn-SOD (panel A) and Mn-SOD (panel B) activities are depicted in Fig. 1. RA treatment of nLP, LP and normal fibroblasts showed only a slight effect on Cu,Zn-SOD activity through 3 h. However, a decrease in Cu,Zn-SOD activity was observed in all three cell types at 96 h of treatment with RA, which was maximal at 18 h (Fig. 1A). Importantly, RA had little effect on the low basal level Mn-SOD activity present in normal fibroblasts through 96 h of treatment, while treatment of both nLP and LP psoriatic fibroblasts with RA for 3 h resulted in a dramatic decrease in Mn-SOD activity (Fig. 1B). This decrease in Mn-SOD activity in response to RA treatment of psoriatic fibroblasts was maintained through 96 h. At this time, the Mn-SOD activity of psoriatic cells was similar to its basal level determined in normal fibroblasts.

3.3. Retinoic acid effects on Cu,Zn-SOD and Mn-SOD protein levels

The protein levels of Cu,Zn-SOD and Mn-SOD present in control and RA-treated cells were analysed by western blot and densitometric scanning of the autoradiographs (Fig. 2). Exposure to RA was found to reduce the level of Cu,Zn-SOD protein in both normal and psoriatic fibroblasts from 18 h through 96 h of treatment (Fig. 2A). In contrast, no alteration in the level of Mn-SOD protein was observed in any of the cell types through 96 h of treatment with RA (Fig. 2B).

In another set of experiments, cells were treated for 18 h and 96 h. While the Mn-SOD protein level was not changed by RA treatment of either normal or psoriatic fibroblasts (Fig. 2D), RA treatment did result in decreased levels of Cu,Zn-SOD protein in all three different human primary cell types ($n = 4$ of each primary cultures, Fig. 2C). Thus, in comparison with untreated cells, the mean values ± SEM of Cu,Zn-SOD protein levels were decreased by 17% ± 4 ($p < 0.02$), 19% ± 2 ($p < 0.05$) and 27% ± 8 ($p < 0.03$) in normal, nLP and LP RA-treated cells, respectively.

3.4. Analysis of Mn-SOD steady-state mRNA levels

Next, the Cu,Zn-SOD and Mn-SOD mRNA levels in all three primary cell types were determined using real time reverse transcription-polymerase chain reaction

Table 1
Down-regulation of Cu,Zn-SOD and Mn-SOD activities by retinoic acid in cultured fibroblasts isolated from skin of normal subjects and from lesional and non-lesional psoriatic patients

SOD activities			
Fibroblasts	Treatment	Mn-SOD (mU/mg prot)	Cu,Zn-SOD (mU/mg prot)
Normal	0	72.3 ± 5.3 (59–102)	231.9 ± 16.0 (180–306)
($n = 7$)	RA 96 h	74.7 ± 6.1 (57–104)	191.9 ± 8.4 (159–222)
Non-Lesional Psoriatic	0	233.7 ± 22.3 (143–300)	364.4 ± 22.0 (293–443.5)
($n = 7$)	RA 96 h	38.8 ± 4.8 (29–61)	270.0 ± 26.6 (192–338.5)
Lesional Psoriatic	0	267.0 ± 19.7 (220–320)	363.6 ± 26.4 (275–452)
($n = 7$)	RA 96 h	57.6 ± 9.7 (40–82)	279.7 ± 6.9 (260–300)

Data are expressed as the mean ± SEM of triplicate determinations carried out with 7 primary cultures of Normal, non-Lesional Psoriatic, and Lesional Psoriatic human fibroblasts with and without treatment by 1 μM RA for 4 days. The range of experimental values is shown in parenthesis. n = number of different primary cultures tested.

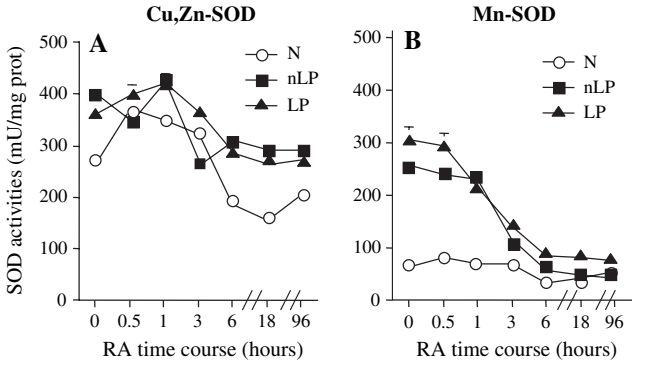

Fig. 1. Time course of retinoic acid (RA) (1 μM) treatment of Normal (N), non-Lesional Psoriatic (nLP) and Lesional Psoriatic (LP) fibroblasts on Cu,Zn-SOD (panel A) and Mn-SOD (panel B) activity. SOD activities were determined as described in Section 2. Data are presented as mean ± SEM ($n = 6$). Each sample was assayed in triplicate in two separate experiments.

(real time RT-PCR) (Fig. 3). To normalize the cDNA concentration in all samples, a housekeeping gene (P0) was amplified in the same experiment along with the Mn-SOD and Cu,Zn-SOD genes. Normalization of the results were obtained by establishing the ratio of Mn-SOD/P0 and Cu,Zn-SOD/P0 [21]. The steady-state level of Cu,Zn-SOD mRNA was not statistically different in psoriatic fibroblasts obtained from non-lesional (nLP, $n = 4$, mean ± SEM, 4.85 AU ± 1.34) and lesional skin (LP, $n = 5$, mean ± SEM, 3.56 AU ± 0.57) compared to normal fibroblasts (N, $n = 4$, mean ± SEM, 4.22 AU ± 0.44) (Fig. 3A, open bars). Conversely, the steady-state level of Mn-SOD was increased by 7 fold ($p < 0.001$) and 48 fold ($p < 0.001$), respectively, in untreated nLP ($n = 4$, mean ± SEM, 9.85 AU ± 0.88) and LP ($n = 4$, mean ± SEM, 68.42 AU ± 7.0) psoriatic fibroblasts compared to normal cells (N, $n = 4$, mean ± SEM, 1.47 AU ± 0.27) (Fig. 4, open bars).

3.5. Effects of retinoic acid treatment on Cu,Zn-SOD and Mn-SOD steady-state mRNA levels

Given our finding that psoriatic fibroblasts had significantly higher levels of Mn-SOD mRNA, we next determined the effects of RA on both Cu,Zn-SOD and Mn-SOD mRNA. As determined by real time RT-PCR, the steady-state levels of Cu,Zn-SOD mRNA found in psoriatic cells were significantly decreased in response to RA treatment for 96 h (nLP 0 = 4.85 AU ± 1.339, nLP RA = 3.07 AU ± 0.21, $p < 0.03$ $n = 4$; LP 0 = 3.56 AU ± 0.57, LP RA = 2.52 AU ± 0.44, $p < 0.015$ $n = 5$; N 0 = 4.22 AU ± 0.44, N RA = 3.27 ± 0.85 $n = 4$). As presented in Fig. 3B, our analysis showed that the Mn-SOD mRNA levels present in normal fibroblasts were not modulated by RA treatment for 96 h. However, exposure to RA for 96 h did significantly decrease steady-state levels of Mn-SOD mRNA in lesional psoriatic fibroblasts ($p < 0.001$, LP 0 = 68.42 AU ± 7.0, LP RA = 32.55 AU ± 4.7 $n = 4$) and in non-lesional psoriatic fibroblasts [$p < 0.001$ nLP 0 = 9.85 AU ± 0.88, nLP RA = 4.16 AU ± 0.35 $n = 4$].

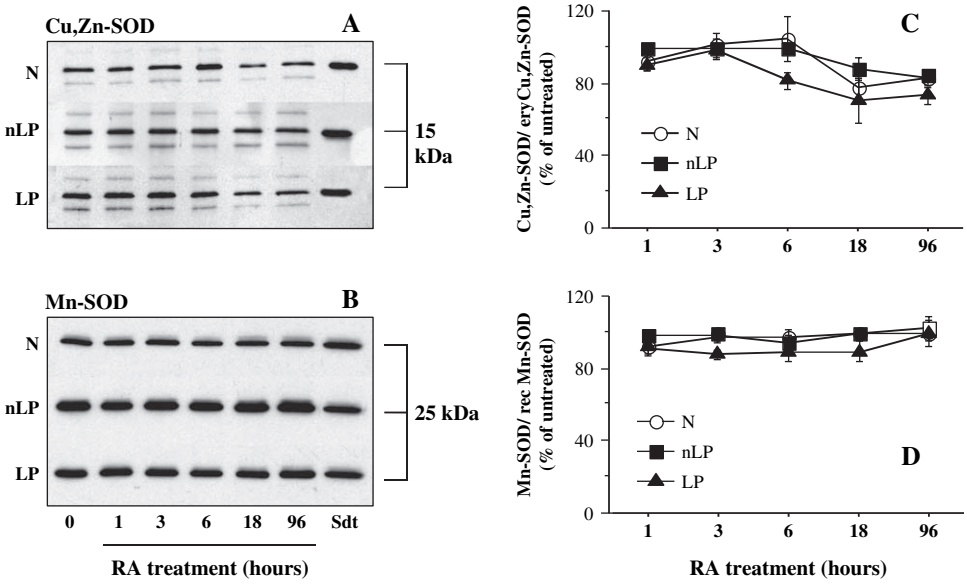

Fig. 2. Effect of RA treatment of Normal (N), non-Lesional Psoriatic (nLP) and Lesional Psoriatic (LP) fibroblasts for increasing periods of time on Cu,Zn-SOD [panels A and B (densitometry)] and Mn-SOD [panels C and D (densitometry)]. Cells were treated with 1 μM RA for the times indicated. 0 indicates untreated cells and sdt indicates the positive standard controls: Controls were erythrocyte Cu,Zn-SOD for panel A, and the recombinant human Mn-SOD for panel C. Data are presented as mean ± SEM ($n = 9$). Each sample was assayed in triplicate in three independent experiments.

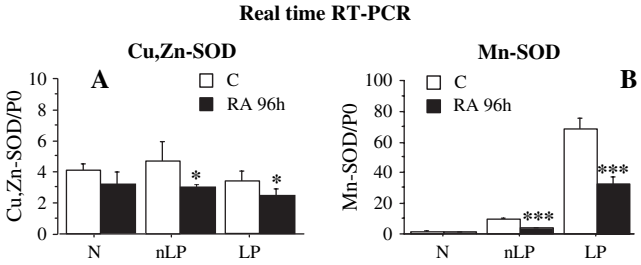

Fig. 3. (A) Effect of RA treatment of Normal (N), non-Lesional Psoriatic (nLP) and Lesional Psoriatic (LP) fibroblasts on Cu,Zn-SOD mRNA levels as determined by real time RT-PCR. The cells were treated without (open bars) and with (filled bars) 1 μM RA. The results of the real time RT-PCR are expressed as a ratio of arbitrary units (AU) of Cu,Zn-SOD/P0 of duplicates of four different N, four and five different nLP and LP primary culture of fibroblasts. P0 is a housekeeping gene used as control. *$p < 0.03$ for nPL, $p < 0.015$ for PL. (B) Effect of RA treatment of Normal (N), non-Lesional Psoriatic (nLP) and Lesional Psoriatic (LP) fibroblasts on Mn-SOD mRNA levels as determined by real time RT-PCR. The cells were treated without (open bars) and with (filled bars) RA. The results of the real time RT-PCR are expressed as a ratio of arbitrary units (AU) of Mn-SOD/P0 of duplicates of three different N, four different nLP and LP primary culture of fibroblasts. P0 is a housekeeping gene used as control.***$p < 0.001$.

3.6. Analysis of Cu,Zn-SOD and Mn-SOD mRNA stability

To determine whether the increase of Mn-SOD mRNA in psoriatic fibroblasts is due to a decrease of Mn-SOD mRNA degradation, the stability of SOD mRNA was measured in the presence of the transcriptional inhibitor actinomycin D (ActD). The cells were treated or not by RA, after which 10 μg/mL ActD was added. Total mRNA was isolated at 0, 1, 3 and 6 h after ActD addition and analysed by real time RT-PCR (Fig. 5). The Cu,Zn-SOD mRNA and Mn-SOD mRNA were stable in the three different types of human dermal fibroblasts (non-lesional, lesional psoriatic, normal) without or after 1 μM RA treatment (Fig. 4, panel A). Thus we can conclude that the increased steady-state Mn-SOD mRNA levels in psoriatic fibroblasts were not due to an increase in mRNA stability.

3.7. Analysis of Cu,Zn-SOD and Mn-SOD protein stability

To investigate whether Cu,Zn- and Mn-SOD protein levels were stabilized by RA, normal and psoriatic fibroblasts were pretreated with 1 μM RA for 48 h and cycloheximide (10 μM/mL) added from 1 to 3 h to inhibit protein synthesis. Cycloheximide alone or with RA (1 μM) did not affect the expression of Cu,Zn-SOD protein in all types of human psoriatic fibroblast primary cultures (non-lesional, lesional psoriatic) (Fig. 5, panels A, B, C). In addition, RA treatment of normal and non-lesional and lesional psoriatic fibroblasts under cycloheximide did not affect Mn-SOD

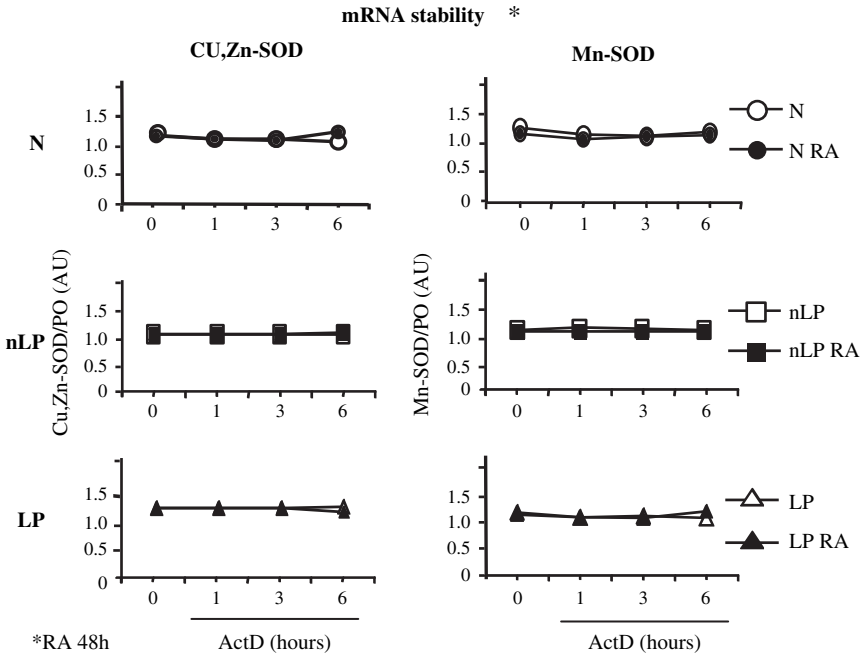

Fig. 4. Increased Mn-SOD steady-state mRNA and decreased expression of Mn-SOD mRNA in response to RA in psoriatic fibroblasts. As shown in Fig. 4, panels A and B, the stability of Cu,Zn- and Mn-SOD mRNA in the presence (open) or absence (filled) of RA was determined utilizing actinomycin D (ActD) as described in Section 2. The results of the real time RT-PCR are expressed as a ratio of arbitrary units (AU) of Mn-SOD/P0 of duplicates of two different N, two different nLP and LP primary culture of fibroblasts. P0 is a housekeeping gene used as control.

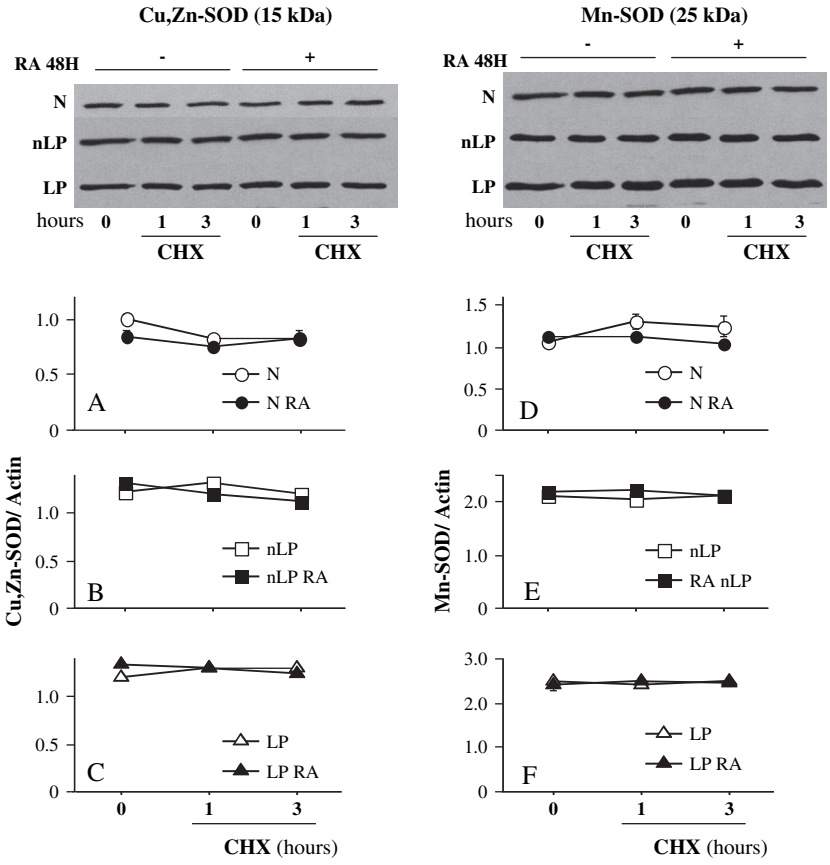

Fig. 5. Analysis of Cu,Zn- and Mn-SOD protein stability in Normal (N), non-Lesional Psoriatic (nLP) and Lesional Psoriatic (LP) fibroblasts. As shown in panels A and B, the three different primary cultures of fibroblasts were untreated or treated with RA for 48 h and cycloheximide (CHX) (10 μg/mL) was added for 1 and 3 h. The molecular mass for Cu,Zn-SOD, Mn-SOD and β-actin is 15, 25 and 45 kDa, respectively. β-Actin serves as loading control as shown in A and B. Immunoblots were quantified by densitometry and expressed as the ratio of the Cu,Zn-SOD/β-actin and Mn-SOD/β-actin for each sample. The band intensities were quantified using ImageQuant software, Amersham Biosciences. Each point in the plot represents the mean, and the error bars represent ±SEM. ($n = 4$).

protein levels (Fig. 5, panels D, E, F). Thus we can rule out an effect of RA on Mn-SOD protein stability.

4. Discussion

The involvement of oxygen free radicals in a growing list of diseases indicates a need for pharmaceutical agents that may inhibit the production of free radicals. These molecules may enhance radical scavenging systems, which may then reverse oxidative damage to important cellular components including those of the signal transduction pathways. All-*trans* retinoic acid (RA), the active metabolite of vitamin A, is critical in the regulation of a number of biological processes, including embryogenesis and cell growth [22,23] and it also has been used in the treatment of psoriasis [24]. Previous results, which demonstrated that RA treatment of cells could reverse the oxidatively modified state of protein kinase C [25], and of PKA [14], indicated that RA might act to alter redox activities within the cell. However, its mechanism of action is still unclear. To assess the role of RA as an important regulator for treatment of psoriasis, we have investigated the effects of RA on Cu,Zn-SOD and Mn-SOD activity as well as mRNA and protein levels in normal and psoriatic fibroblasts.

Retinoic acid treatment of psoriatic (nLP and LP) fibroblasts produced a significant, time-dependent decrease in Mn-SOD activity and mRNA to the basal levels observed in normal fibroblasts. As increased Mn-SOD activity occurs in response to an oxidative stress and particularly in response to superoxide anions, the antioxidant effects of RA treatment results in a reduction of Mn-SOD activity in the psoriatic cells to basal levels. Interestingly, the amount of Mn-SOD protein found in these cells was not modified by RA treatment. However, discrepancies between mRNA and protein levels have also been reported by others. Ahlemeyer et al. [26] showed that staurosporine did not influence the mRNA levels of Cu,Zn-SOD and Mn-SOD, but decreased the protein levels and the activity of both enzymes in cultures from neonatal rat hippocampus. Tsan et al. [27]

showed that hyperoxia-induced increase in the mRNA level of Mn-SOD in the lung was not associated with an increase in the protein level or enzymatic activity of Mn-SOD. Niu et al. [28] found an induction of the Mn-SOD mRNA by lipopolysaccharides in rat astrocytes, with a constant Mn-SOD protein level. These observations suggest that SOD protein levels could be dissociated from their synthesis and from their enzyme activities. The fact that RA decreases mRNA levels of Mn-SOD in psoriatic fibroblasts without a significant decrease in protein levels could be explained by the stability of these enzymes in time course of our experiments. Our results are in agreement with a 5–6 h half-life for Mn-SOD [29]. It is also possible that the RA treatment has altered the subcellular distribution of the protein. For example, if the RA treatment enhanced protein transport into the lysozome or proteosome through post-translational modifications such as ubiqutination, it is possible that while total cellular protein levels were not changed, the enzymatic activity was reduced due to the change in subcellular localization. This hypothesis awaits further investigation.

Enzyme activities often are modulated by post-translational modification of the protein. Covalent modifications such as disulfide formation, phosphorylation, acetylation, glycosylation and nitration all have been reported [30]. An additional modification termed retinoylation also has been observed in cells treated with retinoic acid [31,32]. Following activation of retinoic acid in a coenzyme A-mediated reaction, a thioester bond between protein and RA can be created. The RI and RII regulatory subunits of PKA were found to be retinoylated in HL 60 cells [32], and we also observed retinoylation of RI and RII in normal and psoriatic fibroblasts [33]. Retinoylation of the RII regulatory subunit, as well as total protein retinoylation, was observed to be increased in psoriatic cells. This raises the possibility that Mn-SOD could be covalently modified by retinoylation in response to RA treatment of psoriatic cells, thus explaining our observed decrease in enzyme activity. Finally we recently have shown an increase of carbonylation in psoriatic cells, which could also be responsible for protein modification and decreased enzyme activity [34].

Retinoic acid treatment had a differential effect on the modulation of Cu,Zn-SOD. The basal level of Cu,Zn-SOD mRNA was not significantly altered in psoriatic as compared to normal fibroblasts, whereas the basal Mn-SOD mRNA level was significantly elevated in psoriatic fibroblasts. In studies with psoriatic epidermis, Löntz et al. [35] reported that the expression of Cu,Zn-SOD mRNA was unchanged in psoriatic (nLP and LP) compared to normal skin. In our case, we have determined that retinoic acid treatment resulted in decreased levels of Cu,Zn-SOD mRNA in psoriatic cells. However, the mechanism(s) underlying the down-regulation of Cu,Zn-SOD expression remains unclear. In fact, there is little information concerning factors which may regulate Cu,Zn-SOD transcription, as Cu,Zn-SOD is constitutively expressed in primary cultures of dermal fibroblasts, and is not regulated by cytokines and growth factors [36]. Recently, Frank et al. [37] showed a direct coupling of Cu,Zn-SOD mRNA expression levels to the presence of nitric oxide, since Cu,Zn-SOD levels declined during acute inflammation (endotoxemic shock) in the presence of a selective inhibitor of iNOS and without involvement of the soluble guanylate cyclase. This suggested the possibility that under conditions of chronic inflammation, such as in psoriatic fibroblasts, Cu,Zn-SOD mRNA expression might be regulated by H_2O_2, NO and peroxinitrite.

Results presented here showed that RA treatment of psoriatic fibroblasts for 18 h down-regulated Cu,Zn-SOD mRNA steady-state levels. These results suggest either transcriptional regulation or a modification of mRNA stability of Cu,Zn-SOD mRNA expression with RA treatment of psoriatic cells, but a translational regulation of Cu,Zn-SOD levels in normal cells. Retinoids appear to exert their biological effects through multiple gene regulatory nuclear factors [38–40]. An activated retinoid–receptor complex can regulate gene expression by two distinct mechanisms. It can activate transcription by binding to a retinoid response element in the promoter of specific genes. However, no retinoid response element was found within the promoter regions of the Cu,Zn-SOD and Mn-SOD genes. Alternatively, RA can indirectly repress the activity of other nuclear transcription factors such as AP-1 [41,42]. The activated retinoid receptor complex can interact with cofactor proteins that are required for AP-1 mediated gene transcription factor. However, no binding sequence for the AP-1 transcription factor has been found within the proximal promoter of Cu,Zn-SOD. Thus, RA regulation of Cu,Zn-SOD gene expression may require interaction with other cellular components, such as other cellular retinoic acid binding proteins (CRABPs, see for review Noy [43]). The possible involvement of CRABPs in mediating this down-regulatory effect of RA on Cu,Zn-SOD expression is of interest in view of the finding that CRABP II levels are elevated in psoriatic skin [44] and awaits further analysis. While in this report we studied the effect of RA on psoriatic fibroblasts, as an immunologic sentinel. RA has other cellular targets in psoriasis such as keratinocytes and T lymphocytes. Thus it is quite possible that Cu,Zn-SOD could also be involved in the prevention of T-cell apoptosis [46]. This hypothesis awaits further testing.

In conclusion, RA treatment of primary psoriatic fibroblasts resulted in a significant decrease in the elevated level of Mn-SOD activity found in these cells at a level similar to that in normal fibroblasts. This

regulatory effect of RA could be at the level of protein covalent modification or subcellular localization or at the Mn-SOD mRNA steady-state level, since there was no significant change in total cellular Mn-SOD protein levels. Retinoic acid treatment of both normal and psoriatic fibroblasts also caused a decrease in Cu,Zn-SOD activity that appeared to be at the level of transcriptional regulation. Thus our data begins to provide a biochemical basis for the use of RA in the treatment of psoriasis.

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