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Enhancement of Thioredoxin Production from Nasal Epithelial Cells by the Macrolide Antibiotic, Clarithromycin *In Vitro*

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Abstract. *Low-dose and long-term administration of 14-membered macrolide antibiotics is well-known to be effective in the treatment of chronic airway diseases. Although there is much evidence that the anti-inflammatory action, but not the anti-microbial action of macrolides, is responsible for the clinical efficacy of these agents on chronic airway inflammatory diseases, the precise therapeutic mechanisms are not well-understood. Since thioredoxin (TRX) has now attracted attention as an endogenous peptide with immunomodulatory effects, the present study was undertaken to examine whether macrolide antibiotics could favorably modulate the clinical status of such diseases via the production of TRX from nasal epithelial cells in vitro. Nasal epithelial cells (5×10^5 cells/ml) obtained from five patients were stimulated with 50 μM H_2O_2 in the presence of different concentrations of macrolide antibiotics for 24 h. TRX levels in culture supernatants were examined by enzyme-linked immunosorbent assay. We also examined the influence of macrolide antibiotics on TRX mRNA expression and mRNA translation by RT-PCR and a wheat germ cell-free protein synthesis technique, respectively. The addition of clarithromycin (CAM) to cell cultures caused an increase in the ability of cells to produce TRX in response to H_2O_2 stimulation, and the minimum concentration that caused a significant increase was 0.5 μg/ml. On the other hand, josamycin, a 16-membered macrolide antibiotic, did not increase TRX production induced by H_2O_2 stimulation. Although the treatment of cells with CAM inhibits TRX mRNA transcription, the agent might increase translation of mRNA to produce specific proteins. The ability of CAM to increase TRX production may account, at least in part, for the clinical efficacy of this agent on chronic airway inflammatory diseases, including chronic rhinosinositis.*

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Macrolide antibiotics are a group of anti-bacterial agents with a distinctive macrocyclic lactone ring combined with sugars and are active against many species of Gram-positive and some Gram-negative bacteria. It is now known that in addition to anti-bacterial activity, these compounds exert anti-inflammatory effects *in vitro* and *in vivo* through their inhibitory action on inflammatory cell migration and inflammatory cytokine production, amongst others (1-3). In addition, macrolide antibiotics, especially 14-membered macrolides, are well-known to have beneficial effects in the treatment of chronic airway inflammatory diseases, such as diffuse panbronchiolitis (DPB), chronic sinusitis (CS) and cystic fibrosis (CF) (4). In this regard, much effort has been made to elucidate the therapeutic mechanisms of macrolides and revealed that the anti-inflammatory action, but not the anti-microbial action of macrolides is responsible for the clinical efficacy of the agents against chronic airway inflammatory diseases (1-6).

It is now accepted that inflammatory cells such as neutrophils, which are the most important effector cells in the development of chronic airway diseases, produced not only several types of chemical mediators but also free radicals, including $O_2^{\bullet-}$ and H_2O_2 (3, 4). Although physiological production of free radicals is generally believed to be essential in host defense, overproduction of free radicals and their metabolites are harmful and cause oxidative stress responses, which are implicated in the pathogenesis of conditions such as DPB, CS and pulmonary fibrosis (3, 7). On the other hand, under normal physiological conditions, there are several types of antioxidants such as glutathione, superoxide dismutase and hydrogen peroxide, which prevent the development of oxidative stress responses. Among these, thioredoxin (TRX) has attracted attention as an endogenous antioxidant protein. TRX, which was originally identified as a hydrogen donor for ribonucleotide reductase in *Escherichia coli*, is a 12-kDa protein with two redox active half-cysteine residues (-Cys-Gly-Pro-Cys-) (8-

10). In addition to its anti-oxidative activity, TRX is reported to exert immunomodulatory effects. Administration of exogenous TRX suppresses airway hyper-responsiveness induced by specific allergen challenge through the inhibition of eosinophil accumulation in airways of mouse models of asthma (11, 12). TRX also suppresses the chemotactic response of neutrophils and monocytes induced by stimulation with either lipopolysaccharide or monocyte chemoattractant protein-1 (MCP-1) (13, 14). Moreover, TRX is able to inhibit activity of purified human neutrophil elastase as well as elastolytic activity of sputum prepared from patients with CF (15). Since it is believed that elastase plays an important role in the development of structural lung damage, such as bronchiectasis, progressive pulmonary function decline, and early death, which are observed in CF and DPB (15, 16), the manipulation of the TRX system consisting of TRX, TRX reductase and NADPH, may be good targets in the treatment of chronic airway inflammatory diseases, including CF and DPB. However, the influence of macrolide antibiotics on the function of the TRX system is not clear at present. In the present study, therefore, we investigated the influence of macrolide antibiotics on the TRX system by examining the effect of agents on TRX production from human nasal epithelial cells *in vitro*.

Materials and Methods

Reagents. Clarithromycin (CAM) was kindly donated by Taisho-Toyama Pharmaceutical Co. Ltd. (Tokyo, Japan) as a preservative-free pure powder. The agent was dissolved firstly in 100% ethyl alcohol to a concentration of 10.0 mg/ml and then diluted with phosphate buffered saline (PBS) to a concentration of 10 μg/ml. This was sterilized by passing it through 0.2-μm filters, and stored at 4˚C until used. Josamycin (JM) purchased from Sigma Pure Chemical Co. (St Louis, MO, USA) was also dissolved in PBS in a similar manner.

Cell source and epithelial cell culture. Nasal polyp specimens were surgically-obtained from patients with CS who had not received any medical treatment, including systemic and topical steroid application or oral macrolide antibiotics. Specimens were washed five times with PBS that contained 500 μg/ml streptomycin, 500 U penicillin and 5 μg/ml ampotericin B. These tissues were then treated with 0.1% protease type XIV for 12 h at 4˚C. Epithelial cell layers were then obtained and vigorously mixed with a pipette to obtain a single-cell suspension. The cells were suspended in SABM medium (Lonza Co., Ltd., Walkersville, MD, USA) at a density of 5×10^3 cells/ml. The cell suspension was introduced into triplicate 24-well tissue culture plates coated with human type I collagen and cultured for 48 h, when the number of cells had reached approximately 5 x 10^5 cells/well. The cells were then stimulated with 50 μM H_2O_2 in the presence of different concentrations of either CAM or JM. After 24 h, culture supernatants were collected and stored at –40˚C until used. In the case of examination for mRNA expression, cells were cultured in a similar manner for 12 h. In all experiments, treatment of cells with the agents was started 2 h before H_2O_2 stimulation.

Assay for TRX mRNA expression. Poly A^+ mRNA was separated from cultured cells with oligo(dT)-coated magnetic micro beads (Milteny Biotec, Bergisch Gladbach, Germany). The first-strand cDNA was synthesized from 1.0 μg of Poly A^+ mRNA using a Superscript cDNA synthesis kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was then carried out using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR mixture consisted of 2.0 μl of sample cDNA solution (100 ng/μl), 25.0 μl of SYBR-Green Mastermix (Applied Biosystems), 0.3 μl of both sense and anti-sense primers, and distilled water to a final volume of 50.0 μl. The reaction was conducted as follows: 4 min at 94˚C, followed by 40 cycles of 15 s at 95˚C and 60 s at 60˚C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. mRNA levels were calculated by using the comparative parameter threshold cycle and normalized to GAPDH. The primers used for real-time RT-PCR were 5'-GCCTTGCAAAATGATTCAAGC-3' (sense) and 5'-TTGGCTCCAGAAAATTCACC-3' (anti-sense) for TRX (17), and 5'-TGTTGCCATCAATGACCCCTT-3' (sense) and 5'-CTCCACGACGTACTCAGCG-3' (anti-sense) for GAPDH (18).

Preparation of TRX-specific mRNA. To prepare TRX-specific mRNA for cell-free protein synthesis, the first-strand cDNA synthesized was amplified with a Takara PCR Amplification kit using specific primers for TRX in a final volume of 30 μl. The PCR conditions were as follows: 5 min at 94˚C, followed by 30 cycles of 20 s at 94˚C, 30 s at 58˚C, and 30 s at 72˚C. After measuring mRNA contents, samples were stored at –80˚C until used.

Cell-free protein synthesis. Cell-free protein synthesis was cell-free protein synthesis core kits (Toyobo Co., Ltd., Osaka, Japan). The reaction mixture consisted of 2.0 μl of reaction buffer, 1.7 μl of creatine kinase (10 μg/ml), 1.0 μl of ribonuclease inhibitor (40 U/ml), 10.0 μl of wheat germ extract, 33.5 μl of specific mRNA (0.4 μg/μl) and distilled water containing different concentrations of CAM to a final volume of 50.0 μl. The reaction mixture (50 μl) was then introduced into each well of 96-well plates that contained 250 μl of reaction buffer. The plates were incubated at 26˚C for 24 h and the solutions were stored at –40˚C until use.

Assay for TRX. TRX levels in culture supernatants and protein synthesis solution were examined by the commercially available TRX enzyme-linked immunosorbent assay (ELISA) test kits (BioVendor Lab. Med. Inc., Brno, Czech Republic), according to the manufacturer's recommendations. The minimum detectable level of this kit was 2.13 ng/ml.

Statistical analysis. The statistical significance between control and experimental groups was examined by analysis of variance (ANOVA) followed by the Dunette's multiple comparison test. The level of significance was considered at a *p*-value of less than 0.05.

Results

Influence of H_2O_2 stimulation on TRX production from nasal epithelial cells in vitro. The first experiments were undertaken to examine whether H_2O_2 stimulation increased TRX production from nasal epithelial cells and the optimal concentration of H_2O_2 for stimulation. To do this, the cells

were stimulated with different concentrations of H_2O_2 for 24 h, and the TRX levels in culture supernatants were examined by ELISA. As shown in Figure 1, stimulation of cells with H_2O_2 caused a significant increase in the ability of cells to produce TRX. As little as 2.5 μM of H_2O_2 strongly stimulated TRX production. Maximal production was obtained with 25.0 to 75.0 μM of H_2O_2, and 100 μM was inhibitory (Figure 1).

Influence of macrolide antibiotics on H_2O_2-induced TRX production from nasal epithelial cells in vitro. The second set of experiments was designed to examine the influence of macrolide antibiotics on TRX production from nasal epithelial cells after H_2O_2 stimulation. The cells were stimulated with 50 μM H_2O_2 in the presence of either CAM or JM for 24 h. TRX levels in culture supernatants were examined by ELISA. As shown in Figure 2, treatment of cells with CAM at concentrations of both 0.2 μg/ml and 0.4 μg/ml scarcely affected the ability of cells to produce TRX; TRX levels in culture supernatants were nearly identical to those detected in the control. CAM at 0.5 μg/ml or more significantly increased the TRX levels in culture supernatants, as compared with that in the control. On the other hand, JM did not increase TRX production from nasal epithelial cells in response to H_2O_2 stimulation even when 10.0 μg/ml of the agent was used for treatment.

Influence of CAM on TRX mRNA expression in nasal epithelial cells in vitro. The third set of experiments was undertaken to examine the influence of CAM on *TRX* mRNA expression in nasal epithelial cells *in vitro*. As shown in Figure 3, stimulation of cells with H_2O_2 caused significant increase in *TRX* mRNA level, as compared with the non-stimulated control. On the other hand, the addition of CAM at 0.5 μg/ml but not 0.2 μg/ml caused a significant decrease in *TRX* mRNA expression, which was increased by H_2O_2 stimulation.

Influence of CAM on cell-free protein synthesis. The final set of experiments was designed to examine the influence of CAM on TRX production in cell-free protein systems. As shown in Figure 4, the addition of CAM at 0.1 μg/ml and 0.2 μg/ml into cell-free protein systems did not increase TRX synthesis; TRX level in the solutions were nearly identical ($p>0.05$; not significant) to those observed in the control. On the other hand, CAM at more than 0.5 μg/ml significantly increased the ability of wheat germ extract to produce specific protein as compared with the control.

Discussion

Low-dose and long-term administration of macrolide antibiotics, also called macrolide therapy, is effective in the treatment of DPB, CS and CF (4). In addition to these airway

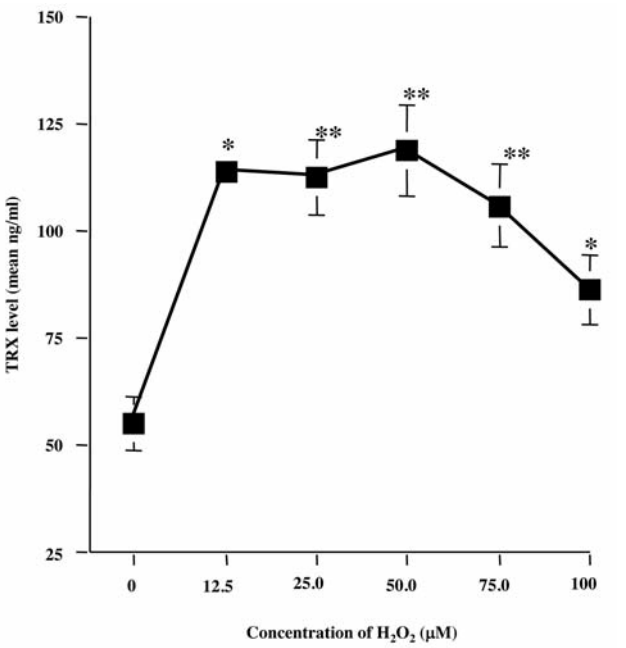

Figure 1. *Influence of H_2O_2 on thioredoxin (TRX) production from nasal epithelial cells in vitro. Nasal epithelial cells (5×10^5 cells) were stimulated with different concentrations of H_2O_2. After 24 h, TRX levels in culture supernatants were examined by ELISA. The data are expressed as the mean±SE of five cultures from different subjects. *Significant at $p<0.05$ vs. 0 μM H_2O_2; **$p>0.05$ vs. 12.5 μM H_2O_2.*

inflammatory diseases, several types of skin diseases characterized by inflammatory disorders are also reported to be susceptible to the macrolide therapy (19, 20). There is considerable evidence to suggest that the inhibitory action of macrolides on the chemotaxis and activation of inflammatory cells such as neutrophils, which are the most important effector cells in the development of chronic airway inflammatory diseases, may account for the clinical efficacy of macrolides in inflammatory diseases (4).

Oxygen-consuming organisms obtain energy through cellular respiration, which is the transformation of carbohydrates and oxygen into carbon dioxide and water. Up to two percent of the oxygen used in this system is transformed into superoxide, a free radical that is toxic to cells. Furthermore, free radicals also produced by phagocytic cells such as macrophages and granulocytes, which are cells essential to the development of airway inflammatory diseases, and function in the intracellular killing of bacteria. Because free radicals are necessary for life, the body has a number of mechanisms to minimize free-radical-induced damage and to repair damage that occurs, such as *via* several types of enzymes and endogenous proteins. Among these, TRX attracts attention as being not only an important antioxidative factor (8-10)

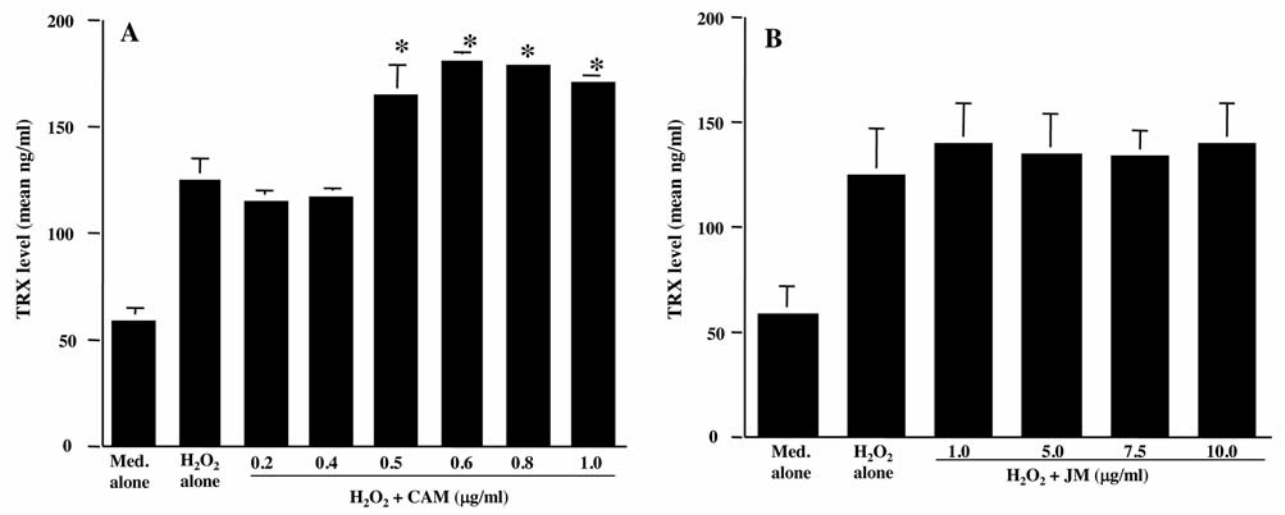

Figure 2. *Influence of macrolide antibiotics on thioredoxin (TRX) production from nasal epithelial cells induced by H_2O_2 stimulation in vitro. Nasal epithelial cells (5×10^5 cells) were stimulated with 50 μM H_2O_2 in the presence of clarithromycin (CAM) or josamycin (JM) for 24 h. TRX levels in culture supernatants were examined by ELISA. The data are expressed as the mean±SE of cells from five different subjects. A: CAM; B: JM; *Significant at $p<0.05$ vs. H_2O_2 alone.*

but also a protective factor in the development of various inflammatory diseases (12-15). However, there is little evidence showing the influence of macrolide antibiotics on TRX. The present study clearly demonstrates that CAM increases the ability of nasal epithelial cells to produce TRX in response to H_2O_2 stimulation, when the cells were treated with the agent at more than 0.5 μg/ml, which is a quite low level compared with therapeutic blood levels (1.03±0.16 μg/ml) (21). On the other hand, JM, which is ineffective in the treatment of DPB and CS (4), did not increase TRX levels in the culture supernatants even when the cells were treated with 10.0 μg/ml. TRX, a well-characterized protein with a highly conserved active site, is reported to play a variety of redox-related roles in organisms from bacteria to Man (12, 13). Intracellular TRX, together with peroxiredoxin, plays essential roles in the scavenging of free radicals and the regulation of redox-sensitive transcription factors such as nuclear factor (NF)-κB and activator protein (AP)-1 (13). In addition to intracellular functions, TRX has been shown to inhibit neutrophil chemotaxis induced by lipopolysaccharide (LPS) and chemokine stimulation (13). It is also reported that TRX directly blocks the adhesion of LPS-stimulated neutrophils on endothelial cells *in vitro* (13). Furthermore, TRX inhibits the down-regulation of L-selectin precursor (CD62L) expression on neutrophils after LPS stimulation *via* the suppression of p38 mitogen-activated protein kinase phosphorylation (13), suggesting that TRX exerts a suppressive effect on neutrophil activation. In the development of airway inflammatory diseases, administration of TRX into mice for three days prevented lung injury induced by exposure by cigarette smoking through the suppression of neutrophil influx (22). Moreover, although chronic exposure by cigarette smoking is reported to cause pulmonary emphysema in normal mice accompanying prominent infiltration of macrophages and neutrophils into the lung, TRX-transgenic mice, which produce much higher levels of human TRX in response to inflammatory stimulation, significantly prevented the development of these pathological changes (22). Taken together, the present data show that the enhancement of TRX production by nasal epithelial cells may be interpreted as part of the therapeutic mode of action of 14-membered macrolides in chronic airway inflammatory diseases.

Forkhead transcription factor-3 (FOXO3) is reported to be an essential transcription factor involved in the up-regulation of *TRX* mRNA expression (17). It is also reported that AMP-activated protein kinase (AMPK) plays a pivotal role in phosphorylation of FOXO3 (23), which is responsible for the promotion of TRX transcription in nuclei, indicating that the AMPK-FOXO3 pathway is essential for *TRX* mRNA expression (17, 23). Together with these reports, the present results showing the inhibitory effect of CAM on *TRX* mRNA expression may be, in part, due to the suppressive effect of the agent on AMPK-FOXO3 pathway activation. This speculation may be supported by the observation that 14-membered macrolide antibiotics, including CAM, exert a suppressive effect on the activation of transcription factors such as NF-κB and AP-1 through the inhibition of activation of MAPKs (3). On the other hand, the present results clearly show that treatment of nasal epithelial cells with CAM

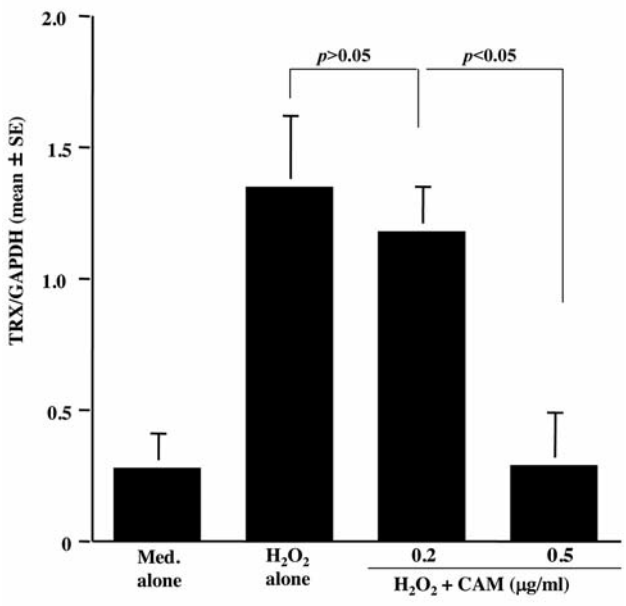

Figure 3. *Influence of clarithromycin (CAM) on thioredoxin (TRX) mRNA expression in nasal epithelial cells in vitro. Nasal epithelial cells (5×10^5 cells) were stimulated with 50 μM H_2O_2 in the presence of CAM. After 12 h, TRX mRNA expression was examined by real-time RT-PCR. The data are expressed as the mean ratio calculated as TRX/GAPDH of cultures from five different subjects. GAPHD: Glyceraldehyde-3-phosphate dehydrogenase.*

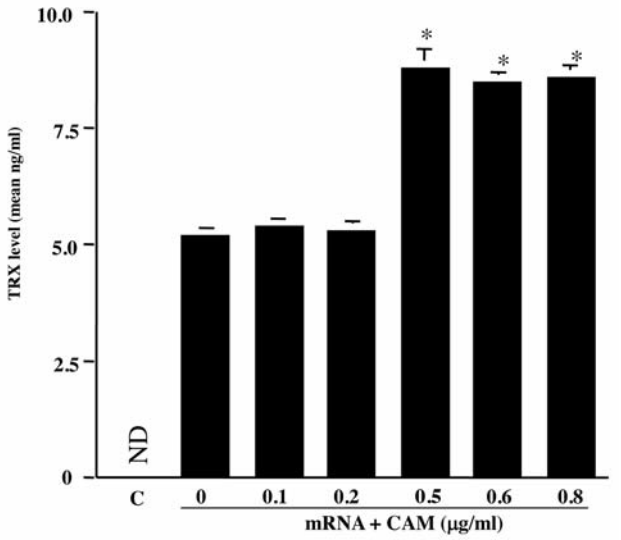

Figure 4. *Influence of clarithromycin (CAM) on thioredoxin (TRX) production in cell-free protein synthesis systems. TRX levels in samples were examined by ELISA. The data are expressed as the mean±SE of five different subjects. ND: Not detected (below 2.13 ng/ml); *Significant at p<0.05 vs. 0 μg/ml CAM.*

caused an increase in TRX protein production in spite of the suppression of its mRNA expression induced by H_2O_2 stimulation. The reasons for this discrepancy are not clear at present. The process of protein synthesis in cells requires two different steps: in the first step, transcription, mRNA is synthesized from DNA in the nucleus. mRNA formed then comes out through the nuclear membrane into the cytoplasm where it binds to mRNA-binding sites on ribosomes and starts protein synthesis translocation. The final experiments, therefore, were undertaken to examine whether pre-treatment of nasal epithelial cells with CAM could increase the translocation activity of TRX mRNA. Cell-free protein synthesis assay revealed that the addition of CAM at more than 0.5 μg/ml could increase TRX synthesis. These results strongly suggest that there is the possibility that CAM could increase the translation of *TRX* mRNA, resulting in an increase of TRX levels in culture supernatants. Further experiments are required to clarify this point.

The present results strongly suggest that macrolide therapy causes an increase in the ability of airway cells, especially epithelial cells, to produce TRX, explaining the favorable modification of clinical symptoms of chronic airway inflammatory diseases (*e.g.* DPB and CS) through the suppression of both neutrophil influx and oxidative stress responses in the area of inflammation.

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