Thymosin beta-4 upregulates anti-oxidative enzymes and protects human cornea epithelial cells against oxidative damage

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ABSTRACT

Background: The ability to scavenge reactive oxygen species (ROS) is crucial for cornea epithelial cells to resist oxidative damage. The authors previously demonstrated that exogenous thymosin beta-4 ($T\beta_4$) was able to protect human cornea epithelial (HCE-T) cells against H_2O_2-induced oxidative damage, and its cellular internalisation was essential. The aim of this study is to further elucidate its protective mechanism.

Methods: HCE-T cells with or without $T\beta_4$ pretreatment were exposed to H_2O_2, and the differences in caspase activity, intracellular ROS levels, cell viability, and the expression of anti-oxidative enzymes, were measured and compared.

Results: Besides reducing caspase-9 activation and intracellular ROS levels induced by H_2O_2, treatment of $T\beta_4$ could also increase cell viability and stimulate the expression of manganese superoxide dismutase (SOD) and copper/zinc SOD. Moreover, both transcription and translation levels of catalase were also upregulated by $T\beta_4$ in the presence of exogenous H_2O_2. Furthermore, it was demonstrated that the addition of catalase inhibitor abrogated the protective effect of $T\beta_4$ against H_2O_2-induced oxidative damage.

Conclusion: To the best of the authors' knowledge, this is the first report to show that $T\beta_4$ was capable of upregulating anti-oxidative enzymes in human corneal epithelial cells, and these findings further support its role in cornea protection.

Apoptosis, also known as programmed cell death, is triggered by a number of stimuli, and its execution is mainly dependent on the activation of various caspases.[1] The activation of caspase-8 following the interaction between Fas-Fas ligand (FasL)[2] as well as caspase-9 activation by releasing of cytochrome *c* (cyt *c*) from mitochondria[3] can initiate a cascade of extrinsic pathway and intrinsic pathway, respectively. Oxidative stress-induced apoptosis is associated with alterations in mitochondrial caspase activity.[4]

Cornea is the first-line defence of the eye, and its survival relies heavily on its ability to remove intracellular reactive oxygen species (ROS)[5 6] because excessive ROS production and accumulation in corneal cells may result in their apoptosis as a consequence of oxidative damage.[7] Consequently, numerous efforts have been made in the search of strategies to protect cornea against oxidative damage.[4 5]

Thymosin β-4 ($T\beta_4$), originally found as a G-actin sequestering protein, has been demonstrated to possess anti-apoptosis function, and its anti-apoptotic effects against ethanol,[8] Fas ligand, and hydrogen peroxide (H_2O_2)[9] have been shown in corneal epithelial cells and conjunctival cells.[10] It is now appreciated that apoptosis triggered by H_2O_2 is mainly mediated by the intrinsic signalling pathway, which is associated with increased intracellular oxidative stress.[11 12] In our previous work, it was found that internalisation of exogenous $T\beta_4$ was essential for its protection of human corneal epithelial (HCE-T) cells from apoptosis.[9] The purpose of the present study is to elucidate further the protective mechanism of $T\beta_4$ against H_2O_2-triggered apoptotic death of corneal epithelial cells. Here, we showed that exogenous $T\beta_4$ protected HCE-T cells from H_2O_2-induced apoptosis by reducing the intracellular ROS levels, and it abrogated H_2O_2-induced caspase-9 activation plausibly by enhancing the expression of several crucial anti-oxidative enzymes.

MATERIALS AND METHODS

Cell culture and preparation of the recombinant $T\beta_4$

The SV-40 immortalised human corneal epithelial (HCE-T) cells[13] were cultured, and histidine-tagged $T\beta_4$ fusion protein (His_6-$T\beta_4$) from *Escherichia coli* was prepared as described previously.[9] The concentration of exogenous $T\beta_4$ in this study was 1 μg/ml.

Detection of the activation of Caspases-9 and Caspase-8

For examining the in situ activation of caspases-9 and -8 in HCE-T cells by H_2O_2, 24 h after H_2O_2 treatment, cells were incubated with the corresponding fluorogenic substrates (OncoImmunin, College Park, MD) for 1 h before being fixed in 4% paraformaldehyde. Fluorescence was detected by flow cytometry (FACS Calibur, BD Bioscience, San Jose, CA). The percentage of cells with activated caspases and intensity of fluorescence were calculated using Modfit software (Verity Software House, Topsham, ME). For analysing the activity of caspase-9 in cell lysate, a colorimetric substrate cleavage assay (R&D systems, Minneapolis, MN) was performed as described previously.[9]

Measurement of intracellular ROS levels

For measuring the levels of intracellular ROS, HCE-T cells after various treatments were incubated with 40 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFH-DA, Molecular Probes, Eugene, OR) at 37°C in the dark for 30 min as previously described.[14] Cells were then harvested and resuspended in 50 mM HEPES buffer (5 mM HEPES,

pH 7.4; 5 mM KCl, 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM glucose) before their fluorescence intensities were analysed by flow cytometry.

Western blot analysis

For western blot analysis, HCE-T cells were treated with or without exogenous $T\beta_4$ or incubated with exogenous $T\beta_4$ for 2 h before treated with 200 μM of H_2O_2. Cells were collected and resuspended in 50 μl lysis buffer (4 mM EDTA, 2 mM EGTA, 1% Triton X-100 and an aliquot of complete protease inhibitors mixture (Roche Diagnostics, Mannheim, Germany), pH 7.4). Cell lysate was incubated on ice for 30 min and then centrifuged at 12 000×*g* at 4°C for 20 min. The supernatant was collected, and protein concentration was measures by Bradford assay (Bio-Rad, Hercules, CA). Fifteen micrograms of total lysates after being separated by 10% SDS-PAGE were blotted onto PVDF membranes (Amersham Biosciences, Uppsala, Sweden). Non-specific bindings were blocked by 5% skim milk in TBST buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 0.1% Tween 20) at room temperature for 1 h. The membrane was then probed with the following primary antibodies: manganese-superoxide dismutase (Mn-SOD) and copper/zinc-superoxide dismutase (Cu/Zn-SOD) (1:5000, Upstate Biotechnology, Charlottesville, VA), catalase (1:3000, Calbiochem, San Diego, CA), and β-actin (1:5000, Chemicon, Temecula, CA). After three washes with TBST (5 min each), the membrane was incubated with a HRP-conjugated secondary antibody (Amersham Biosciences) at room temperature for 1 h. After another washes of TBST, protein signals were detected by ECL (NEN Life Science, Boston), and their intensities were measured by densitometry (LabWorks, UVP).

RNA isolation and quantitative RT-PCR analysis

HCE-T cells were treated with or without exogenous $T\beta_4$ for 6 h or pretreated with $T\beta_4$ for 2 h followed by incubation with H_2O_2 (200 μM) for 4 h. Total RNAs were extracted from 1 to 5×10^5 HCE-T cells using RNEasy (Qiagen, Stanford, Valencia, CA) as per the manufacturer's instructions. For real-time RT-PCR, 5 μg of total RNAs was reverse-transcribed to cDNA using Advantage RT-for-PCE (Clontech, Palo Alto, CA) following the manufacturer's protocols. Quantitative PCR was then carried out in a LightCycler 480 system (Roche, Basel, Switzerland), using 2 μl (20 ng) of cDNA template, 1 nmol of primers, and 10 μl of iQ SYBR green supermix (Bio-Red, Hercules, CA) in a total volume of 20 μl. The primers used for Mn-SOD were 5′-CACCAGCAGGCAGCTGGCTCC-3′ (sense) and 5′-TCCACC-ACCGTTAGGGCTGAGG-3′ (antisense); Cu/Zn-SOD were 5′-CAGT GCAGGTCCTCACTTTA-3′ (sense) and 5′-CCTGTC-TTTGTACTTTCTTC-3′ (antisense); catalase were 5′-TTTCCCAGGAAGATCCTGAC-3′ (sense) and 5′-AC CTTGG-TGAGATCGAATGG-3′ (antisense); and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5′-CCAGGTGGT CTCCTCTGACTTC-3′ (sense) and 5′-GTGGTCGTTGAGGG-CAATG-3′ (antisense). The cDNA were amplified with initial denaturation at 95°C for 5 min, followed PCR by 40 cycles of: 95°C 30 s, 60°C 30 s, 72°C 40 s and finally 1 cycle of melting curve following cooling at 40°C for 10 s. Each experiment was repeated three times, and the amount of target mRNA was normalised with that of the GAPDH.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science-10 software (SPSS, Chicago). Data from caspase-9 colorimetric substrate cleavage, intracellular ROS assays and real-time RT-PCR of anti-ROS enzymes were analysed by ANOVA tests with Tukey's post-hoc tests at 95% confidence intervals. Results from fluorogenic assays for caspase-9, -8 activity, and densitometry of western blotting of anti-ROS enzymes were determined by two-tailed Student t tests, and a p value less than 0.05 was considered as statistically significant.

RESULTS

$T\beta_4$ protects HCE-T cells against H_2O_2-induced apoptosis

To determine the H_2O_2 concentration that could effectively induce apoptosis of human corneal epithelial cells, the viability of HCE-T cells after being incubated with various concentrations of H_2O_2 for 24 h was first measured by MTS assay. We found that the 50% lethal dose (LD_{50}) of H_2O_2 on these cells was between 100 and 200 μM (data not shown). The activities of caspases-9 and -8 as well as the intracellular ROS levels in HCE-T cells 24 h post-H_2O_2 (200 μM) treatment were subsequently measured by flow-cytometric analyses. As expected, the activity of caspase-9 but not that of caspase-8 was significantly increased after H_2O_2 treatment (fig 1A,B). HCE-T cells were then pretreated with exogenous $T\beta_4$ (1 μg/ml) for 2 h before the addition of H_2O_2. Twenty-four hours later, the viability and caspase-9 activity of these cells were measured. In agreement with our previous finding that exogenous $T\beta_4$ protected HCE-T cells from H_2O_2-triggered death,[8] this peptide also effectively abrogated H_2O_2-induced caspase-9 activation in these cells (fig 1C).

$T\beta_4$ reduces intracellular oxidative stress in HCE-T cells elicited by H_2O_2

Since the increase in intracellular ROS may be responsible for the activation of caspase-9 as well as the consequential death triggered by H_2O_2, we asked whether exogenous $T\beta_4$ could also diminish the increase in intracellular ROS elicited by H_2O_2. Intracellular ROS levels were significantly lower in cells with a 2 h pretreatment of $T\beta_4$ at 1.5, 4 and 24 h after exposure to 200 μM H_2O_2 (fig 2A). The ROS scavenging ability of $T\beta_4$ was evaluated by comparing it with that of N-acetylcysteine (NAC), a precursor of glutathione (GSH) and a well-known ROS scavenger[15] in the initial 0.5 and 1.5 h after exposure to H_2O_2. Even though neither $T\beta_4$ nor NAC (1 mM) altered the baseline ROS levels in HCE-T cells (fig 2B), the latter significantly reduced intracellular ROS levels after these cells were treated with H_2O_2 for 30 min (fig 2C). Moreover, the intracellular ROS level in $T\beta_4$ treated HCE-T cells also decreased significantly 90 min after exposure to H_2O_2 (fig 2D).

$T\beta_4$ increases the expression of anti-oxidative enzymes in HCE-T cells

Because $T\beta_4$ did not have any immediate anti-ROS effects on HCE-T cells, the above-mentioned anti-apoptotic effect of this peptide may be attributed to its influence on antioxidant enzymes. To examine this possibility, a western blot analysis was then performed. Indeed, expression of Mn-SOD and Cu/Zn-SOD in HCE-T cells was significantly upregulated after being incubated with exogenous $T\beta_4$ for 24 h (fig 3A,B). Moreover, more than threefold increase in total SOD activity was detected in these cells under a similar treatment (data not shown). Intriguingly, although the protein level of catalase was not affected by $T\beta_4$ alone (fig 3C), it was nonetheless upregulated by $T\beta_4$ when HCE-T cells were subsequently

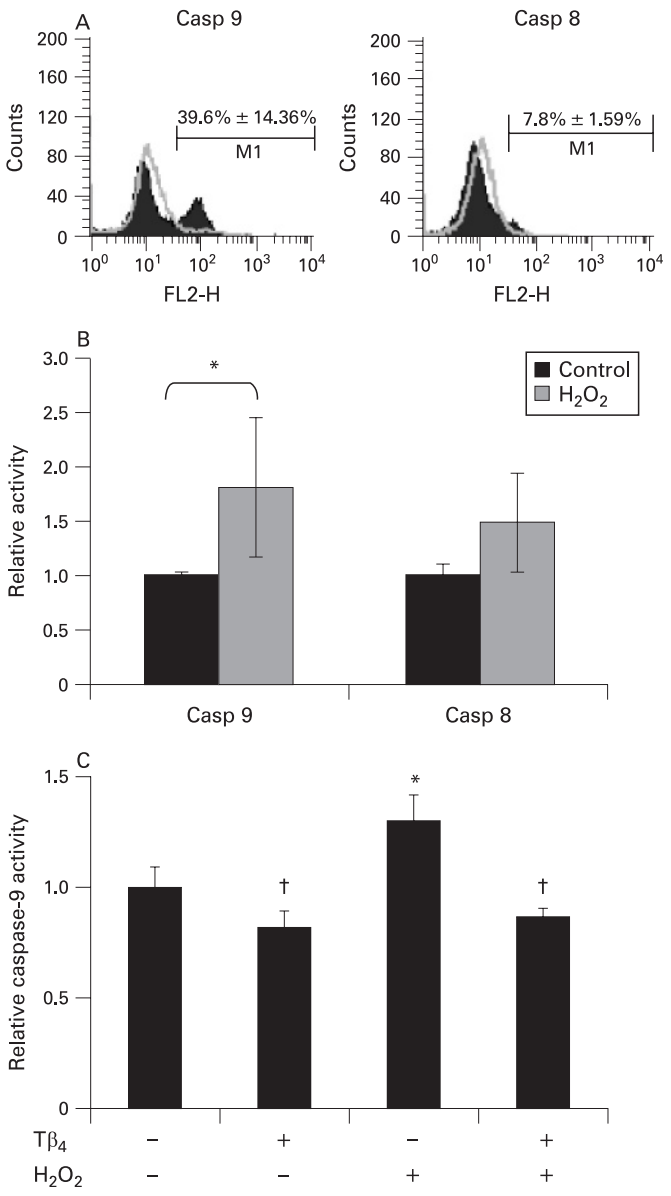

Figure 1 $T\beta_4$ inhibition of the activation of caspase-9 (Casp 9) and elevation of intracellular ROS induced by H_2O_2 treatment. (A) Activation of caspase-8 (Casp 8) and -9 in human cornea epithelial cells after being treated with 200 μM H_2O_2 for 24 h, examined by *in situ* fluorogenic substrate cleavage assays. Shown are the percentages of cells with caspase-9 (left panel) and caspase-8 (right panel) activities after H_2O_2 treatment. (B) Quantitative analysis of (A). *$p<0.05$ by Student t test ($n = 3$). (C) Colorimetric substrate cleavage assay applied to examine the activation of caspase-9 in human cornea epithelial cells incubated without or with exogenous $T\beta_4$ (1 μg/ml) for 2 h before being exposed to 200 μM H_2O_2 for 24 h. †Significantly lower and *significantly higher than the control group by ANOVA test ($n = 3$).

exposed to H_2O_2 (fig 3D). Similar increases in Mn-SOD and Cu/Zn-SOD protein levels were also found in HCE-T cells incubated sequentially with $T\beta_4$ and H_2O_2 (data not shown). Surprisingly, while RNA levels of Mn-SOD, Cu/Zn-SOD and catalase were all upregulated in HCE-T cells after H_2O_2 treatment (fig 4A–C), only Mn-SOD expression was upregulated by exogenous $T\beta_4$ alone (fig 4A). However, RNA levels of Mn-SOD and catalase in these cells were further stimulated by $T\beta_4$ after they were subsequently incubated with H_2O_2 (fig 4A,C).

Catalase inhibitor counteracts the ROS-reducing effects of exogenous $T\beta_4$

To further confirm the role of anti-oxidative enzyme upregulation in the ROS-reducing effects elicited by exogenous $T\beta_4$, 3-amino-1,2,4-triazole (AT), a catalase inhibitor,[16] was added to HCE-T cells at the concentration of 50 mM. Such concentration was without cytotoxicity (data not shown). It was found that the addition of AT increased the basal intracellular ROS level regardless of $T\beta_4$ pretreatment (fig 5A). Besides, AT abrogated the ROS-reducing effect of $T\beta_4$ in HCE-T cells in the presence of H_2O_2 (fig 5B).

DISCUSSION

We previously reported that $T\beta_4$ reduced the activity of caspase-3 in HCE-T cells upon treatment of H_2O_2.[9] The current study aimed to further dissect the molecular pathways governing such phenomenon. First of all, it was demonstrated that apoptosis induced by H_2O_2 (200 μM) was primarily through the intrinsic pathway because of a dramatic activation of caspase-9 but not caspase-8 (fig 1A,B), as well as a marked increase in intracellular ROS (fig 2A).

Two major molecular mechanisms have been proposed to elaborate the apoptosis triggered by ROS-initiated intrinsic signalling.[3] First, ROS causes Bax dimerisation by inducing the formation of disulfide bonds between critical cysteine residues within these molecules.[17] Bax dimers then translocate to the mitochondrial outer membrane where they aggregate to form pores that allow cyt *c* to efflux from this organelle.[18] Second, by peroxidation of cardiolipin, a mitochondria-specific cyt *c*-anchoring phospholipid, ROS may facilitate the release of cyt *c* from mitochondria by disrupting their interactions.[19] Hence, preventing or reducing the production of intracellular ROS is important to protect the cells from apoptosis triggered by the intrinsic pathway, as were the findings in the current study that exogenous $T\beta_4$ reduced intracellular ROS levels as well as intrinsic pathway-induced apoptosis. Even though our results have not directly demonstrated a reduction in cyt *c* efflux by $T\beta_4$ in HCE-T cells, they nevertheless supported the postulation made by Sosne *et al* that this peptide may function as an anti-apoptotic agent for human corneal epithelial cells by inhibiting the release of cyt *c* from mitochondria and by suppressing the activation of caspases.[8] $T\beta_4$ per se, on the other hand, did not affect the expression of Bax and Bcl-2 (Supplementary Figure).

Regarding the extreme effectiveness of exogenous $T\beta_4$ in reducing intracellular ROS in H_2O_2-treated HCE-T cells (fig 2A), two critical intracellular defence systems, the non-enzymatic system and enzymatic system, need to be taken into account. While the former includes reduced glutathione (GSH), ascorbic acid (vitamin C), α-tocopherol (vitamin E) and thioredoxin, all acting as antioxidants, the so-called ROS scavengers, the latter consists of cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, catalase, and a variety of peroxidases.[20] In this study, the intracellular ROS level was not affected by pretreating HCE-T cells with exogenous $T\beta_4$ (1 μg/ml) followed by 30 min exposure to H_2O_2 (fig 2C), indicating that this peptide may not be a strong ROS scavenger. On the other hand, intracellular ROS level was markedly reduced after these cells were incubated with H_2O_2 for 90 min (fig 2D), suggesting that it might activate the enzymatic defence mechanism. Both Mn-SOD and Cu/Zn-SOD have been reported to play a crucial role in protecting ocular cells from oxidative damage.[21] Indeed, $T\beta_4$ upregulated the protein levels of Mn-SOD and Cu/Zn-SOD in HCE-T cells (fig 3A,B).

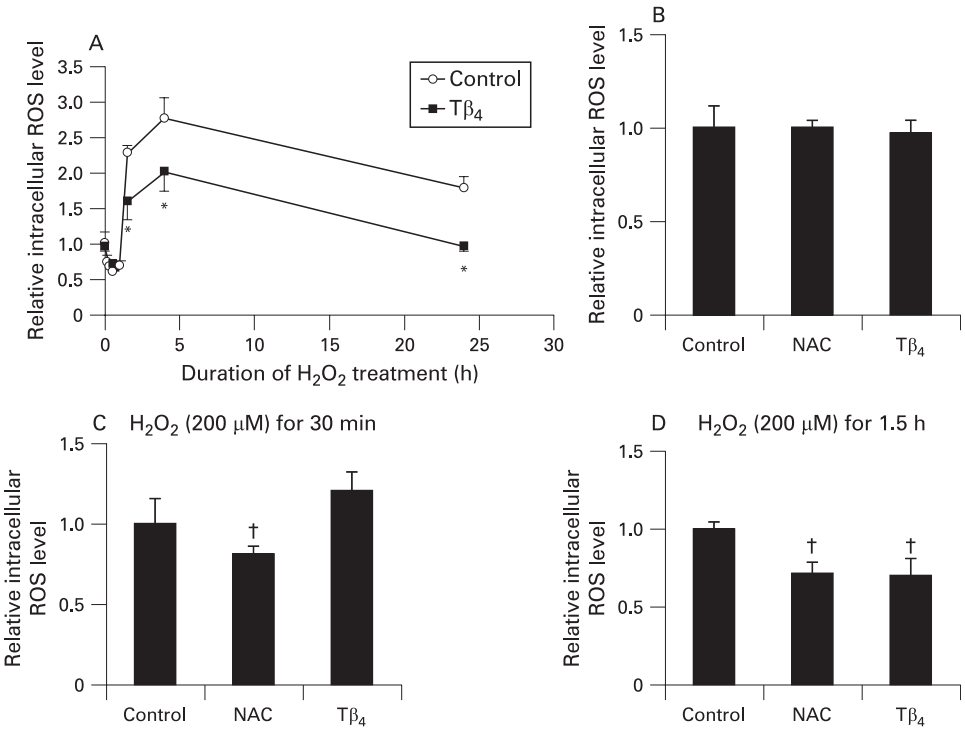

Figure 2 Exogenous $T\beta_4$ suppressed elevation of intracellular reactive oxygen species (ROS) induced by H_2O_2, and such an effect is not immediate. (A) Relative reactive oxygen species levels in human cornea epithelial cells pretreated without or with 1 μg/ml $T\beta_4$ for 2 h before being exposed to 200 μM H_2O_2 for 0.5, 1.5, 4 and 24 h were measured by analysing the DCF intensity using flow cytometry. *$p<0.05$ by the Student t test ($n = 3$). (B) Reactive oxygen species levels in human cornea epithelial cells treated without or with $T\beta_4$ or 1 mM N-acetylcysteine (NAC) for 2 h were measured as described above. Reactive oxygen species levels in human cornea epithelial cells treated without or with $T\beta_4$ or NAC for 2 h before being exposed to 200 μM of H_2O_2 for 30 (C) and 90 (D) min were measured. †$p<0.05$ when compared with the control group by ANOVA test ($n = 3$).

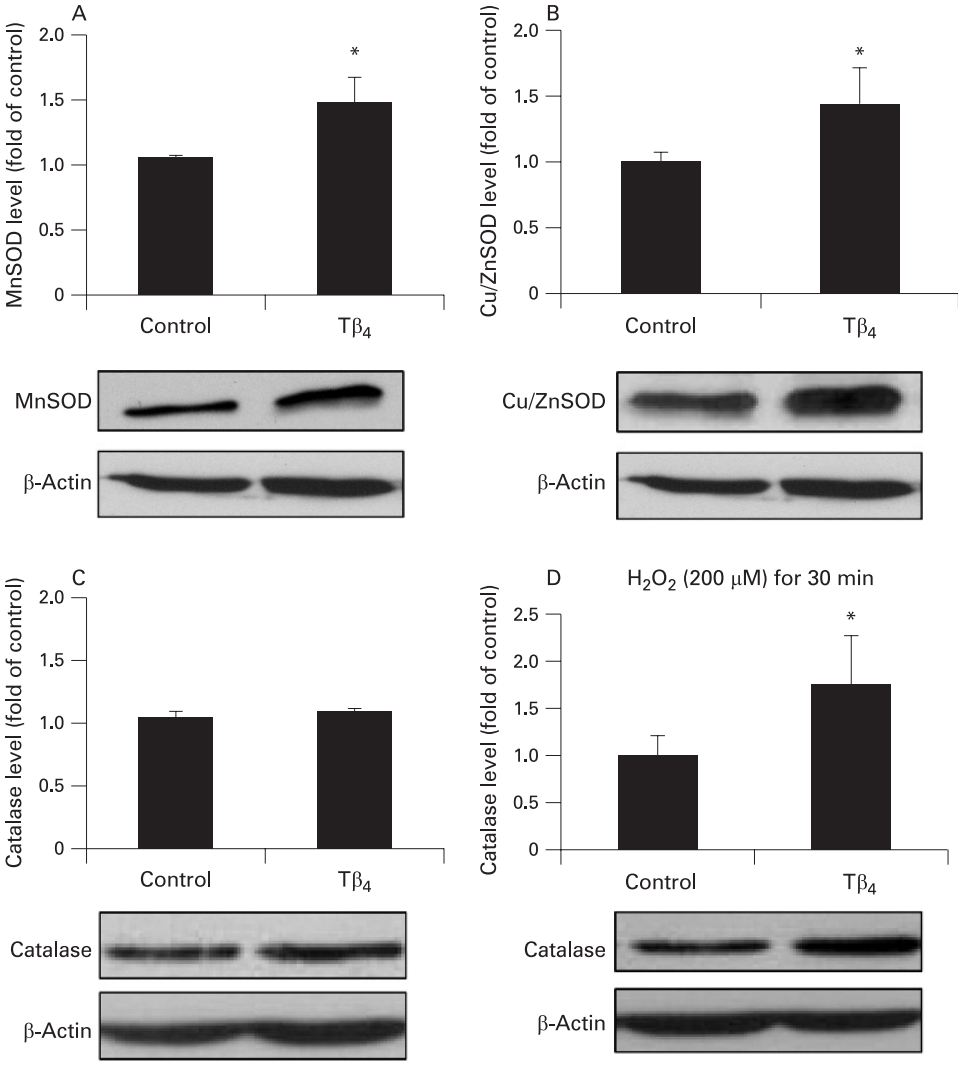

Figure 3 $T\beta_4$ increased protein expression of anti-oxidative enzymes. Protein levels of manganese-superoxide dismutase (MnSOD) (A), copper/zinc-superoxide dismutase (Cu/ZnSOD) (B) and catalase (C) in human cornea epithelial cells incubated without and with 1 μg/ml $T\beta_4$ for 24 h examined by western blot analysis. Catalase levels in human cornea epithelial cells pretreated with $T\beta_4$ for 2 h following 200 μM H_2O_2 for 30 min were also analysed (D). Beta-actin signals were used as loading controls. *$p<0.05$ by Student t test ($n = 3$). ROS, reactive oxygen species.

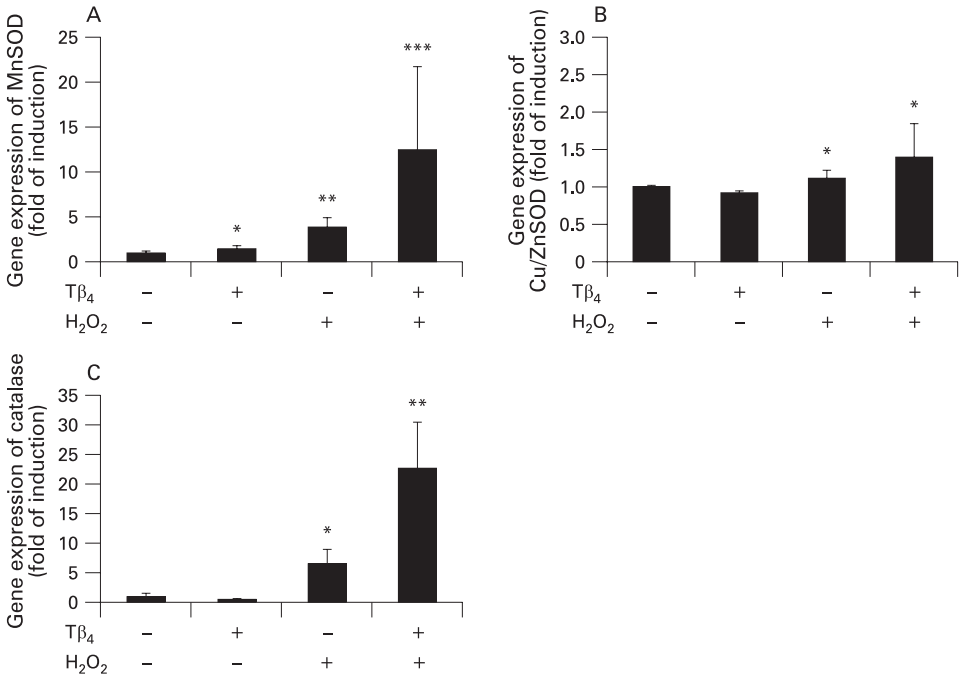

Figure 4 $T\beta_4$ upregulated gene expression of anti-oxidative enzymes. Messenger RNA levels of manganese-superoxide dismutase (MnSOD) (A), copper/zinc-superoxide dismutase (Cu/ZnSOD) (B) and catalase (C) in human cornea epithelial cells, incubated with and without 1 μg/ml $T\beta_4$ added 2 h before a 4 h exposure to 200 μM H_2O_2, examined by real-time RT-PCR analysis. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. Numbers of asterisks represent the levels of significance higher than the control group by ANOVA test (n = 6).

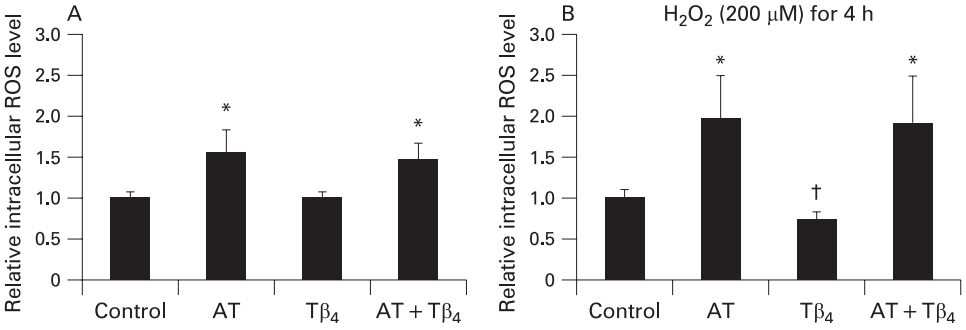

Figure 5 Influence of catalase inhibitor on the reactive oxygen species (ROS)-reducing effects of $T\beta_4$. ROS levels in HCE-T cells treated without or with 1 μg/ml $T\beta_4$, 50 mM 3-amino-1,2,4-triazole (AT), or both for 2 h before (A) and after (B) being exposed to 200 μM of H_2O_2 for 4 h measured by flow cytometry. †Significantly lower and *significantly higher than the control group by ANOVA test (n = 6).

Although the exact mechanism of SOD upregulation by exogenous $T\beta_4$ is currently unknown, transcriptional activation of their corresponding genes is worth further investigation, since nuclear entry of this peptide in HCE-T cells occurs rapidly [9] and a transcription factor activity of $T\beta_4$ has also been postulated.[22] In this study, even though the protein levels of both Mn-SOD and Cu/Zn-SOD were increased by $T\beta_4$, this peptide upregulated the gene encoding the former more efficiently (fig 4A,B). Catalase, which was directly responsible for H_2O_2 clearance,[23] was upregulated by $T\beta_4$ at both the protein level and gene expression in the presence of H_2O_2 (figs 3D, 4C). Also, the anti-ROS capacity of $T\beta_4$ could be abrogated by catalase inhibitor (fig 5B), which demonstrated that the decrease intracellular ROS level by $T\beta_4$ resulted from upregulation of this crucial anti-oxidative enzyme. It has been reported that activator protein 1 (AP-1) stimulated Mn-SOD expression.[24] Moreover, enhancement of AP-1 activity by exogenous $T\beta_4$ has already been demonstrated in endothelial cells.[25] We therefore are in the process of investigating the involvement of AP-1 in Mn-SOD activation in $T\beta_4$-treated HCE-T cells as well as the mechanism underlying $T\beta_4$-enhanced upregulation of catalase by H_2O_2 in these cells.

It has been postulated that exogenous $T\beta_4$ can be used ophthalmologically, as preclinical studies indicated that it promotes corneal wound healing and modulates inflammatory mediators in vivo,[26] inhibits corneal epithelial cell apoptosis after ethanol exposure[8] and inhibits benzalkonium chloride-mediated apoptosis in corneal and conjunctival epithelial cells.[10] By demonstrating, for the first time, that exogenous $T\beta_4$ protects human corneal epithelial cells against intrinsic pathway-mediated apoptosis by reducing intracellular oxidative stress through enhancing the expression of several anti-oxidative enzymes, our results not only offered more mechanistic explanation about the protective mechanism of $T\beta_4$ but also supported further investigation towards the clinical use of this small molecule in treating and preventing oxidative damage of cornea.

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Competing interests: None.

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