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The Protection and Antioxidant Mechanisms of Corticosteroids in LPS-Treated Retinal Pigment Epithelial Cells

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ABSTRACT

Dexamethasone (DEX) and triamcinolone acetonide (TA) are two corticosteroids as anti-inflammatory agents that have been used as an anti-inflammation in several diseases to protect against oxidative damage. The present study examined the anti-oxidant effect of DEX and TA on lipopolysaccharide (LPS)-induced intracellular reactive oxygen species (ROS) in human retinol epithelium ARPE-19 cells. DEX and TA markedly inhibited the LPS-induced intracellular ROS level. DEX and TA also inhibited the expression of NADPH oxidase subunit gp91 and p22. Moreover, the expression of two antioxidant enzymes, heme oxygenase-1 (HO-1) expression and gamma-glutamylcysteine synthetase (gamma-GCS), were increased by DEX and TA treatment in LPS-treated ARPE-19 cells. These results indicate that DEX and TA inhibits the intracellular ROS response by blocking the NADPH oxidase pathway and may increase some antioxidant enzymes in LPS -induced ARPE-19 cells. Therefore, DEX and TA may be useful as antioxidant agents against oxidative damage in anti-inflammatory diseases.

Keywords

Introduction

Inflammation and oxidative stress involve in many diseases including age-related macular degeneration and diabetic retinopathy [1]. The current clinical treatment includes surgery, laser treatment, photodynamic therapy, intraocular injection of anti-angiogenic drugs [2]. The retinal pigment epithelium (RPE) is a monolayer as a selective barrier and maintains the function of the neural retina and photoreceptor (PR) survival by secreting growth factors, cytokines and nutrients to the retina. Retinal pigment epithelium also participates in immune response by releasing pro- and anti-inflammatory cytokines. Inflammation and oxidative stress are vital factors in the pathogenesis of retinal diseases.

Reactive oxygen species (ROS) is produced from mitochondria and causes cell damage, oxidative stress [3,4]. ROS is superoxide, H_2O_2 and hydroxyl radicals, most of which from the electron transport chain in mitochondria play a key role in the regulation of cell death [5-7]. When mitochondrial damage also increases oxidative stress, an excess of ROS generation can cause intracellular glutathione depletion, lipid peroxidation, enzyme activity and DNA damage, and finally result in cell death or plan necrosis [8-13]. NADPH oxidase composes of four cytosolic components (p47phox, p67phox, p40phox, and

G protein) and two transmembrane components (gp91phox and p22phox) [14,15]. Activation of NADPH oxidase stimulated by inflammatory molecules, such as LPS and cytokines, produce ROS and then regulate a series of cell signal transductions [16,17]. The macula has the highest amount of oxygen and fat density in the central portion of the retina, and it will bait conducts the most effective pro-oxidant effect after receiving light.

Dexamethasone (DEX) is widely used in the field of ophthalmic corticosteroids for the treatment of anterior chamber of infection, uveitis, and macular edema [18]. DEX suppresses the microglia from producing reactive oxygen species by inhibiting the activation of the MAPK pathway [19]. In addition, DEX inhibited IL6 and VEGF-A in the corneal angiogenesis and reducd the occurrence of corneal neovascularization [20]. Triamcinolone acetonide (TA) is a corticosteroid (adrenal corticosteroid) that aids in the inhibition of inflammatory ocular diseases. Recently, TA has been used in medicine treatments for several illnesses, such as diabetic retinopathy with macular edema, retinal vein occlusion, proliferative vitreoretinopathy, and exudate age-related macular retinopathy [21-23]. Oxidative stress-induced tight junction disruption can be prevented by TA treatment in retinal pigment epithelial cells [24].

Steroid agents have anti-inflammation and antioxidant activities. However, the antioxidant mechanisms of dexamethasone (DEX) and triamcinolone acetonide (TA) on human retinol epithelium ARPE-19 cells remain unclear. Clarification of the mechanism of steroids on inhibiting the intraocular generation of reactive oxygen species (ROS) is an urgent task. In this study, we used LPS (lipopolysaccharide) to induce human retinal epithelial cells (ARPE-19 cell) for ROS production and to gain more knowledge of the oxidative mechanism of DEX and TA in human retinal pigment epithelium ARPE-19 cells.

Experimental
Cell Lines and Reagents
ARPE-19 cells were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Dulbecco's modified Eagle's medium (DMEM) was obtained from Hyclone. Ham's F-12 medium (F12) and fetal bovine serum (FBS) was obtained from GIBCO. Primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). DEX and TA were respectively purchased from *Tai Yu Chemical & Pharmaceutic*al Co., Ltd (Hsinchu, Taiwan) and Taiwan Biotech Co., LItd (Taoyuan, Taiwan). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell Culture and Treatment
ARPE-19 cells were cultured in DMEM/F12 medium containing 100 units/ml penicillin G, 100 µg/ml streptomycin and 10% FBS. Cells were maintained at 37°C in 5% CO_2 humidified air. Culture medium was replaced with fresh culture medium every two days. LPS (10 µg/ml) was dissolved in the cultured medium for 2 hours then various concentrations (µg/ml) of dexamethasone (DEX)

and triamcinolone acetonide (TA) were added to the cultured medium for the designated time points. After treatment, cells were then accordingly added with various probes or extracted from the total proteins for the measurement of different cellular parameters.

Measurement of Intracellular ROS by Flow Cytometry
The level of intracellular ROS was evaluated with flow cytometry using DCFH-DA. After drug treatment, the cells were washed twice with PBS, treated with 20 µM DCFH-DA for 30 min in the dark, washed again with PBS, centrifuged for collection, and then suspended in PBS. The intracellular ROS levels, which were indicated by the fluorescence of dichlorofluorescein (DCF) by excitation at 488 nm and measured by a 530/22-nm barrier filter, was evaluated using a Becton-Dickinson FACSan flow cytometer.

Western Blotting Analysis
After drug treatment, cells were washed with PBS. To obtain total extracted proteins (supernatant), cells were resuspended in a protein extraction buffer for 10 min, and centrifuged at 12,000 g for 10 min at 4°C. A protein assay reagent was employed for measuring protein concentrations (Bio-Rad, Richmond, CA, USA). The extracted cellular proteins were heated in loading buffer, and an aliquot corresponding to 50–100 µg of protein was divided on a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were electrotransferred onto a polyvinylidene fluoride transfer membrane. After blotting, the membranes were immersed with various primary antibodies overnight and then washed with PBST solution (0.05% Tween 20 in PBS). Following washing, the secondary antibodies labeled with horseradish peroxidase were added to the membrane for 1 h, and then washed with PBST solution (0.05% Tween 20 in PBS). The antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a chemiluminescence analyzer.

Statistical Analysis
Data are presented as the mean (SD) of at least 3 independent experiments and were analyzed using Student's *t*-test. A *P* value <0.05 was considered statistically significant.

Results
Evaluation of ROS induced by LPS in ARPE-19 cells
To evaluate a suitable concentration of lipopolysaccharide (LPS) to induce the production of ROS in ARPE-19 cells, cells were treated with 1, 5, 10, 20, 30 µg/ml LPS for 24 h and then stained with DCFH-DA probe for 30 min. The intracellular ROS was evaluated with flow cytometry. A significant increase in intracellular ROS of ARPE-19 cells was observed when LPS was over 10 µg/ml in concentration (Figure 1). However, 1 and 5 µg/ml LPS did not stimulate ARPE-19 cells to produce intracellular ROS. These results indicate that concentrations over 10 µg/ml LPS were able to induce the production of reactive oxygen species in ARPE-19 cells.
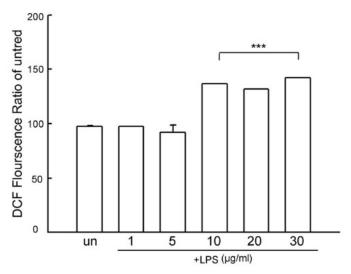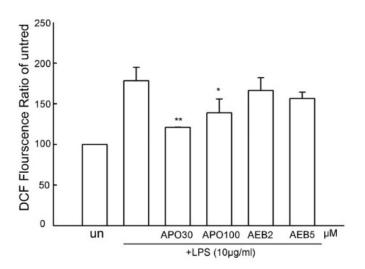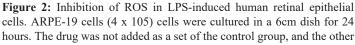
Figure 1: Evaluation of ROS in LPS-induced human retinal epithelial cells. ARPE-19 cells (4 x 105) cells were cultured in a 6cm dish for 24 hours. The drug was not added as a set of the control group, and the other group was treated separately LPS (1, 5, 10, 20, 30 µg/ml) for 24 hours. The intracellular ROS content was measured by DCF analysis. The DCF fluorescence intensity of the untreated control group was set to 100% performance, the fluorescent intensity setting of the other cells in the experimental group and the control group is a table representing the percentage.

The ROS sources of LPS treatment from NADPH oxidase in ARPE-19 cells

To confirm the ROS sources of LPS-treated from NADPH oxidase ARPE-19 cells, two concentrations of apocynin (APO, 30 and 100 µM) and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 2 and 5 µM) were pre-incubated 30 min to the cells, then co-cultured with LPS for 24 h. The LPS-induced intracellular ROS production, which was evaluated using the DCFH-DA probe and flow cytometry, was significantly decreased when cells were pre-incubated with APO, but AEB did not scavenge the LPS-induced intracellular ROS production (Figure 2).

Figure 2: Inhibition of ROS in LPS-induced human retinal epithelial cells. ARPE-19 cells (4 x 105) cells were cultured in a 6cm dish for 24 hours. The drug was not added as a set of the control group, and the other

group was treated separately with 10 µg/ml LPS for 24 hours and the addition of various concentrations of iNOX inhibitor (APO 30, 100 µM, AEB 2,5 µM) for 2 hours prior to LPS. The intracellular ROS content was measured using a DCF analysis. The DCF fluorescence intensity of the untreated control group was set to 100% performance, the fluorescent intensity setting of the other cells in the experimental group and the control group is a table representing the percentage.

LPS-induced ROS inhibited by DEX and TA in ARPE-19 cells

We measured whether the DEX and TA could suppress ROS using cytometry in LPS-treated ARPE-19 cells. As shown in Figure 3A, there was a dose-dependent inhibition of LPS-induced ROS by DEX. It is worth noting that 100 µg/ml DEX had the same effect of ROS suppression compared with the untreated group. In contrast, lower concentrations of TA could not significantly decrease the LPS-induced ROS generation in ARPE-19 cells, and only 200 µg/ml TA had a slight inhibitory effect (Figure 3B).

DEX and TA inhibits NADPH oxidase expression in LPS-treated ARPE-19 cells

We found that gp91 and p22, both major catalytic and regulatory components of NADPH oxidase, generated ROS. The inhibition of ROS production by DEX and TA may be related to NADPH oxidase; the gp91 and p22 expression during LPS treatment of ARPE-19 cells were evaluated with western blotting. Figure 4A shows that the gp91 expression was increased after LPS treatment. The cotreatment LPS with DEX or TA inhibit the gp91 expression in a dose-dependent manner: There was no notable repression to p22 protein by DEX or TA (Figure 4B). We confirmed that DEX had a better inhibitory function than TA via suppressing the gp91 protein to down-regulate NADPH oxidase activity during LPS treatment.

DEX and TA up-regulated antioxidative enzymes expression in LPS-treated ARPE-19 cells

As ROS scavengers, γ-GCS and HO-1 are two important antioxidative enzymes protecting cells from damage. We examined whether DEX or TA up-regulated the expression of γ-GCS and HO-1 to increase antioxidant activity in LPS-treated ARPE-19 cells. As shown in Figure 5A, we demonstrated that treatment with either DEX or TA up-regulated expression of γ-GCS in comparison to the LPS treatment group but did not markedly increase HO-1 expression (Figure 5B). These results suggest the involvement of γ-GCS up-regulation in LPS-induced ROS generation in ARPE-19 cells.

Discussion

Macular degeneration is becoming the major cause of blindness. Retina exposed ROS for long-term resulting in oxidative damage [17,25]. Previous study reported that LPS induced oxidative stress and cellular adhesion molecules [16]. Another study indicated that over 10µg/ml LPS stimulation can effectively cause mitochondrial damage in ARPE-19 cells and ultimately result in more cell death [26]. We observed that LPS (10 µg/ml) significantly increased ROS production compared to untreated cells. Low level of inflammation and ROS may result in tissue damage.
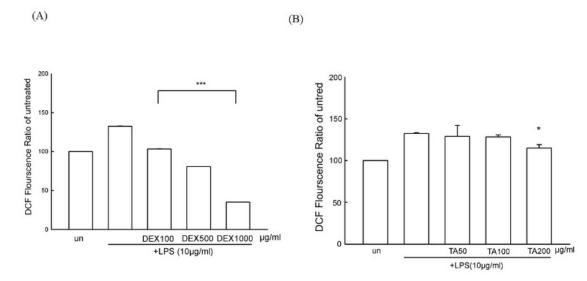
Figure 3: Evaluation of Dexamethasone (DEX) and Triamcinolone (TA) on anti-LPS-induced human retinal epithelial cells ROS. ARPE-19 cells (4 x 105) cells were cultured in a 6 cm dish for 24 hours. The drug was not added as a set of the control group, and one group was only treated with 10 μg/ml LPS as a positive control group. The other three groups were treated separately with LPS (10 μg/ml) for two hours prior and then different concentrations of (A) Dexamethasone (DEX) and (B) Triamcinolone (TA) were added for 24 hours. The intracellular ROS content was measured by DCF analysis. The DCF fluorescence intensity of the untreated control group was set to 100% performance, and the fluorescent intensity setting of the other cells in the experimental group and the control group is a table representing the percentage.
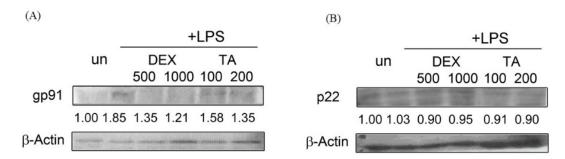

Figure 4: Expression of gp91 and p22 in Dexamethasone (DEX) and Triamcinolone (TA) treatment in LPS-induced human retinal epithelial cells. ARPE-19 cells (4 x 105) cells were cultured in a 6 cm dish for 24 hours. The drug was not added as a set of the control group, and one group was only treated with 10 μg/ml LPS as a positive control group. The other three groups were treated separately with LPS (10 μg/ml) for two hours prior and then different concentrations of Dexamethasone (DEX) and Triamcinolone (TA) were added for 24 hours. The expressions of gp91 and P22 were estimated using Western blot analysis. Visualization of the actin band was used as the control for normalization.
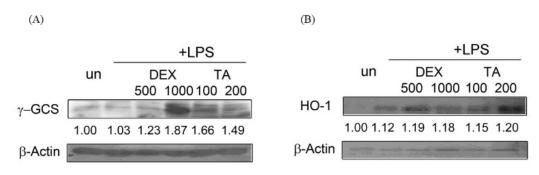

Figure 5: Assessing Dexamethasone (DEX) and Triamcinolone (TA) on anti-LPS-induced human retinal epithelial cells ROS. ARPE-19 cells (4 x 105) cells were cultured in a 6 cm dish for 24 hours. The drug was not added as a set of the control group, and one group was only treated with 10 μg/ml LPS as a positive control group. The other three groups were treated separately with LPS (10μg/ml) for two hours prior and then different concentrations of Dexamethasone (DEX) and Triamcinolone (TA) were added for 24 hours. The expression ofγ-GCS and HO-1 were estimated using Western blot analysis. Visualization of the actin band was used as the control for normalization.

LPS increased NADPH oxidase activity and generation of O_2^- and H_2O_2 in a murine model [16]. These ROS were generated by NADPH oxidase as signaling molecules to mediate biological responses, initiate angiogenesis, and they additionally play a critical role in intracellular signaling during angiogenesis. We demonstrated that apocynin (APO) inhibited ROS production, but this was not observed in the 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF) group (Figure 2). The different inhibitory effect of APO and AEB may be because they target different active sites of NADPH oxidase [27].

DEX and TA are two very common drugs often used to treat AMD, and they are typical corticosteroids that have anti-inflammatory, anti-angiogenic, and anti-edematous properties [28]. Prior research reported that DEX inhibits iNOS expression and NO production in LPS-induced macrophages [29]. TA prevents oxidative stress-induced tight junction disruption of retinal pigment epithelial cells [24]. The inhibition of LPS-induced ROS production by DEX in a dose-dependent manner.

gp91 is also known as Nox-2, a subunit of NADPH oxidases, for generating ROS induction [30]. Stabilizing p22 (also a subunit of NADPH) regulates NADPH oxidase activities. In the present study, an abundant expression of gp91 was found in LPS-induced ARPE-19 cells, indicating that the main source of large amounts of ROS production derives from the expression of gp91, instead of p22 in ARPE-19 cells. In agreement with our findings, Huo et al. also demonstrated that a significant inhibition of Nox-dependent ROS production was achieved via the suppression of MKP-1-dependent MAPK pathways in activated microglia [19]. MAPK participates in the signaling pathway of NADPH oxidase activation (superoxide production) and phagocytosis [31]. These results clearly show that treatment of DEX suppressed the LPS-induced gp91 expression compared to the untreated cells.

Our current results demonstrate that inhibition of LPS-induced ROS was related to the expression of γ-GCS in DEX and TA treatments. The γ-GCS is a rate-limiting enzyme of GSH that is an important antioxidant to scavenge ROS. The expression of γ-GCS would maintain the intracellular GSH level to inhibit the LPS-induced ROS in DEX and TA treatment.

In the present study, we obtained valuable information in our gross examination with five major findings: 1.) LPS dose-dependently stimulated ARPE-19 cells to generate ROS; 2.) LPS induced ROS in ARPE-19 cells via active NADPH oxidase; 3.) NADPH oxidase inhibitor (APO) decreased ROS in LPS-induced ARPE-19 cells; 4.) DEX and TA inhibited the ROS production by suppressing the gp91 and p22 subunits of NOX; and 5.) DEX and TA increased the expression of γ-GCS and HO-1 to inhibit ROS production. This study suggests that DEX and TA may provide potential antioxidant activity against oxidative damage in anti-inflammatory diseases (Figure 6).
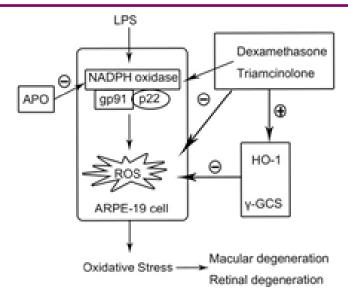

Figure 6: The pathways of LPS-induced ROS and antioxidant of DEX and TA in ARPE-19 cells.

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