Original Article

Vincamine prevents lipopolysaccharide induced inflammation and oxidative stress via thioredoxin reductase activation in human corneal epithelial cells

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Abstract: Lipopolysaccharide (LPS) induced keratitis is a progressive infectious ocular disease in which innate inflammatory responses often cause clinical tissue damage and vision loss. In this study, the potential protective effects of vincamine, a plant alkaloid used clinically as a peripheral vasodilator, against LPS induced inflammation and oxidative stress were investigated on human corneal epithelial cells (HCECs). HCECs were treated with LPS and vincamine at various concentrations. Cell viability, reactive oxygen species (ROS) levels, and the gene expression levels of interleukin-6 (IL-6), IL-8, IL-1β, TNF-α, transforming growth factor-β (TGF-β) in HCECs, were assessed. The antioxidant potential of vincamine was evaluated by measuring the levels of malondialdehyde (MDA), total antioxidant capacity (T-AOC), and superoxide dismutase (SOD). The effects of vincamine on intracellular activities of thioredoxin reductase (TrxR) as well as other anti-oxidant proteins were also investigated in LPS treated HCECs. The results showed that vincamine protected HCECs from LPS induced cell viability reduction and ameliorated the inflammation. Vincamine exhibited a strong antioxidant activity, decreasing ROS levels and regulating the levels of SOD, T-AOC and MDA. Vincamine also exerted anti-inflammatory activities by decreasing IL-6, IL-8, IL-1β, TNF-α, TGF-β expression. Intracellular TrxR activity was significantly activated by vincamine. These findings suggest that vincamine exerts positive effects against LPS induced oxidative stress and inflammation and may be useful in protecting corneal epithelial cells from LPS induced keratitis.

Keywords: Vincamine, lipopolysaccharide, human corneal epithelial cells, oxidative stress, inflammation, thioredoxin reductase

Introduction

Microbial keratitis is a common ocular infection caused by bacteria, fungi, viruses or parasites and is the second most significant cause of monocular blindness, particularly in certain developing countries and, generally, in the tropics [1]. Clinically, this infection requires aggressive antimicrobial management to eliminate the causative organisms, suppress destructive reactions, and restore normal ocular structure and vision [2, 3]. However, despite timely and correct therapeutic strategies, infective keratitis remains clinically challenging, in which approximately 50% of eyes have poor visual outcomes [4, 5], because conventional therapies, such as anti-biotic treatment, often fail to control the tissue damage caused by excessive local inflammation, even if viable bacteria are cleared from the cornea [6]. Hence, in addition to antibiotic treatment, it is also important to develop new therapeutic modalities to control the inflammatory response in microbial keratitis. Lipopolysaccharide (LPS) is one of the most common causes of microbial keratitis to eyes [7]. As a well-characterized pathogen-associated molecular pattern found in the outer leaflet of the outer membrane of the bacteria, LPS induced keratitis is a rapidly progressive infectious ocular disease [8]. A number of key factors were found in the pathogenesis of LPS induced injury including inflammation and oxidative stress. Previous studies have shown that inflammatory cells were recruited to the cornea to produce various pro-inflammatory cytokines (e.g., IL-6 and IL-1β) and modulate anti-inflam-
Vincamine prevents inflammation via activating thioredoxin reductase

Cytosolic thioredoxin (Trx), thioredoxin reductase (TrxR) and nicotinamide adenine dinucleotide phosphate (NADPH) comprise the mammalian Trx system, which plays powerful roles in defense mechanism against oxidative stress, nitrosative stress and in redox regulation [17, 18]. Trx is a small redox-active protein that is ubiquitously present in mammalian and is one of the defense proteins induced in response to various oxidative stress conditions [19, 20]. The reduction of oxidized Trx by NADPH is catalyzed by seleno protein TrxR. TrxR may catalyze the NADPH-dependent reduction of H₂O₂, lipid hydroperoxides and dehydroascorbate as well [21]. In addition to its potent anti-oxidative effect, Trx system also has anti-inflammatory properties, mainly because of its ability to inhibit neutrophil chemotaxis to inflammatory sites and to suppress the expression and activation of the macrophage migration-inhibitory factors [22, 23]. Because of its desirable anti-oxidative and anti-inflammatory properties, Trx system could be a new and potentially effective therapeutic target for anti-inflammation and anti-oxidant.

Herbal medicines have been proven to be a major source of novel agents with various pharmaceutical activities [24-28]. Vincamine, the chemical structure of which is given in Figure 1, is an indole alkaloid of clinical use against the brain sclerosis, as well as in post-operative states of the central nervous system [29]. Vincamine seems to act as an oxygen vector in living cells. Also, it has been proposed for the treatment of drepanocytosis (sicklemia) [30]. In addition, vincamine possesses a selective vasoregulator action on the microcapillary circulation, especially in the brain. Vincamine is a peripheral vasodilator that increased cerebral blood flow and used as a nootropic agent to combat the effect of aging [31]. Vincamine has been shown to be a cerebral metabolic enhancer through its effect on ATP production, efficient utilization of glucose and oxygen, while at the same time providing increased protection against ischemia and hypoxia [32]. Vincamine enhanced dopaminergic, serotonergic, and noradrenergic functions probably through its anti-oxidant capacity, comparably to vitamin E [33-35].

In the present study, the effects of vincamine on HCECs were investigated. Here we showed that vincamine presented potent protective effects against LPS induced oxidative damage and inflammation in HCECs. The underlying regulatory mechanisms associated with the potential anti-inflammatory and anti-oxidant effects of vincamine were also investigated.

Materials and methods

Reagents

Vincamine, *Pseudomonas aeruginosa* LPS, glutamine, fetal bovine serum (FBS), trypsin, 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA). Dulbecco’s Modified Eagle’s medium (DMEM) was obtained from Gibco BRL (Grand Island,
Vincamine prevents inflammation via activating thioredoxin reductase

NY, USA). Fetal calf serum (FCS) and RPMI 1640 medium were purchased from HyClone (USA). The antibodies to TrxR, Trx, GR, GPx and GAPDH were purchased from Cell Signaling Technology (USA). Other routine laboratory reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of vincamine

Vincamine was dissolved in sterile PBS to a stock concentration of 0.1 M, and stored at 4°C in the dark to be used within 2 days after preparation.

Cell line and cultures

HECEs were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 2 mM glutamine, 10% fetal bovine serum, and antibiotics, incubated in 5% CO₂ incubator at 37°C. Cells were passaged at a 1:3 ratio with trypsin, every 5 to 7 days. In indicated experiments, cells were seeded into 6-well plates at a density of 2 × 10³ cells per well. After incubation overnight, cells were treated as indicated concentration of LPS or vincamine and assessed by CCK-8 assay at 6 and 24 h respectively. 10 μL of CCK-8 reagent was added to each well and incubated for 1 h. The difference in absorbance between 450 and 630 nm was measured by a microplate reader (BioTek, Winooski, VT, USA) as an indicator of cell viability. Independent experiments were done in triplicate. IC₅₀ values were calculated as the concentration of compound that inhibited the viability of cells by 50% as compared with control cells grown in the absence of LPS or vincamine.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequence</th>
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<tr>
<td>IL-6</td>
<td>F: 5'-TGGCTGAAAAGATGGATGCT-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TCTGCACAGCTGCTGCT-3'</td>
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<td>IL-8</td>
<td>F: 5'-TTGCGACGCTCTGTTTTTCCC-3'</td>
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<tr>
<td></td>
<td>R: 5'-TTGCTCCTGCTCTCACCACACCTCA-3'</td>
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<tr>
<td>IL-1β</td>
<td>F: 5'-CCCTGGCTGGTGGTGAAGAGA-3'</td>
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<tr>
<td></td>
<td>R: 5'-GGGAACCTGGCGACTCAA-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: 5'-TGGACGCTGTTGAGTGGATAAGGT-3'</td>
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<tr>
<td></td>
<td>R: 5'-GAGGACCTGGGAGTGATGAGGT-3'</td>
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<tr>
<td>TGF-β</td>
<td>F: 5'-CGCCAGAGTCGTTACCTTTTGA-3'</td>
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<td></td>
<td>R: 5'-CGGATGGAACCGTTGATG-3'</td>
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<tr>
<td>β-actin</td>
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<tr>
<td></td>
<td>R: 5'-CCCTTTAGGATGCGAAGGG-3'</td>
</tr>
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RNA extraction and quantitative real-time PCR

To investigate the change of the mRNA expression of inflammatory factors including interleukin-6 (IL-6), IL-8, IL-1β, TNF-α, transforming growth factor-β (TGF-β) in HECEs, RT-PCR analyses were performed. Total RNA was extracted from cells using an MN-total RNA isolation kit (Machereynagel, Germany) according to the manufacturer’s instructions. First-strand cDNA were synthesized using the transcriptor first strand cDNA synthesis kit (Roche, Switzerland). The primers used to amplify IL-6, IL-8, IL-1β, TNF-α, TGF-β and β-actin are described in Table 1. Real-time studies were carried out using the SYBR Premix Ex TaqTM kit (TaKaRa, Japan) with β-actin as the reference gene.

Measurement of oxidative stress

Oxidative stress was assessed by measuring malondialdehyde (MDA), total antioxidant capacity (T-AOC), and superoxide dismutase (SOD) levels. HECEs cultured in six-well plates (4 × 10³ cells/well) for 24 h were treated with vari-
Vincamine prevents inflammation via activating thioredoxin reductase

Figure 2. Vincamine increased the viability of LPS treated HCECs. A. Effect of LPS on cell viability by CCK-8 assay in HCECs. Viability of untreated control cells was 100% and it decreased with increase in LPS concentration administered for 24 h. The results are expressed as mean ± SD (n = 6). *P < 0.05, **P < 0.01 as compared with the control cells. B. Effect of vincamine on cell viability by CCK-8 assay in LPS treated HCECs at 24 h. Viability of HCECs was increased after vincamine administration in a dose-dependent manner. The results are expressed as mean ± SD (n = 6). #P < 0.05, *P < 0.05, **P < 0.01 as compared with the control cells. **P < 0.01 as compared with the LPS treated cells.

Figure 3. Effects of vincamine on the production of ROS, SOD, MDA, and T-AOC in LPS treated HCECs. LPS treatment significantly increased the values of the intracellular ROS and MDA levels in the HCECs. The T-AOC and SOD levels were decreased significantly. However, vincamine administration reduced the values of the intracellular ROS and MDA, while increased the levels of T-AOC, and SOD in a dose dependent manner. All data were shown as mean ± SD of eight rats. #P < 0.05 vs. control group, *P < 0.05 vs. LPS treated group.

Western blot analysis

After treatments, HCECs were harvested, and homogenized in 200 μL RIPA lysis buffer. Then were extracted and the protein concentration was determined by Lowry method. Protein lysates (40 μg) from each sample were subjected to SDS-PAGE on 10% acrylamide gel and the separated proteins transferred to a PVDF membrane. After transfer, the membranes were blocked with 5% non-fat dry milk in TBS for 1 h at room temperature, then the membranes were incubated with primary antibodies to TrxR, Trx, GR, GPx, and GAPDH overnight at 4°C followed by secondary horseradish peroxidase-labeled antibody (1:2000). The bound antibodies were visualized using the ECL blotting detection system. The relative expression of proteins was quantified densitometrically with the software ImageJ and calculated according to the reference bands of GAPDH.
Vincamine prevents inflammation via activating thioredoxin reductase

**Determination of TrxR and Trx activity in cell lysates by insulin reduction assay**

Freshly collected cell lysates were used to determine cellular Trx and TrxR activities as described previously [38]. Briefly, to measure TrxR activity, in each well of a 96-well plate, 25 μg of cell lysate was incubated in a final volume of 50 μL containing 85 mM Hepes (pH 7.6), 0.3 mM insulin, 10 μM Trx, 2.5 mM EDTA and 660 μM NADPH for 40 min at 37°C. 200 μL of 1 mM DTNB in 6 M guanidine-HCl, 200 mM Tris-HCl pH 8.0 solution was added to quench the reaction. The amount of free thiols generated from insulin reduction was determined by DTNB reduction at 412 nm using the VersaMax microplate reader. To measure Trx activity, procedures were carried out similarly to those for determining cellular TrxR activity, except that the cell lysates were incubated with TrxR in place of Trx. Controls containing lysates and all reaction reagents except TrxR for each lysate sample were also set up. For each sample, Trx or TrxR activity was calculated as the absorbance at 412 nm subtracted from that of the corresponding control and expressed as a percentage of the activity measured in DMSO-treated cells.

**Determination of GPx activity in cell lysates by GPx activity assays**

For determination of cellular GPx activity, vincamine (20, 40, 80 μM) was incubated with lysates of HCECs (25 μg protein), 20 nM GR, 1 mM GSH and 200 μM NADPH in a volume of 100 μL phosphate buffer (0.1 M sodium phosphate, 2 mM EDTA pH 7.5) for 1 h at room temperature. H₂O₂ solution in phosphate buffer was added to initiate the reaction (final concentration 1.5 mM). NADPH consumption was monitored at 340 nm using the VersaMax microplate reader. The results were calculated based on change in absorbance in the initial 3 min and presented as a percentage of GPx activity of drug-treated sample over that of DMSO-treated sample.

**Determination of GR activity in cell lysates by glutathione reduction assay**

To determine cellular GR activity, 25 μg of cell lysate was mixed with a solution of GSSG and NADPH in phosphate buffer to a final volume of 200 μL (final GSSG and NADPH concentrations 1 mM and 200 μM respectively). The enzyme activity was determined by measuring the decrease in absorbance at 340 nm for 10 min at 37°C and expressed as a percentage of the enzyme activity of that of the DMSO-treated sample.

**Statistical analysis**

The data were expressed as means ± standard deviation (SD). T-test was used to compare the difference between the two groups. Statistical analyses between three or more groups were analyzed by one-way analysis of variance (ANOVA) by using SPSS software version 16.0 (IBM, Armonk, NY). Values for *P* < 0.05 were considered statistically significant.

**Results**

**Effect of the vincamine on cell viability in LPS treated HCECs**

Figure 2A shows the relationship between LPS concentration and percentage of cell survival relative to the control. Exposure of HCECs to various concentrations of LPS resulted in a concentration-dependent decrease in cell viability. Specifically, after a 24 h exposure with 10 μg/mL LPS, about 52.2% of the viability was remained, while in the cells exposed to 20, 50 and 100 μg/mL LPS, the relative viability was 32.3, 20.1 and 14.6%, respectively (Figure 2A). The results in Figure 2B show that vincamine (20, 40 and 80 μM) administration exerted a significant, concentration-dependent protective effect.

**Vincamine reduced ROS levels in LPS treated HCECs**

ROS levels were significantly increased in LPS treated HCECs (Figure 3). Vincamine significantly reduced ROS level in a dose-dependent manner (Figure 3). ROS levels in the vincamine 40 and 80 μM groups were significantly lower than the level in the model control group (*P* < 0.01).

**Vincamine attenuated the oxidative stress in LPS treated HCECs**

As shown in Figure 3, compared to untreated control HCECs, the intracellular MDA levels were significantly elevated in LPS treated cells,
Vincamine prevents inflammation via activating thioredoxin reductase

while T-AOC, and SOD levels were decreased significantly. In contrast, after vincamine administration, the levels of MDA and were significantly reduced while the levels of T-AOC, and SOD were increased in a dose-dependent manner.

**Effects of vincamine on the expressions of IL-6, IL-8, IL-1β, TNF-α and TGF-β mRNA in LPS treated HCECs**

As shown in Figure 4, the RT-PCR results revealed that treatment with LPS could significantly increase the mRNA expression of inflammatory factors IL-6, IL-8, IL-1β, TNF-α and TGF-β. Vincamine treatment in HCECs elicited a significant reduction of IL-6, IL-8, IL-1β, TNF-α and TGF-β, compared to LPS treated cells.

**Vincamine activated the activity of TrxR in HCECs**

The effect of vincamine on Trx, TrxR, GR and GPx in LPS treated HCECs was evaluated. After 30 min of incubation in the presence of NADPH, the activation of mammalian TrxR by LPS, LPS + vincamine was evaluated in the DTNB reduction assay. As shown in Figure 5, LPS significantly inhibited the intracel-
Vincamine prevents inflammation via activating thioredoxin reductase

The cellular activity of TrxR, while vincamine rescued TrxR activity in a dose-dependent manner. However, the intracellular activities of Trx, GR, and GPx were neither inhibited nor activated by both LPS and vincamine. These results suggested that TrxR activation could potentially serve as an underlying mechanism for at least part of the antioxidant effects of vincamine.

**The effect of vincamine on the expression levels of Trx, TrxR, GR and GPx in HCECs**

The effect of vincamine on the expression levels of Trx, TrxR, GR, and GPx in LPS and vincamine treated HCECs was evaluated. Western blotting analysis revealed that the expression levels of Trx, TrxR, GR, and GPx were not affected either by LPS or by vincamine administration, as shown in Figure 6. These results suggested that the activation of TrxR activity is not related to the expression changes of this enzyme.

**Discussion**

Eye infection is one of the major causes of visual impairment and blindness. Well-conserved structural motifs of different microorganisms including LPS of the gram-negative bacteria can mediate innate immune responses leading to either activation or suppression of inflammatory processes and eventually cell death. LPS administration increased the number of apoptotic cells in corneal injury models and induced the expression of autophagic related genes. LPS, through its receptor, toll-like receptor 4 (TLR4), can induce cell migration and proliferation.

Dietary phytochemicals consist of a wide variety of biologically active compounds that are ubiquitous in plants, many of which have been reported to have pharmaceutical properties. Epidemiological studies have shown that natural components may play an important role in preventing human diseases [39, 40]. Among them, vincamine, which is abundant in *Vinca minor L.*, has been reported to have therapeutic potential for treating many human diseases [41, 42]. Vincamine is employed from 1970s in the therapy of cerebral metabolic and circulatory disorders, since it combines cerebrovascular and hemodynamic properties [29, 30]. However, no anti-oxidative and anti-inflammatory effects of vincamine on LPS treated cells were reported so far.

In the present work, we found that vincamine could protect HCECs from LPS induced injury. LPS decreased the viability of HCECs significantly, while vincamine treatment increased HCECs viability in a dose-dependent manner. Following LPS administration, the mRNA expression levels of IL-6, IL-8, IL-1β, TNF-α and
Vincamine prevents inflammation via activating thioredoxin reductase

TGF-β in HCECs were significantly raised, which represented a stimulation of inflammatory responses [43]. Reduction of these inflammatory factors in HCECs by vincamine was also detected in a dose-dependent manner.

Accumulating evidence has indicated that oxidative stress is a potential cause of corneal inflammation, and there is an imbalance between the generation of ROS and the capacity to detoxify these intermediates [44]. In addition to looking at indicators of oxidative damage, we also examined the concentrations of several important antioxidants ROS, SOD, T-AOC and MDA levels in HCECs. SOD is an important member of the antioxidant enzymatic defense system and the level of T-AOC reflects the overall cellular endogenous antioxidative capability [45]. And MDA level reflects the degree of organic lipid peroxidation, which indicated the severity of damage to cell membranes [46]. It was shown that in the LPS treated cells, MDA and ROS levels were substantially increased while SOD and T-AOC levels were reduced. In contrast, vincamine remarkably decreased the levels of MDA and ROS, and simultaneously enhanced the levels of SOD and T-AOC. Those results suggested that vincamine could significantly suppress LPS-induced inflammation and oxidative stress, which might explain the protective mechanisms against microbial keratitis induced by LPS.

Trx system is a very important anti-oxidative system which has been reported many effects, such as regulating cellular reduction/oxidation (redox) status and cell proliferation/cell survival processes [47]. Trx system has also been reported to regulate the pathologic processes of several kinds of tumors [17], as well involved in cardiovascular disease, heart failure, stroke, inflammation, metabolic syndrome, and other diseases [48]. However, Trx's function in microbial keratitis has not been thoroughly investigated. In the present study, we found that vincamine activated the activity of TrxR without affecting the activities of GR, Trx, and GPx. Interestingly, the expression levels of all these proteins were not affected significantly by both LPS and vincamine. These results indicated that the intracellular TrxR activity was specifically activated by vincamine administration. Its selectivity towards cellular TrxR activation over related antioxidant enzymes GR, Trx and GPx suggested that vincamine showed anti-oxidant activity via targeting TrxR. TrxR activation mediated by vincamine led to cellular Trx reduction, decreased oxidative stress and inhibition of inflammation.

Conclusions

In summary, our study provided direct evidence to prove that LPS induced inflammation and oxidative stress in HCECs. Both inflammatory mediators’ production and oxidative stress could be significantly inhibited by vincamine, through an activation of TrxR activity. Further investigation should be carried out to identify the accurate mechanism of vincamine's impact on Trx system in microbial keratitis.

Disclosure of conflict of interest

None.

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Vincamine prevents inflammation via activating thioredoxin reductase


Vincamine prevents inflammation via activating thioredoxin reductase


