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厦门大学

博 士 学 位 论 文

SERPINA3K 在角膜抗氧化应激与抗新生血管作用及其机制研究

Study on the anti-oxidant and anti-neovascularization effects of SERPINA3K on cornea and its underlying mechanisms

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摘 要

SERPINA3K 是丝氨酸蛋白酶抑制剂家族成员之一。研究表明，SERPINA3K 在眼底具有抗氧化应激、抗炎以及抗新生血管的作用，是一类新型 Wnt 信号通路抑制剂。本课题组前期研究也证实了 SERPINA3K 在碱烧伤诱导的角膜新生血管中具有抗炎和抗新生血管作用，但其详细分子作用机制及其对角膜的其他作用尚未深入探究。本课题主要围绕 SERPINA3K 在角膜的抗氧化应激和抗新生血管作用及其机制进行系列、深入研究。

第一部分研究了 SERPINA3K 在角膜上皮的抗氧化应激作用及其机制。首先建立了 H_2O_2 诱导的人角膜上皮细胞氧化应激模型以及大鼠在体角膜氧化应激模型，通过一系列实验手段验证 SEPRINA3K 的抗氧化应激活性，包括 CCK-8 和流式细胞术检测角膜上皮细胞的活性，TUNEL 检测细胞凋亡情况，ROS 探针和 3-NT 的表达测定氧化产物生成情况。通过酶活性检测、免疫染色、免疫印迹以及实时定量 PCR 方法和技术检测和分析氧化产物生成和降解系统的相关酶和信号通路。

实验结果表明，SERPINA3K 能剂量依赖性和时间依赖性地保护 H_2O_2 诱导的人角膜上皮细胞（HCE）氧化应激损伤。SERPINA3K 显著抑制 H_2O_2 诱导的 HCE 细胞中氧化产物的增加。其机制可能通过抑制氧化产物生成酶，例如 NOX4，并且提升氧化产物降解酶，例如超氧岐化酶 SOD 以及过氧化氢酶 Catalase。除此以外，SERPINA3K 下调 H_2O_2 诱导的 HCE 细胞中 Nrf2-Keap1 信号通路的激活，同时下调 H_2O_2 诱导的下游靶基因如 NQO1 和 GSTP 等的表达增强。同样地，在大鼠在体模型中也观察到类似的现象。

总结第一部分结果，SERPINA3K 在角膜上具有抗氧化应激作用，其机制可能是通过调控氧化应激系统中氧化产物产生和降解途径的关键酶，以及 Nrf2-Keap1 信号通路来实现。

第二部分研究了 SERPINA3K 在角膜的抗新生血管作用及其机制。首先建立缝线诱导的大鼠角膜新生血管模型。建模后按不同分组给药 7 天，每天 3 次，在第 2、5、7 天利用裂隙灯检测新生血管面积以及角膜炎症指数。给药 7 天后处死大鼠并

取眼球进行一系列实验，包括组织切片形态学观察、免疫组织化学染色、蛋白印迹以及实时定量 PCR。体外实验利用原代人脐静脉内皮细胞（HUVEC），给予 Wnt3a 配体诱导 Wnt 信号通路的激活，检测细胞迁移和相关蛋白表达。另外，使用 $VEGF_{165}$ 作为诱导物建立体外血管内皮细胞模型，检测细胞活性、细胞迁移情况以及体外管腔形成情况。

实验结果表明，SERPINA3K 显著抑制缝线诱导的角膜新生血管及角膜炎症。SERPINA3K 调节 Wnt 信号通路重要因子的水平，如下调缝线诱导的 β-catenin、non-pi-β-catenin 以及转录因子 4 (TCF4)的表达，上调 pi-β-catenin 的表达。与此同时，SERPINA3K 抑制 Wnt3a 诱导的 HUVEC 的迁移和 VEGF 的水平。SERPINA3K 也抑制 $VEGF_{165}$ 诱导的 HUVEC 的增殖、迁移以及管腔形成。

综合第二部分实验结果，SERPINA3K 具有抑制缝线诱导的角膜新生血管作用，其机制可能通过抑制 Wnt 信号通路来实现。

综上所述，本研究在原有基础上，对 SERPINA3K 在角膜的抗氧化应激以及抗新生血管作用和分子机制进行了系统、深入研究。研究结果将有助于开拓、研发新型治疗眼表疾病的药物以及深入了解眼表疾病的发病机理。

关键词：SERPINA3K；抗氧化应激；抗新生血管

ABSTRACT

It has been demonstrated that SERPINA3K, a member of the serine proteinase inhibitor (SERPIN) family, has anti-oxidant, anti-inflammation and anti-neovascularization effects in the retina and the mechanisms are associated with the Wnt signaling pathway. Our previous study showed the anti-neovascularizaion and anti-inflammation effects of SERPINA3K on cornea in a rat alkali burn model. However, the underlying mechanism and its other beneficial effects need to be further investigated. The present research focuses on the anti-oxidant and anti-neovascularization effects of SERPINA3K and the underlying mechanisms in cornea.

In the first part of the study, we investigated the antioxidant effects of SERPINA3K in the corneal epithelium and the mechanism underlying its action. We established the oxidative stress models induced by hydrogen peroxide (H_2O_2) in cultured human corneal epithelial (HCE) cells and in rat corneal epithelium in vivo. Cell viability, flow cytometry, and TUNEL analysis were conducted to detect viable cells and cell death; reactive oxygen species (ROS) and 3-Nitrotyrosine fluorescent assay was applied to measure ROS levels. Enzyme activity measurement, immunostaining, Western blot, and quantitative RT-PCR assay were performed to analyze the factors of the ROS generation/degradation system and pathway.

The results demonstrated that SERPINA3K protected the HCE cells from H_2O_2-induced oxidative stress in a dose- and time-dependent manner. SERPINA3K also significantly reduced the production of ROS. Towards the mechanism underlying these effects, SERPINA3K downregulated ROS generation by inhibiting NOX4 and upregulated ROS degradation by increasing the activity of superoxide dismutases (SOD) and catalase. Furthermore, H_2O_2 induced activation of the Kelch-like ECH-associated protein 1 (KEAP1)/NF-E2–related factor-2 (NRF2) pathway, while SERPINA3K inhibited H_2O_2-induced activation of KEAP1 and NRF2 and their downstream factors, including NAD(P)H quinone oxidoreductase and glutathione Stransferase. In the H_2O_2-induced rat corneal epithelium, SERPINA3K alleviated the oxidative stress and downregulated

NOX4 and NRF2.

Collectively, we concluded from results in the first part that SERPINA3K protects against oxidative stress by targeting the ROS generation/degradation system and modulating the KEAP1-NRF2 signaling pathway.

In the second part of this study, we aimed to evaluate the anti-neovascularization effects and investigate the possible mechanisms of SERPINA3K, using a specific rat model of suture-induced corneal neovascularization. After the rat corneal suture model was set up, SERPINA3K was topically administered thrice daily for 7 days. The clinical indications were evaluated on day 2, 5 and 7, including area of neovascularization and inflammation index. The global specimens were collected after day 7 and the following examinations were performed: histological investigation, immunostaining, western blot and quantitative real-time polymerase chain reaction (PCR) assay. Wnt3a, a Wnt pathway ligand, was added to cultured Human Umbilical Vein Endothelial Cells (HUVEC), followed by detecting cell migration and western blot. Meanwhile, an in vitro $VEGF_{165}$-stimulated HUVEC model was applied and the following measurements were conducted: cell proliferation, cell migration and tube formation.

The results revealed that SERPINA3K significantly suppressed corneal neovascularization and inhibited corneal inflammation. SERPINA3K downregulated the levels of β-catenin, non-pi-β-catenin and transcription factor 4 (TCF4), but upregulated the level of pi-β-catenin of the corneas induced by suture. SERPINA3K also decreased the gene expression and protein level of VEGF. Meanwhile, induction of Wnt3a increased the cell migration, activated the Wnt signaling and upregulated VEGF in cultured HUVEC, which were antagonized by SERPINA3K. In addition, SERPINA3K significantly inhibited $VEGF_{165}$-induced cell proliferation and migration of HUVEC, SERPINA3K also specifically suppressed the $VEGF_{165}$-induced tube formation of HUVEC.

The conclusion of the second part of this study is that the SERPINA3K has therapeutic potential for corneal neovascularization. The underlying mechanism may be through inhibiting Wnt signaling pathway.

Taken together, we performed a continuous series study to investigate the beneficial effects of SERPINA3K in cornea and its underlying mechanism. Our experimental evidence will provide a new direction of the development of new agents to treat ocular surface diseases and better understanding about the pathogenesis of ocular surface diseases.

Key Words: SERPINA3K；anti-oxidants；anti-neovascularization

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