

学校编码：10384

密级_____

学号：24520130154310

厦 门 大 学

博 士 学 位 论 文

**MK2 抑制剂对角膜碱烧伤的抗炎和抗新生
血管作用机制研究**

**MK2 inhibitor selectively reduces alkali burn-induced
inflammation and neovascularization in cornea**

陈艳丰

指导教师姓名：刘祖国教授

专业名称：生理学

论文提交日期：2016年5月

论文答辩时间：2016年5月

学位授予日期：2016年5月

答辩委员会主席：_____

评 阅 人：_____

2016年5月

抑制剂对角膜碱烧伤的抗炎和抗新生血管作用机制研究

陈艳丰

指导教师

刘祖国
教授

厦门大学

厦门大学学位论文原创性声明

本人呈交的学位论文是本人在导师指导下,独立完成的研究成果。本人在论文写作中参考其他个人或集体已经发表的研究成果,均在文中以适当方式明确标明,并符合法律规范和《厦门大学研究生学术活动规范(试行)》。

另外,该学位论文为(刘祖国)课题(组)的研究成果,获得(刘祖国)课题(组)经费或实验室的资助,在(厦门大学医学院眼科研究所)实验室完成。(请在以上括号内填写课题或课题组负责人或实验室名称,未有此项声明内容的,可以不作特别声明。)

声明人(签名):

年 月 日

厦门大学学位论文著作权使用声明

本人同意厦门大学根据《中华人民共和国学位条例暂行实施办法》等规定保留和使用此学位论文，并向主管部门或其指定机构送交学位论文（包括纸质版和电子版），允许学位论文进入厦门大学图书馆及其数据库被查阅、借阅。本人同意厦门大学将学位论文加入全国博士、硕士学位论文共建单位数据库进行检索，将学位论文的标题和摘要汇编出版，采用影印、缩印或者其它方式合理复制学位论文。

本学位论文属于：

1.经厦门大学保密委员会审查核定的保密学位论文，
于 年 月 日解密，解密后适用上述授权。

2.不保密，适用上述授权。

（请在以上相应括号内打“√”或填上相应内容。保密学位论文应是已经厦门大学保密委员会审定过的学位论文，未经厦门大学保密委员会审定的学位论文均为公开学位论文。此声明栏不填写的，默认为公开学位论文，均适用上述授权。）

声明人（签名）：

年 月 日

摘要

眼化学烧伤是一类常见的眼外伤疾病，特别是在发展中国家。与酸性烧伤相比，碱性烧伤更为常见。碱性物质可快速穿透眼前房，导致慢性炎症和角膜浑浊，从而导致严重且持久的视觉缺损。尽管角膜移植手术是一项有效的治疗方法，然而其成功与否取决于角膜的炎症和新生血管是否得到控制。尽管目前有许多用于治疗角膜炎症和新生血管的药物，然而由于其有一定的副作用或仅仅通过缓解症状而非从根本途径进行治疗，因此亟须从发病机制入手寻找新的治疗办法。

持久的角膜炎症与新生血管，不仅会导致永久的视觉缺失，还会使行角膜手术的时机推迟。碱烧伤常伴随着大量炎症细胞浸润和炎症因子表达上调，促新生血管因子和抗新生血管因子的不平衡是导致新生血管生成的重要原因。目前碱烧伤抗炎和抗新生血管治疗方法包括激素、非甾体类抗炎药、柠檬酸盐、氩激光、光动力疗法等。然而以上治疗方法效果有限，特别是对于较大的炎性新生血管。此外，还有一些新兴的治疗方法，例如将相关炎症因子和促新生血管因子作为治疗靶点，但由于疗效有限且有一定副作用，因此其临床应用得到一定限制。

丝裂原活化蛋白激酶激酶 2 (Mitogen-activated protein kinase-activated protein kinase 2, MK2) 是 p38 丝裂原活化蛋白激酶 (Mitogen-activated protein kinase, MAPK) 的下游，可直接被 p38 MAPK 活化，其在术后肠粘连、胰腺炎、动脉粥样硬化、风湿性关节炎、肿瘤等疾病中发挥着重要的作用。由于 MK2 特异性增强炎症因子、趋化因子和细胞黏附因子 mRNA 翻译效率并提高其稳定性，因此 MK2 是许多炎症性疾病的治疗靶点。目前有关 MK2 在角膜炎症和新生血管形成中的作用还未有文献报道。

因此，本实验通过建立大鼠碱烧伤模型，研究 MK2 抑制剂对碱烧伤的抗炎和抗新生血管作用。我们在治疗的第 1、4、7 天通过裂隙灯观察 MK2 抑制剂对大鼠角膜炎症、新生血管、上皮修复的影响情况，采用免疫组织化学方法观察各组炎症细胞浸润，采用实时荧光定量-聚合酶链式反应 (Real-time polymerase chain reaction, Real-time PCR) 和蛋白印迹 (Western blot) 法检测各组单核细胞趋化蛋白(monocyte chemotactic protein, MCP)-1、巨噬细胞炎症蛋白(macrophage inflammatory protein, MIP)-1 α 、细胞间粘附分子(intercellular adhesion molecule,

ICAM)-1、血管细胞粘附分子(vascular cell adhesion molecule, VCAM)-1、白细胞介素(interleukin, IL)-6、IL-1 β 和血管内皮细胞生长因子(vascular endothelium growth factor, VEGF) 等促炎和促新生血管因子和抗新生血管因子色素上皮细胞衍生因子(pigment epithelium derived factor, PEDF) 的表达情况。通过细胞增殖实验和划痕实验检测 MK2 抑制剂对人角膜上皮细胞(human corneal epithelial cells, HCECs) 增殖和迁移的影响。此外, 采用 Draize 实验和免疫组织化学方法评估 MK2 抑制剂对正常角膜的毒副作用。

我们发现, MK2 抑制剂可抑制碱烧伤导致的 MK2 活化, 且具有抗炎和抗新生血管作用。一方面, MK2 抑制剂可减少角膜炎症指数、ED1 阳性巨噬细胞和多形核白细胞(polymorphonuclear, PMN) 阳性中性粒细胞浸润。另一方面 MK2 抑制剂可下调 IL-6、IL-1 β 、MIP-1 α 、ICAM-1 和 VCAM-1 等细胞因子表达。此外, MK2 抑制剂还可下调促新生血管因子和上调抗新生血管因子 PEDF 的表达。同时, 我们还发现 MK2 抑制剂不影响角膜上皮的增殖和迁移, 且对正常角膜无毒副作用。

以上结果表明, MK2 抑制剂对大鼠碱烧伤角膜具有抗炎和抗新生血管作用, 因此其有望成为临床上治疗角膜碱烧伤的新药。

关键词: MK2; 角膜碱烧伤; 炎症

Abstract

Ocular chemical burns are a common trauma encountered worldwide particularly in the developing countries. They are more frequently a cause of injury than exposure to injurious acids. Caustic agents can readily penetrate into the anterior ocular surface and produce chronic inflammation and opacification resulting in severe and permanent visual impairment. Even though keratoplasty is a viable therapeutic option, the success of this procedure depends on first resolving inflammation and neovascularization (NV) with drugs. Currently, many of the drugs used for this purpose are somewhat problematic since they can have side effects and only provide symptomatic relief rather than target the mechanisms underlying inflammation and NV. This limitation has prompted numerous studies to delineate mechanisms underlying the pathogenesis of chronic inflammation and NV.

Unrelenting and dysregulated corneal inflammation and NV, are common sequels of an alkali burn that can lead to persistent visual impairment and delay performing penetrating keratoplasty. Alkali burns induce chemokine driven immune cell corneal infiltration accompanied by rises in proinflammatory cytokines levels. In addition, the tenuous balance between pro-angiogenic and anti-angiogenic factors can be disrupted leading to corneal NV. Thus, suppression of these maladaptive injury-induced responses is essential for reducing losses in corneal transparency and hastening wound healing. Various medical and surgical options such as steroids, nonsteroidal inflammatory agents, citrate, argon laser photocoagulation, and photodynamic therapy are used to treat corneal inflammation and inflammatory NV induced by an alkali burn⁴; however, sometimes, these therapies are ineffective, especially for large inflammatory NV. The novel approaches under investigation to improve treatment of chemical burns include manipulating the inflammatory and angiogenesis-related factors by means of monoclonal antibodies, receptor modification, aptamers, and inhibitors of candidate inflammatory and/or angiogenesis pathways. Even though

some of these options look promising, each one of them can have side effects that limit their usefulness in restoring corneal transparency and optical properties needed for normal vision.

Mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK2 or MK2) is an intracellular serine/threonine kinase substrate downstream from p38 mitogen-activated protein kinase (MAPK) and its activation by p38 is implicated in many inflammatory diseases including postoperative ileus, pancreatitis, atherosclerosis rheumatoid arthritis and cancer. Accordingly, it is an established drug target for treating many inflammatory diseases since its activation selectively induces the translation and increases stability of proinflammatory cytokine, chemokine and cell adhesion factor mRNA. There are no reports describing a role for MK2 in mediating corneal inflammation and inflammatory NV.

We determined if a MK2 inhibitor, MK2i, improves cornea wound healing by inhibiting inflammation and neovascularization (NV) caused by burning rat corneas with alkali. The effects of MK2i on inflammation, NV and epithelial damage were assessed under a slit lamp microscope 1, 4 and 7 days after injury. Immune cell infiltration was evaluated with immunohistochemistry. Real-time PCR and Western blot analysis were performed to assess pro-angiogenic and proinflammatory cytokine expression levels including monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), IL-6, IL-1 β and VEGF as well as anti-angiogenic cytokine PEDF levels. Cell proliferation assay and scratch wound assays were conducted to measure cell proliferation and migration in human corneal epithelial cells (HCECs). Draize test and immunohistochemistry were performed to determine if MK2i altered ocular surface health.

MK2i inhibited alkali-induced MK2 activation as well as rises in inflammation and NV based on: a) blunting rises in inflammatory index, inflammatory cell infiltration, ED1+ macrophage and PMN+ neutrophil infiltration; b) suppressing IL-6 and IL-1 β gene expression along with those of MIP-1 α , ICAM-1 and VCAM-1; c) reducing angiogenic gene expression levels and NV whereas anti-angiogenic PEDF

levels increased. In contrast, MK2i did not affect HCEC proliferation and migration and had no detectable side effects on ocular surface integrity.

We show here that MK2i selectively inhibited alkali-induced corneal inflammation and NV. MK2i may be a viable option for clinical management of corneal alkali burns.

Keywords: mitogen-activated protein kinase-activated protein kinase 2 (MK2); cornea alkali burn; inflammation

厦门大学博硕士学位论文摘要

目 录

摘 要	I
Abstract	III
第一章 课题背景	1
1.1 碱烧伤的病因学	2
1.2 碱烧伤的临床表现	2
1.3 碱烧伤的炎症反应	3
1.3.1 炎症细胞在炎症反应中的作用	3
1.3.1.1 多形核白细胞(polymorphonuclear, PMN)	3
1.3.1.2 巨噬细胞	4
1.3.2 炎症因子在炎症反应中的作用	4
1.3.2.1 白细胞介素(interleukin, IL)-1 β	4
1.3.2.2 IL-6	5
1.3.2.3 肿瘤坏死因子(tumor necrosis factor, TNF)- α	5
1.3.3 趋化因子在炎症反应中的作用	6
1.3.3.1 单核细胞趋化蛋白(MCP, monocyte chemotactic protein)-1	6
1.3.3.2 巨噬细胞炎性蛋白(macrophage inflammatory protein, MIP)-1 α	6
1.3.4 黏附分子在炎症反应中的作用	6
1.3.4.1 细胞间粘附分子(intercellular adhesion molecule, ICAM)-1	6
1.3.4.2 血管细胞粘附分子(vascular cell adhesion molecule, VCAM)-1	6
1.4 碱烧伤的炎性新生血管	7
1.4.1 新生血管的形成机制	7
1.4.2 促血管生成因子	8
1.4.2.1 VEGF	8
1.4.2.2 IL-1	10
1.4.2.3 IL-6	11

1.4.2.4	TNF- α	11
1.4.2.5	ICAM-1.....	11
1.4.2.6	MCP-1.....	11
1.4.3	抗血管生成因子：色素上皮细胞衍生因子(pigment epithelium-derived factor, PEDF).....	12
1.5	碱烧伤的抗炎和抗新生血管治疗.....	12
1.5.1	药物治疗.....	13
1.5.1.1	激素.....	13
1.5.1.2	非甾体类抗炎药.....	14
1.5.1.3	柠檬酸盐.....	14
1.5.1.4	环孢霉素.....	15
1.5.1.5	抗 VEGF 药物.....	15
1.5.1.6	实验性基因治疗.....	17
1.5.2	激光和常规手术治疗.....	18
1.5.2.1	氩激光(Argon laser, AL).....	18
1.5.2.2	黄激光.....	19
1.5.2.3	钕铝石榴石晶体 (neodymium yttrium-aluminium-garnet, Nd:YAG) 激光.....	19
1.5.2.4	光动力疗法(photodynamic therapy, PDT).....	19
1.5.2.5	角膜表层切除术.....	20
1.5.2.6	针透热疗法和烧灼法.....	20
1.6	p38/MK2 信号通路炎症.....	21
1.6.1	p38 MAPK 成员.....	21
1.6.2	p38 MAPK 信号通路及生物学作用.....	22
1.6.3	MK2 与炎症.....	22
1.7	研究目标、内容及意义.....	23
第二章	实验材料与方法.....	25
2.1	实验材料.....	25
2.1.1	动物和细胞系.....	25

2.1.2	哺乳动物细胞培养试剂	25
2.1.3	实验试剂与耗材	25
2.1.4	抗体	27
2.1.5	仪器	27
2.2	实验方法	29
2.2.1	大鼠角膜碱烧伤模型的建立及分组	29
2.2.2	眼表炎症指数测定	29
2.2.3	角膜新生血管测定	30
2.2.4	角膜荧光素染色	30
2.2.5	大鼠角膜苏木素和伊红(Hematoxylin & eosin, HE)染色	30
2.2.5.1	主要溶液配制	30
2.2.5.2	石蜡切片	30
2.2.5.3	HE 染色步骤	32
2.2.6	大鼠角膜巨噬细胞表面抗原(ED-1)、多形核白细胞(PMN)、增殖细胞核抗原(Ki-67)的免疫荧光染色	33
2.2.6.1	主要溶液配制	33
2.2.6.2	冰冻切片	34
2.2.6.3	免疫荧光染色步骤	35
2.2.7	实时荧光定量-聚合酶链式反应 (Real-time polymerase chain reaction, Real-time PCR) 法测定小鼠角膜中炎症介质的 mRNA 表达水平	35
2.2.7.1	大鼠角膜总 RNA 提取	35
2.2.7.2	测定 RNA 的浓度和纯度	35
2.2.7.3	逆转录	35
2.2.7.4	Real-time PCR	36
2.2.7.5	数据分析	38
2.2.8	蛋白印迹 (western blot) 法	38
2.2.8.1	主要溶液配制	38
2.2.8.2	BCA 法测定蛋白浓度及样品处理	39
2.2.8.3	蛋白免疫印迹实验	40

2.2.9 HCECs 培养	43
2.2.9.1 主要溶液配制	43
2.2.9.2 HCECs 复苏	44
2.2.9.3 HCECs 传代	44
2.2.9.4 HCECs 冻存	44
2.2.9.5 HCECs 计数	45
2.2.10 细胞增殖实验	45
2.2.11 细胞划痕实验	46
2.2.12 MK2 抑制剂对大鼠眼表毒性的影响	46
2.2.13 原位末端标记法 (in situ terminal deoxynucleotidyl transferase dUTP nick end labeling, TUNEL) 测定大鼠角膜上皮细胞凋亡	48
2.2.14 数据统计分析	49
第三章 实验结果	50
3.1 验证 MK2 抑制剂的作用方式	50
3.2 MK2 抑制剂可改善碱烧伤大鼠的临床体征	53
3.3 MK2 抑制剂可抑制炎症细胞浸润	57
3.4 MK2 抑制剂可抑制炎症因子和趋化因子表达的上调	61
3.5 验证 MK2 抑制剂的选择性抑制作用	63
3.6 MK2 抑制剂对眼表无毒性作用	66
第四章 讨论	70
第五章 总结	75
第六章 展望	77
参考文献	78
附录 英文缩略词索引	105
攻读博士学位期间发表的论文	109
致 谢	110

Table of Contents

Abstract in Chinese	I
Abstract in English	III
Chapter 1 Background	1
1.1 Etiology of alkali burn	2
1.2 Clinical manifestation of alkali burn	2
1.3 The role of inflammation in cornea alkali burn	3
1.3.1 The role of immune cells in the pathogenesis of cornea alkali burn	3
1.3.1.1 Polymorphonuclear(PMN)	3
1.3.1.2 Macrophage.....	4
1.3.2 The role of proinflammatory cytokines in the pathogenesis of cornea alkali burn.....	4
1.3.2.1 Interleukin(IL)-1 β	4
1.3.2.2 IL-6	5
1.3.2.3 Tumor necrosis factor(TNF)- α	5
1.3.3 The role of chemokines in the pathogenesis of cornea alkali burn	6
1.3.3.1 Monocyte chemotactic protein(MCP)-1	6
1.3.3.2 Macrophage inflammatory protein(MIP)-1 α	6
1.3.4 The role of adhesion molecules in the pathogenesis of cornea alkali burn	6
1.3.4.1 Intercellular adhesion molecule(ICAM)-1.....	6
1.3.4.2 Vascular cell adhesion molecule(VCAM)-1.....	6
1.4 Inflammatory corneal neovascularization after alkali burn	7
1.4.1 Pathogenesis of neovascularization	7
1.4.2 Pro-angiogenic factors	8
1.4.2.1 VEGF	8
1.4.2.2 IL-1	10

1.4.2.3	IL-6	11
1.4.2.4	TNF- α	11
1.4.2.5	ICAM-1	11
1.4.2.6	MCP-1.....	11
1.4.3	Anti-angiogenic factors: pigment epithelium-derived factor (PEDF).....	12
1.5	Treatment of corneal inflammation and neovascularization.....	12
1.5.1	Medical treatments	13
1.5.1.1	Corticosteroids	13
1.5.1.2	Non-steroidal Anti-inflammatory Drugs (NSAID).....	14
1.5.1.3	Citrate.....	14
1.5.1.4	Cyclosporine	15
1.5.1.5	Anti-VEGF agents	15
1.5.1.6	Experimental gene therapy strategies	17
1.5.2	Laser and conventional surgical treatments.....	18
1.5.2.1	Argon laser (AL).....	18
1.5.2.2	Yellow laser	19
1.5.2.3	Neodymium yttrium-aluminium-garnet (Nd:YAG)	19
1.5.2.4	Photodynamic therapy (PDT)	19
1.5.2.5	Superficial keratectomy	20
1.5.2.6	Needle diathermy and cautery	20
1.6	p38/MK2 pathway and inflammation	21
1.6.1	p38 group of mitogen-activated protein kinases	21
1.6.2	The p38 pathway and its biological function	22
1.6.3	MK2 and inflammation	22
1.7	Research aim, contents and significance.....	23
Chapter 2	Materials and Methods	25
2.1	Materials.....	25
2.1.1	Animals and cell line	25
2.1.2	Reagents for mammal cell culture.....	25

2.1.3	Experimental reagents and consumables	25
2.1.4	Antibodies	27
2.1.5	Experimental instruments	27
2.2	Methods	29
2.2.1	Establishment of rat cornea alkali burn model and grouping	29
2.2.2	Evaluation of ocular surface inflammatory index	29
2.2.3	Corneal neovascularization evaluation	30
2.2.4	Corneal fluorescein staining	30
2.2.5	HE staining	30
2.2.5.1	Preparation of major solutions	30
2.2.5.2	Paraffin section preparation	30
2.2.5.3	HE staining procedures	32
2.2.6	Immunofluorescent staining of ED1, PMN and Ki67	33
2.2.6.1	Preparation of major solutions	33
2.2.6.2	Frozen section preparation	33
2.2.6.3	Immunofluorescent staining	34
2.2.7	Real-time PCR for mRNA expression of inflammatory mediators	35
2.2.7.1	Corneal RNA isolation	35
2.2.7.2	Measurement of RNA concentration and purity	35
2.2.7.3	Reverse transcription	35
2.2.7.4	Real-time PCR	36
2.2.7.5	Data analysis	38
2.2.8	Western blot analysis for p38、 p-p38、 MK2、 p-MK2、 VEGF and PEDF	38
2.2.8.1	Preparation of major solutions	38
2.2.8.2	BCA assay for protein concentration and sample preparation	39
2.2.8.3	Western blot	40
2.2.9	HCECs culture	43
2.2.9.1	Preparation of major solutions	43
2.2.9.2	Cell resuscitation	44

Degree papers are in the “[Xiamen University Electronic Theses and Dissertations Database](#)”.

Fulltexts are available in the following ways:

1. If your library is a CALIS member libraries, please log on <http://etd.calis.edu.cn/> and submit requests online, or consult the interlibrary loan department in your library.
2. For users of non-CALIS member libraries, please mail to etd@xmu.edu.cn for delivery details.