行政院國家科學委員會專題研究計畫 成果報告

bradykinin 受體在腎缺氧再灌流傷害後期可能功能的初步探討

研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-2314-B-002-187-
執 行 期 間 ：95 年 08 月 01 日至 96 年 07 月 31 日
執 行 單 位 ：國立臺灣大學醫學院內科

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處 理 方 式 ：本計畫可公開查詢

中 華 民 國 96 年 08 月 17 日

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

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中文摘要

本研究在探討缺氧再灌流腎臟傷害後期遲緩激肽 B2 受體的分佈及其可能的功能。遲緩激肽 B2 受體的分佈係在缺氧再灌流腎臟傷害 2、7、14 及 28 天後以免疫組織染色法偵測，結果顯示：在這些時間點遲緩激肽 B2 受體廣泛於各腎小管特別是近端小管，在缺氧再灌流腎臟傷害第 7 及 14 天，緩激肽 B2 受體在再生的近端腎小管有特別的濃染。為探討缺氧再灌流腎臟傷害後期遲緩激肽 B2 受體可能的功能，吾人將大白鼠分為偽病組、對照組、血管增滲酶組及 HOE 組，大白鼠於缺氧再灌流腎臟傷害第 2 天開始治療直到 7 天後量測血壓結束犧牲，取腎臟組織分析。結果顯示：各組間的血壓及腎小管囊狀擴大情形並無差別。缺氧再灌流腎臟傷害後對照組皮質及外髓質出現增生細胞及發炎細胞浸潤，但 HOE 組細胞增生及發炎細胞浸潤則較其它組要顯著。這些結果意味著：缺氧再灌流腎臟傷害後增加的遲緩激肽 B2 受體表現係來自再生的近端小管，可能是將恢復期細胞功能做準備，而與心血管表現型無關。至於 HOE 組細胞增生及發炎細胞浸潤則較其它組要顯著的原因，可能為此組的樣本太小(等於 4)，形成實驗誤差。或者，抑制遲緩激肽 B2 受體會加強再灌流後期傷害，而有較多的增生細胞及發炎細胞浸潤。若要區分原因，則需要更多實驗加以釐清。

英文摘要

This study aimed to study the distribution and role of bradykinin receptor in the later phase of ischemic reperfusion (I/R) renal damage. The distribution of bradykinin B2 receptor was examined with immunohistochemistry staining on the 2, 7, 14, and 28 days after I/R injury. The results revealed that bradykinin B2 receptor was intensively stained in proximal tubular cell and less stained in distal and connecting tubule on the 2, 7, 14, and 28 days after I/R injury. There were much more staining in the regenerated tubular cells 7 and 14 day. To study the role of bradykinin B2 receptor, rats were divided into sham (N = 4), control (N = 12), kallikrein (N = 12) and HOE (N = 4) groups. The treatment was begun from day 2 and continued to sacrifice. Severn days after I/R injury, there was no difference in the blood pressure of change of tubular cystic dilatation between control, kallikrein and HOE groups. There were proliferating cells and inflammatory cells infiltration in cortex and outer medulla in control rats after I/R injury. The proliferation was more prominent in HOE group. Rats in HOE group also had more inflammatory cell infiltration in outer medulla. These results indicated that the increased bradykinin B2 receptor seems to be the phenomenon of regaining function of proximal tubular cell, but have no influence on cardiovascular phenotype. The cause of more prominent cell proliferation and inflammatory cells infiltration in bradykinin B2 receptor inhibiting rats (HOE group) was unknown. It may be due to the lab variation (small sample number in HOE group: 4). More initial I/R injury leads to more cell proliferation and inflammation in later phase. , so the other possibility is that it is the true detrimental effect of antagonizing bradykinin B2 receptor in later phase (on day 2 to 7) of I/R renal injury. Further studies are needed to clarify this point.

報告內容：

前言及文獻探討

Ischemic injury is an important cause of acute renal failure relating to septic shock, operation of aorta, and renal transplantation in clinical practice. The renal injury brings higher morbidity and mortality to patient. However, the pathogenesis of injury and physiology of regeneration were not fully understood. This leads to the limited improvement in developing the prevention of injury or accelerating the recovery from renal ischemic damage.

The tissue kallikrein-kinin system includes tissue kallikrein, kininogen, kinin, and bradykinin B2 or B1 receptor. Tissue kallikrein cleaves kininogen substrate to release vasoactive kinin peptide via limited proteolysis. Binding of intact kinin to the kinin B2 receptor activates second messengers such as nitric oxide (NO)/cGMP and prostacyclin/cAMP and triggers many biological effects, such as vasodilation, natriuresis and edema formation. There is increasing awareness that in addition to its vasoactive and inflammatory actions, bradykinin exerts significant effects on cellular growth. For example, bradykinin is mitogenic in murine and human fibroblasts, where it appears to facilitate the stimulation of DNA synthesis produced by epidermal growth factor or platelet derived growth factor[1], [2], [3]. Bradykinin also stimulates DNA synthesis and proliferation of quiescent rat aortic smooth muscle cells, suggesting that bradykinin may act as a growth modulator in the vessel wall[4],[5]. Bradykinin, acting via B2 receptors, stimulates immediate early gene expression, AP- 1 binding activity, and DNA synthesis in cultured rat mesangial cells[6]. In addition, Bascands *et al.*[7] also reported that bradykinin is mitogenic in quiescent mesangial cells. The proliferative effect may help kidney recover from ischemic injury.

In previous studies, through real-time RT-PCR analysis at different time points after ischemic reperfusion (I/R) renal injury we found that the B2R began to be upregulated 2 days after I/R and reached high level 7~14 days after I/R injury. Two to seven days after I/R is a time point when renal tubules regenerates and regain the structure and function of normal tubule. The up-regulation of bradykinin receptor indicates that bradykinin receptor might participate in the repair process after acute tubular necrosis induced by I/R injury or regain the tubular function. Reviewing the literature, there was no study evaluating the roles of bradykinin receptor in tubular regeneration after acute tubular necrosis. But in nephrogenesis, recent studies indicating that the developing kidney expresses all the components of the kallikrein-kinin system and that kininogen and B2R are highly expressed in the differentiating distal nephron[8],[9] support the hypothesis that kinins may act as developmental factors. The B2R gene is abundantly expressed during the latter half of fetal metanephrogenesis and that the terminal ureteric bud branches and immature glomeruli are the principal sites of B2R expression. Gestational B2R blockade combined with high salt causes aberrant fetal nephrogenesis. The resulting renal dysgenesis is dysplastic in nature and the aberrant tubules exhibit excessive apoptosis[10].The ability of B2R blockade to alter

the blood pressure phenotype in young animals suggests that kinins play a role in renal and cardiovascular maturation[11]. B2R blockade from birth to 11 weeks of age in rats causes an increase in blood pressure associated with cardiac hypertrophy and sodium retention[12]. Treatment of newborn rats with high-dose Icatibant for the first 2 weeks of postnatal life compromises renal growth[13]. We believe that the nephrogenesis in embryonic or early postnatal stage, and the tubular regeneration after acute tubular necrosis might share some common process in renal tubular formation. The findings in pre-natal renal growth, combined with our recent observations that renal bradykinin receptors gene transcription are upregulated during regeneration phase after I/R injury, prompted us to evaluate the potential role of bradykinin receptors after post I/R renal repair.

研究目的

In this study, we will first localize the distribution of bradykinin receptor by immunohistochemistry in kidney at the time when receptors are up-regulated after I/R injury. Then we will activate or antagonize the action of bradykinin B2 receptor with suitable antagonists at certain time point after I/R renal injury. To study the impact of bradykinin receptor on renal repair we will focus on the examination of histology, inflammation and proliferation status. The functional change such as renal function, and cardiovascular phenotype (including blood pressure and heart rate) between control and treated groups will be examined. This study will help us understand the role of bradykinin receptor in the renal regeneration process after I/R injury.

研究方法

Animal model construction

For all groups male Wistar rats weighing 240 to 280 g were obtained from the Experimental Animal Center in our institute. All rats are housed in a constant-temperature room with a consistent light cycle (light from 07:00 to 18:00) and fed a standard rat chow (protein 23.4%). The animal care and treatment were conducted according to the guidelines of the National Science Council of the Republic of China (NSC 1997). At the beginning of day 0, to establish ischemia/reperfusion renal injury, were anesthesialized intraperitoneally with 50 mg/kg of Phenobarbital. After left nephrectomy right renal artery was clamped with vessel clamp (not injury to the artery) for 40 minutes, followed by reperfusion.

Localization of bradykinin B2 receptor in different time point after I/R renal injury

Rats that receiving I/R renal injury as above were sacrificed at 2, 7, 14, and 28 days after operation. Kidneys were removed for immunohistochemistry staining for bradykinin B2 receptor.

Evaluation of the role of bradykinin B2 receptor in the later phase of I/R renal injury

Rats were divided into 4 groups. All rats received I/R renal injury as described above. On the 2nd day after reperfusion, rats in the group A (sham group, N = 4) received left nephrectomy only

without I/R injury of right kidney. Rats in group B (control group, N = 12)received PBS treatment only. Rats in group C (kallikrein group, N = 12) received kallikrein through subcutaneous osmotic pump (Alza Corporation, Palo Alto, California, 0.208 μg/hr at 1 μL/hr, minipump rate). Rats in group D (HOE group, N = 4) received HOE140 at a rate of *31.3* μg/day (1.3 μg/hr at 1 μL/hr, minipump rate). All rats were sacrificed 7 days after I/R renal injury.

Histological examination

Kidneys were fixed by immersion in 10% buffered formalin, dehydrated in ethanol and embedded in paraffin blocks. Frontal corticomedullary sections (5 μm) taken through the hilus were stained with H&E. Upon examination of the stained sections, particular attention was focused on tubular cystic dilatation..

Immunohistochemistry

Sections were immunostained by the immunoperoxidase technique. The kidney specimens are immersed in 30% sucrose/PBS at 4°C overnight after fixation in 4% paraformaldehyde/PBS at 4°C. The specimens are then embedded in Tissue-Tek OCT compound (Miles Inc., Elkhart, IN) in isopentane in liquid nitrogen and stored at -70°C until cryostat sectioning Briefly, 5-μm renal sections are microwaved (Tatung TMO-6810) in 0.01 M citrate buffer (pH 6.0) for 5 min at 800 W. They are allowed to cool for 15 min, rinsed in distilled water twice and in PBS for 5 min. The sections are then treated with 0.5% hydrogen peroxidase/PBS for 20 min at room temperature to block the endogenous peroxidase. Subsequently, tissue sections were sequentially incubated with: (1) normal serum for 20 min for blocking, (2) primary antibody at 4^{o}C for overnight, (3) secondary antibody, biotin-conjugated goat anti-rabbit or anti-mouse IgG at room temperature for 1 hr, and (4) avidin DH, biotinylated horseradish peroxidase H complex for 1 hr. Peroxidase activity was visualized with 0.01% 3,3'-diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin or PAS, washed with tap water, dehydrated, and mounted. Consecutive control sections were incubated in the absence of the primary antibody. The primary antibodies included: (1) B2R, (Santa Cruz) (2) a monoclonal antibody against proliferating cell nuclear antigen (PCNA, clone PC-10, DAKO) (3) ED-1 antibody (Serotec, Oxford, United Kingdom) diluted 1/50.

結果

The renal function 24 hours after I/R renal injury in all rats

Twenty four hours after I/R renal injury, the serum creatinine increased in control group. But there was no difference between control rats and kallikrein or HOE treated rats. (Figure 1)

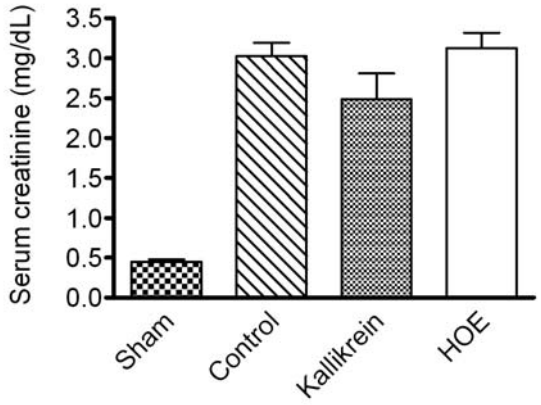


Figure 1. Serum creatinine level 24 hours after I/R renal injury in all rats. There was no difference between control and kallikrein or HOE treated rats.

Blood pressure 7 days after I/R renal injury in all rats

There was no difference of blood pressure in rats between sham, control, kallikrein and HOE groups.

Distribution of bradykinin B2 receptor after I/R renal injury

In normal kidney, bradykinin B2 receptor is extensively distributed over proximal tubule, and is less in distal and connecting tubule. At the 2, 7, 14 and 28 days after I/R injury, the distribution bradykinin B2 receptor was similar to the normal kidney. But the expression was more prominent in newly regenerated proximal tubular cells 7 days after I/R injury (Figure 2).

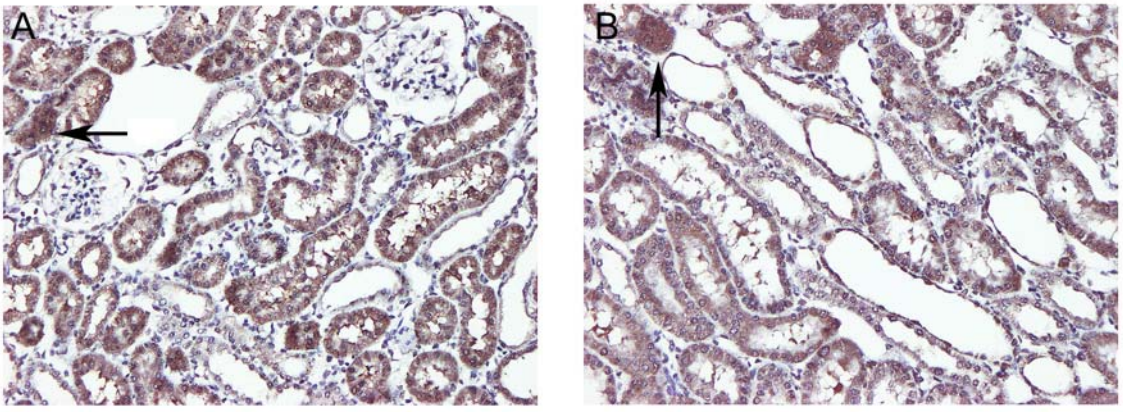
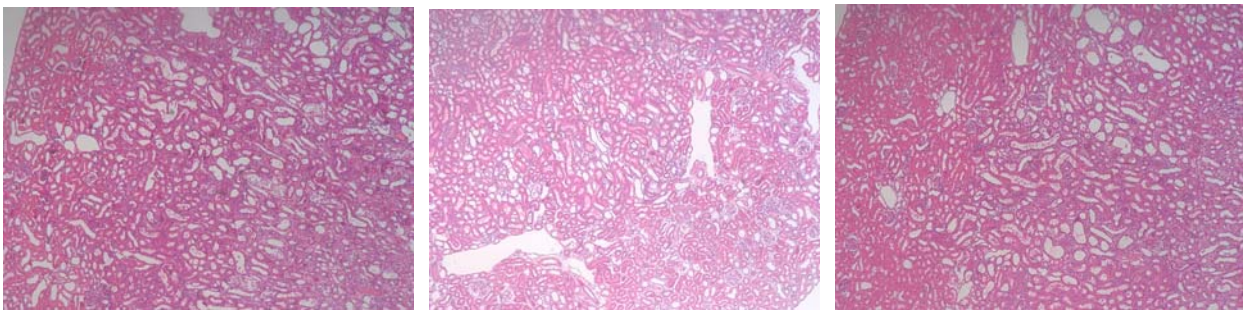


Figure 2. The distribution of bradykinin B2 receptor 7 days after I/R injury in A) cortex and B) outer medulla. Arrow indicated the strongly stained bradykinin B2 receptor in the newly regenerated proximal tubular cells going to form tubular structure.

Bradykinin B2 receptor activation or blockade did not change the morphology after I/R renal injury

After 7days of I/R injury, the tubular necrosis disappeared. New tubular formation with flatten cells were noted and there were numerous cystic change of tubule in cortex and outer medulla.

However, there is no difference in tubular cystic dilatation between control, receiving bradykinin B2 receptor activation or blockade rats. (Figure 3)



Control Kallikrein HOE140

Figure 3: Renal histological change 7 days after I/R injury in control, kallikrein and HOE140 treated rats. The degree of cystic dilation of renal tubule was similar in all groups.

Role of bradykinin B2 receptor in cell proliferation after I/R renal injury

Seven days after I/R renal injury, proliferating cells were found over cortex and more intensively in outer medulla. The activation of bradykinin receptor by kallikrein administration did not increase the proliferating cells in cortex or outer medulla. However, more proliferating cells in cortex and outer medulla were seen in HOE treated rats (figure 4).

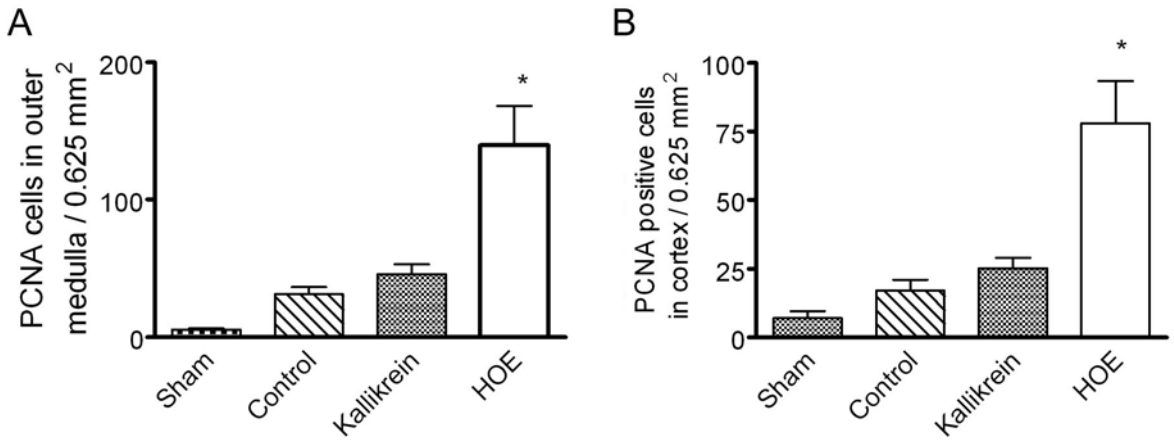


Figure 4. PCNA positive cells appeared in cortex and outer medulla 7 days after I/R renal injury. There were more PCNA positive cell in HOE treated rats. A) outer medulla, * $P <$ 0.001 compared to control and kallikrein groups. B) Cortex, * $P < 0.001$ compared to control and kallikrein groups.

Role of bradykinin B2 receptor in inflammation after I/R renal injury

Seven days after I/R renal injury, many ED-1 positive cells were found over cortex and more intensively in outer medulla. The activation of bradykinin receptor by kallikrein administration did not increase the infiltration of ED-1 positive cells in cortex or outer medulla. However, more infiltration of ED-1 positive cells in outer medulla was seen in HOE treated rats when compared

to control or kallikrein treated rats (figure 5).

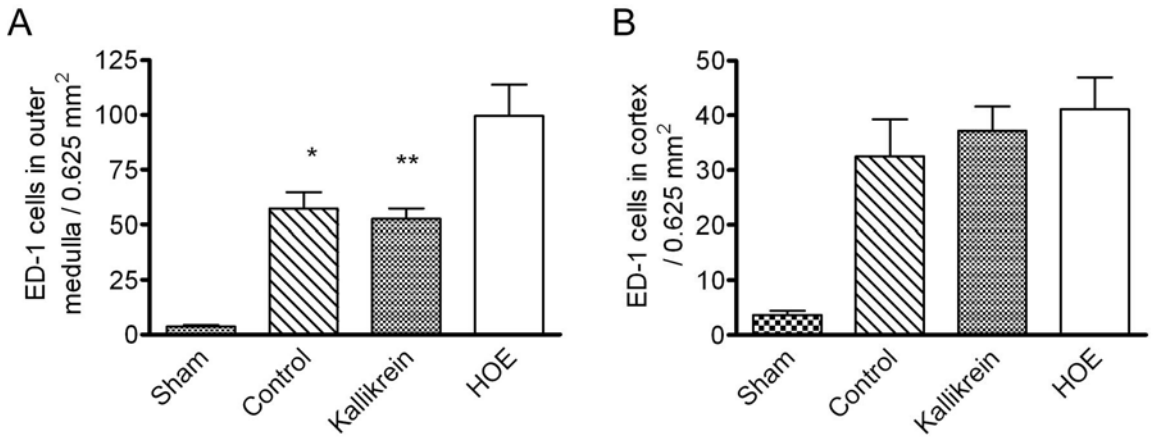


Figure 5. Many ED-1 positive cells appeared in cortex and outer medulla 7 days after I/R renal injury. There were more PCNA positive cell in HOE treated rats. A) Outer medulla, * $P < 0.05$ compared to HOE group, ** $P < 0.01$ compared to HOE group. B) Cortex.

討論

From immunohistochemistry staining, we demonstrated the regenerated proximal tubular cell regain the expression bradykinin B2 receptor 7 days after I/R injury. This finding is compatible with our previous results that gene expression of bradykinin B2 receptor is up-regulated 7~14 days after I/R injury. However, the increased bradykinin B2 receptor seems to have no influence on blood pressure regulation since our result did not reveal any difference in all rats. It may be a process that regenerated proximal tubular cell regaining their tubular function.

It is interesting that there was more prominent tubular cell regeneration and inflammatory cell infiltration in HOE treated rats. There are two possibilities. One is the sample size in HOE group is too small, so the result comes from laboratory variation. We have to conduct more studies to expand the sample size in HOE group to uncover the truth. The other possibility is that blocking of bradykinin B2 receptor in later phase of I/R renal injury will result in more damage. More I/R renal injury may initiate more tubular regeneration and inflammation in the recovery stage. Our result revealed that the renal function one day after I/R injury was not different between control, kallikrein and HOE group. So the more cell proliferation and inflammation in HOE rats does not come from more severe initial I/R damage, but is resulting from more renal damage due to the effect of bradykinin B2 receptor inhibition on day 2 to day 7 after I/R injury. However, activation of bradykinin receptor by kallikrein infusion did not attenuate renal damage. It needs more other evidence to clarify the possibility.

In summary, the increased expression of bradykinin B2 receptor did not influence the cardiovascular phenotype after I/R injury. It seems to be the regaining of tubular cell function in regenerated tubule. Whether bradykinin B2 receptor influence the renal damage or recovery in

later phase of I/R injury need further studies to clarify.

計畫成果自評：

We have completed most of the work that we planed to do in this one year. Although there is no significant function of bradykinin B2 receptor in later phase of I/R renal injury as we previously predicted, this result still give us some information about the regaining process bradykinin B2 receptor after I/R injury. Because the expense of HOE140, we did not conduct a study with large sample size of HOE group. This the weak point in our study.

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