行政院國家科學委員會專題研究計畫　期中進度報告

以抗氧化物減低腎臟缺血再灌流誘發腎小管細胞凋亡的效應(I):細胞凋亡基因的研究(2/3)

計畫主持人： 賴明坤

計畫參與人員： 鄭劍廷 楊坤忠

報告類型： 精簡報告

處理方式： 本計畫可公開查詢

中 華 民 國 92 年 5 月 23 日

行政院國家科學委員會專題研究計畫執行進度報告

計畫題目:以抗氧化物減低腎臟缺血再灌流誘發腎小管細胞凋亡的效應(I):細胞凋亡基因的研究(2/3)

計畫編號：NSC 91-2314-B-002-322
執行期限：91 年 08 月 01 日至 92 年 07 月 31 日
　　主持人：賴明坤
　　執行機構及單位名稱:台大醫學院醫學系

一、中文摘要

　　腎臟移植或缺血灌流造成自由基的釋放可能是造成腎細胞死亡之主因。為驗證此一假說我們已利用不同時間之腎缺血觀察腎自由基與細胞凋亡的產生。我們也以超冷光儀偵測活體腎臟及離體腎小管與全血之自由基表現，TUNEL 免疫染色, DNA 階梯生化證據及西方墨點法探討致死基因 bax/bcl-2, CPP32, and PARP 的細胞凋亡的分子機轉。結果發現增加缺血再灌流引發自由基的釋放量增加, 增加 TUNEL 正染色細胞(apoptotic cell), 與 DNA 階梯之表現。此機轉是因活化 bax/bcl-2, CPP32, and PARP的細胞凋亡的分子機轉有關。我們亦發現 Mn SOD, CuZn SOD與 catalase 會隨著缺血與再灌流時間的增加而逐漸降低。為進一步探究自由基的增加與抗氧化酵素的降低是造成腎細胞凋亡與腎功能的產生主因。我們以添加抗氧化物 SOD, C60(FC4S)或以缺氧預處理誘發內源性抗氧化酵素來評估腎細胞凋亡的反應。結果發現內源性或外源性抗氧化物的誘發可降低腎細胞凋亡數目, DNA 階梯現象與 Bax/Bcl-2, CPP32 , PARP的反應。此結果證明內、外源性抗氧化物的添加可以改善腎細胞凋亡與自由基的清除是正相關。

關鍵詞：自由基；細胞凋亡; 缺血再灌流

Abstract

We have examined ischemia-induced oxidative damage to the reperfused kidney. A modified chemiluminescence (CL) method, an *in situ* nitro blue tetrazolium (NBT) perfusion technique, and a DNA fragmentation/apoptosis-related protein assay were adapted for demonstration *de novo* and co-localization of reactive oxygen species (ROS) production and apoptosis formation in rat kidneys subjected to ischemia/reperfusion (I/R) injury. The results showed that prolonged ischemia potentiated proapoptotic mechanisms, including increases in the Bax/Bcl-2 ratio, CPP32 expression, and PARP fragments, and subsequently resulted in severe apoptosis, including increases in DNA fragmentation and apoptotic cell number in renal proximal tubules (PT) and distal tubules (DT) in a time-dependent manner. The increased level of ROS detected on the renal surface was correlated with that in blood and was intensified by a prolonged interval of ischemia. The source of ROS synthesis was mainly the PT epithelial cells. Furthermore, three important endogenous antioxidant enzymes including Mn superoxide (MnSOD), Cu-Zn SOD, and catalase were all decreased after I/R injury. We found that the ROS and apoptotic nuclei

detected in the PT cells can be ameliorated by exogenous SOD and C60(FC4S) and upregulation of endogenous Mn superoxide (MnSOD), Cu-Zn SOD, and catalase by hypoxia preconditioning, indicating a therapeutic potential of antioxidants for I/R injury.

Key words: Reactive Oxygen Species, Apoptosis, Kidney, Ischemia, Reperfusion injury

二、緣由與目的

Complete or partial cessation (ischemia) followed by restoration of blood flow (reperfusion) is a serious event that affects many organs, such as the heart, brain, liver, and kidney [1-7]. Ischemia/reperfusion (I/R) contributes to abnormal signal transduction or cellular dysfunction [8,9] and initiates the cascade of apoptosis/necrosis, with subsequent inflammatory infiltration [6,7,10].

Apoptosis, or programmed cell death, which can be distinguished based on morphologic and biochemical criteria, is different from necrosis [11-13]. The mechanism responsible for post-I/R apoptosis is attributed to the burst release of reactive oxygen species (ROS) [13,14-16]. ROS induce apoptosis by causing DNA damage, oxidation of lipid membranes, and/or direct activation and expression of the genes/proteins responsible for apoptosis [17]. For example, increases in the Bax/Bcl-2 ratio [18], expression of caspase and its activity [19,20], and caspase-mediated cleavage of poly-(ADP-ribose)-polymerase (PARP) [21] have been found in organs subjected to I/R injury or in cells after a cytotoxic insult.

The sources of ROS generated after I/R may be circulatory macrophages/neutrophils [22] or the resident cells [23]. In the liver, the sources of ROS appear to be Kupffer cells, sinusoidal cells, and infiltrating leukocytes [24]. In the kidney, we have demonstrated increased renal and renal venous ROS after I/R by an ultrasensitive chemiluminescence method [Chien et al., 2001] and the efficacy of antioxidants or free-radical scavengers in minimizing I/R injury [7,14,16] suggest a role of ROS in I/R injury. However, the precise origin of ROS in the I/R kidney has yet to be defined.

In this study, we adapted a modified chemiluminescence (CL) method for direct measurement of the amount of ROS produced from the kidney surface and from renal veins in I/R kidney. CL emission when specific chemical compounds {e.g., N,N'-dimethyldiacridinium (lucigenin) and 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a]-pyrazin-3-one hydrochloride (MCLA)} react with ROS is a well-established method for monitoring ROS production *in vitro* [25] and *in vivo* [26,27]. In addition, we determined the cellular location of *de novo* ROS synthesis in the I/R kidney by employing an *in situ* vascular perfusion technique, whereby nitro blue tetrazolium (NBT) was perfused

through the kidney for depicting cells in which superoxide was formed [28]. The onset of apoptosis and its severity in the I/R kidney were determined by examination of the presence of DNA fragmentation and an increase in cellular proteins associated with apoptosis. Our study showed that prolonged ischemia resulted in severe apoptosis in the reperfused kidney in a time-dependent manner. Cells in both proximal (PT) and distal tubules (DT) were subjected to I/R injury, whereas only PT cells produced measurable ROS by the NBT method. Not unexpectedly, the apoptosis in vivo and in vitro induced by I/R and hypoxia/reoxygenation (H/R) in PT cells, but not in DT cells, can be prevented by superoxide dismutase (SOD) or a novel free-radical scavenger, hexa(sulfobutyl)fullerene (FC$_4$S) [14].

三、方法

Surgery

Female Wistar rats (250 g) were housed at the Experimental Animal Center, National Taiwan University, at a constant temperature and with a consistent light cycle (light from 07:00 to 18:00 o'clock). The animal care and experimental protocol were in accordance with the guidelines of the National Science Council of the Republic of China (NSC 1997). On the day of the experiments, all rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and were tracheotomized. Catheters were placed in the left carotid artery for blood sampling and in the left femoral vein for anesthetic supplement and blood administration. After surgery, the left renal artery was cannulated by introduction of a length of stretched PE10 tubing from the left femoral artery via the aorta. A catheter was placed in the left renal vein for blood sampling. The rat was then placed on its right side, and the left kidney was exposed via a flank incision and dissection from the surrounding tissue.

Induction of unilateral renal ischemia

SOD treatment or hypoxic preconditioning

Some rats were subjected to 10% altitude hypoxia for 4 weeks. Another set experimental rats (n=4 for each ischemic group) was treated with intravenous SOD (500 U per rat, Sigma, St. Louis, MO, USA) after the ischemic period (before reperfusion). In this experiment, all ischemic kidneys were reperfused for 4 h. The experiment was intended for confirmation of the role of ROS in apoptosis formation in the I/R kidney.

Tissue preparation

For evaluation of the effect of ischemic intervals on apoptosis formation and apoptosis-related protein expression, twelve rats in each ischemic group (I15, I45, and I60) were used. Four rats were sacrificed with an overdose of anesthetics at the end of

ischemia (i.e., no reperfusion) and at 1 h or 4 h after reperfusion. For comparison, three control rats were used. The kidney was resected and divided into three parts. One part was stored in 10% neutral buffered formalin for routine histology and *in situ* apoptotic assay; another was prepared for DNA fragmentation electrophoresis, and the third was quickly frozen in liquid nitrogen and stored at −70°C for protein isolation.

In situ apoptotic assay (ISAA)

The method for ISAA (i.e., terminal deoxynucleotidyl transferase-mediated nick-end labeling method, TUNEL) was performed according to the method of Gavrieli et al. [29] with minor modifications [30]. The distal tubules were recognized by their smaller (the nuclei are close together) and more regular cells [31]. The number of apoptotic cells was expressed per 100 of the PT or DT cells in each section.

In vivo CL recording

The method for detection of CL from the organ surface after intrarenal arterial MCLA (TCI-Ace, Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) injection [26] was adapted for demonstration of ROS production in the I/R kidney. The rat was maintained on a respirator (tidal volume: 1.5-2.0 ml; rate, 80-90 cycles/min; inspiratory pressure, 20-30 cm H_2O) and a circulating water pad at 37°C during photon detection. For excluding photon emission from sources other than the kidney, the animal was housed in a dark box with a shielded plate. Only the renal window was left unshielded and was positioned under a reflector, which reflected the photons from the exposed kidney surface onto the detector area.

A single dose (1 mM in 0.1 ml) of lucigenin (Sigma, St. Louis, MO, USA), or a continuous infusion (0.2 mg/ml/hr) of MCLA was administered into the control or I/R kidney via an intrarenal arterial catheter. The lucigenin- or MCLA-enhanced CL signal from the kidney surface was measured continuously during administration by use of a CL Analyzing System (CLD-110, Tohoku Electronic Industrial Co., Sendai, Japan). Eight rats (5 for lucigenin and 3 for MCLA treatment) in each ischemic group (i.e., I15, I45, and I60) were used in this part of the experiment.

Preparation of whole-blood sample for lucigenin-enhanced CL determination

Four groups of rats (I15, I45, I60, and control; 5 rats in each group) were used for measurement of ROS in whole-blood samples. A series of blood samples (0.2 ml) from the left renal vein or carotid artery was obtained immediately after reperfusion for various times. The blood samples were immediately wrapped in aluminum foil and kept on ice until CL measurement, usually done within 2 h [32].

In situ demonstration of superoxide formation by NBT

The vascular perfusion of NBT was performed as described previously by

Chien et al. [32].

Immunoblot analysis for Bax, Bcl-2, CPP32, and PARP

We measured the amounts of CuZn SOD, MnSOD, Catalase, Bax/Bcl-2, caspase 3, and PARP [18,19,21] in renal tissues of I/R rats. For protein analysis, left kidney samples were homogenized with a prechilled mortar and pestle in extraction buffer, which consisted of 10 mM Tris-HCl (pH 7.6), 140 mM NaCl, 1 mM PMSF, 1% NP-40, 0.5% deoxycholate, 2% β-mercaptoethanol, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin. The mixtures were homogenized completely by vortexing and kept at 4°C for 30 min. The homogenate was centrifuged at 12,000×g for 12 min at 4°C, the supernatant was collected, and the protein concentrations were determined by BioRad Protein Assay (BioRad Laboratories, Hercules, CA, USA).

四、結果與討論
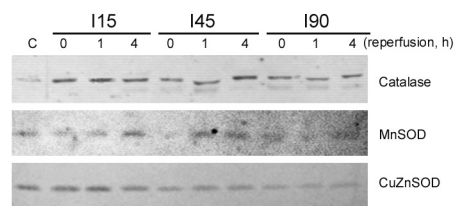
Figure 1. Increased ischemic interval and reperfusion time decreased the expression in catalase, MnSOD, and CuZnSOD of rat kidneys subjected to I/R injury.
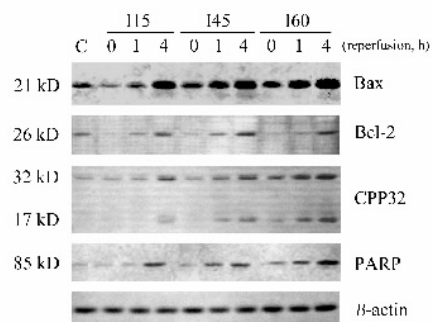

Figure 2. Western blot analysis with specific antibodies to Bax, Bcl-2, CPP32, PARP, and β-actin of homogenates of rat kidney subjected to I/R injury. Note the increased expression of Bax, CPP32 (32 kD proenzyme and 17 kD cleaved product), and PARP after reperfusion. The expression of Bcl-2 appeared to be decreased after ischemic insult, and returned to its preischemic level after reperfusion. 15-min ischemia plus reperfusion was sufficient to cause an increase in the Bax/Bcl-2 ratio (see text). Equal

protein loading was displayed by β-actin.
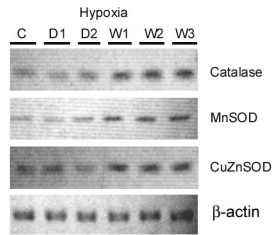
Figure 3. Increased endogenous expression in catalase, MnSOD, and CuZnSOD of rat kidneys subjected to hypoxic preconditioning.
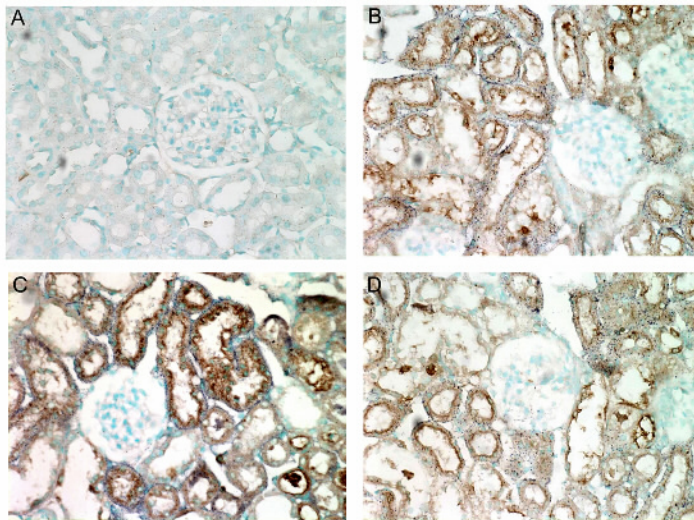
Figure 4. Increased ROS formation (blue deposits) as well as bax expression (brown color) in the I/R kidney (C), but not displayed in the control kidney (A). However, the enhanced ROS and bax expression are depressed by SOD treatment (B) and hypoxic preconditioning (D).

DISCUSSION

During the stage of prolonged ischemia during surgery or organ harvest for renal transplantation, hypoxia and the following reperfusion could initiate the cascade of cellular apoptosis, tissue necrosis, and subsequent inflammatory cell infiltration [6,7,10]. In this study, we explored the antioxidant gene and enzymes on ischemia/reperfusion induced oxidative stress in the kidneys. Our study showed that prolonged ischemia resulted in severe apoptosis in the fused kidney in a time-dependent manner. This was demonstrated by increases in DNA fragmentation, in apoptotic cell number, and in "proapoptotic" proteins such as Bax, CPP32, and PARP. Prolonged ischemia appeared to potentiate the proapoptotic mechanism. In

addition, the endogenous antioxidants like catalase, MnSOD, and CuZnSOD were all decreased followed by ischemia/reperfusion injury. By employing an *in situ* vascular NBT perfusion technique, we showed that the cellular source of ROS synthesis was mainly the PT epithelial cells in the I/R kidney. The increased bax expression as well as ROS formation were consistently reduced by SOD or hypoxia preconditioning. Therefore, the apoptotic cell death as well as the ROS production in PT cells can be ameliorated by SOD treatment or hypoxia preconditioning, which can upregulate antioxidant gene expression. Our results indicate that, as in many organs, the increased Bax, CPP32, and PARP levels could contribute to apoptotic cell death in I/R kidneys. The expression patterns of these proteins in kidneys subjected to I/R as well as in PT and DT cultures subjected to H/R are similar.

In summary, the present study indicates that ROS are produced in significant amounts in PT epithelium under reperfusion conditions and may therefore be responsible for the apoptotic death of these cells. Treatment with exogenous SOD and other free-radical scavengers as well as upregulation of endogenous antioxidant genes may be effective for the prevention of ROS-mediated apoptosis in renal tubules.

五、成果自評

　　本研究使用之技術及數據與實驗前之推論極為相近。本實驗提供直接證據即自由基的數量與缺血時間成正相關同時造成細胞凋亡數目也成正相關。我們的實驗結果證明內、外源性抗氧化物的添加可以改善腎細胞凋亡與自由基的清除。給以抗氧化物於器官缺血或保存液以減低傷害是具有非常重要之醫學應用價值。

六、參考文獻

1. **Kilgore KS, Lucchesi BR:** Clin Biochem 1993, 26: 359-370.
2. **Kontos CD, Wei EP, Williams J, Kontos HA, Polvisshock JT:** Am J Physiol 1992, 263: H1234-H1242.
3. **Okuda M, Lee HC, Kumar C, Chance B:** Circ Shock 1992, 38:228-237.
4. **Strassberg SM, Howard TK, Molmenti EP, Hertl M:** Hepatology 1994, 20:829-838.
5. **Bonventre JV, Weinberg JM:** Annu Rev Med 1992, 43:523-553.
6. **Schumer M, Colombel MC, Sawczuk IS, Gobe G, Connor J, O'Toole KM, Olsson CA, Wise GJ, Buttyan R:** Am J Pathol 1992, 140:831-838.
7. **Zager RA, Fuerstenberg SM, Baehr PH, Myerson D, Torok-Storb B:** J Am Soc Nephrol 1994, 4:1588-1597.
8. **Hunter T:** Cell 1995, 80:225-236.
9. **Stamler JS:** Cell 1994, 78:931-936.
10. **Ueda N, Walker PD, Hsu SM, Shah SV:** Proc Natl Acad Sci USA 1995, 92:7202-7206.

11.   **Majno G, Joris I:** Am J Clin Pathol 1995, 146:3-15.

12. **Farber E:** Mod Pathol 1994, 7:605-609.

13. **Hockenbery D:** Am J Pathol 1995, 146:16-19.

14. **Chien CT, Chen CF, Chiang LY, Lai MK:** Fullerene Sci Techn 1999, 7: 529-540.

15. **Wei EP, Povlishock JT, Kontos HA, Moskowitz MA:** Am J Physiol 1992, 263:H1356-H1362.

16. **Paller MS, Hoidal JR, Ferris TF:** J Clin Invest 1984, 74:1156-1164.

17. **Buttke TM, Sandstrom PM:** Immunol Today 1994, 15:7-10.

18. **Basile DP, Liapis H, Hammerman MR:** Am J Physiol 1997, 272:F640-F647.

19. **Kaushal GP, Singh AB, Shah SV:** Am J Physiol 1998, 274:F587-F595.

20. **Cohen GM:** Biochem J 1997, 326:1-16.

21. **McGown AJ, Ruiz-Ruiz MC, Gorman AM, Lopez-Rivas A, Cotter TG:** FEBS Lett 1996, 39:299-303.

22. **Rembish SJ, Trush M:** Free Radic Biol Med 1994, 17:117-126.

23. **Mochida S, Masaki N, Ohta Y, Matsui A, Ogata I, Fujiwara K:** J Pathol 1992, 167:83-89.

24. **Calabrese F, Valente M, Pettenazzo E, Ferraresso M, Burra P, Cadrobbi R, Cardin R, Bacelle L, Parnigotto A, Rigotti P:** J Pathol 1997, 183:477-485.

25. **Kricka LJ:** *Anal Chem* 1993, 65:460R-462R.

26. **Takahashi A, Nakano M, Mashiko S, Inaba H:** FEBS 1990, 261:369-372.

27. **Dirnagl U, Lindauer U, Them A, Schreiber S, Pfister HW, Koedel U, Reszka R, Freyer D, Villringer A:** J Cereb Blood Flow Metab 1995, 15:929-940.

28. **Hagen TM, Huang S, Curnutte J, Fowler P, Martinez V, Wehr CM, Ames BN, Chisari FV:** Proc Natl Acad Sci USA 1994, 91:12808-12812.

29. **Gavrieli Y, Sherman Y, Ben -Sasson SA:** I J Cell Biol 1992, 119:493-501.

30. **Hsu SM, Raine L, Fanger H:** J Histochem Cytochem 1981, 29:577-580.

31. **Kiyama S, Yoshioka T, Burr IM, Kon V, Fogo A, Ichikawa I:** Kidney Int 1995, 47:536-546.

32. **Chien CT, Lee PH, Chen CF, Ma MC, Lai MK, Hsu, SM :** J Am Soc Nephrol 2001, 12:973-982.