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The role of NO in renal ischemia-reperfusion injury in rats.

Go HIRABAYASHI
(Director: Prof. Atsushi ISSHIKI)

Department of Anesthesiology, Tokyo Medical University

ABSTRACT

INTRODUCTION: Direct and real-time measurement of nitric oxide (NO) production has not been possible until recently because of its extremely short half-life. Therefore, the role of NO remains unclear and we do not know whether and when NO production is increased or decreased in renal ischemia-reperfusion injury. In this study, we used a special device with a NO sensitive electrode to determine NO levels by measuring real-time production of NO-related electron current, and measured renal NO production and renal cortex tissue blood flow (TBF) in real time to elucidate the role of NO in renal ischemia-reperfusion injury.

METHODS: Male Wistar rats were anesthetized with pentobarbital sodium, and renal artery occlusion was done with a forceps. The occlusion was released to allow reperfusion of renal vasculature for the next 60 min. Renal NO production, TBF, arterial blood pressure and heart rate were measured in real time through the study. The experimental models were divided into 4 groups; 30 or 60 min renal artery occlusion without and with L-arginine (300 mg/kg) administration before reperfusion.

RESULTS: 1) During ischemia; NO production increased sharply and peaked at 15 minutes, then gradually decreased. 2) During the early phase of reperfusion; In the non-L-arginine administered groups, the release of renal artery occlusion caused the sudden return of NO production to baseline values with a sudden increase in TBF within two or three minutes. On the other hand, groups given L-arginine showed significantly lower values of TBF than groups not given L-arginine. 3) During the late phase of reperfusion; NO production and TBF returned to baseline values in 30 min ischemia groups without and with L-arginine. On the other hand, TBF decreased significantly with no increase in NO production from the pre-occlusion values in 60 min ischemia groups without and with L-arginine.

CONCLUSIONS: It was indicated that low values of TBF during the late phase of reperfusion might be due to reduced NO activity. L-arginine administration before release of occlusion did not improve TBF.

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Key words : Nitric oxide, Renal ischemia, Renal reperfusion, L-arginine

Reprint requests to : Go Hirabayashi, Department of Anesthesiology, Tokyo Medical University, 6–7–1, Nishi-Shinjuku, Shinjuku-ku, Tokyo, 160–0023, Japan

INTRODUCTION

The endothelium derived relaxing factor (EDRF), discovered by Furchgott in 1980[1], was proved to be nitric oxide (NO) by Moncada in 1987[2]. Since then various studies have been undertaken on the physiological effects of NO and its role in pathological conditions. NO regulates blood vessel tone as a potent vasodilator, has antiplatelet adhesive properties, and can inhibit neutrophil aggregation and interaction with the endothelium[3]. In the study of renal ischemia-reperfusion injury, it has been hypothesized that ischemia-induced impairment of vascular sensitivity is due mainly to reduced EDRF activity. This hypothesis is supported by the observation that there is a blunted renal vascular response to endothelium-dependent vasodilators such as acetylcholine, bradykinin, serotonin, and histamine[4]. In a recent study, however, it has been reported that not only NO but inducible nitric oxide synthase (iNOS), and peroxinitrite (metabolic production of NO and superoxide) play important roles in renal ischemic-reperfusion injury[5~9]. The role of NO remains unclear and we do not know whether or when NO production is increased or decreased in renal ischemia-reperfusion injury. This ambiguity may be attributable to differences in experimental models and pathological stages, and differences in methods of NO determination. The molecule of NO is unstable with a short half time of 3-6 seconds. There are several methods to measure NO production, but only a few studies of real-time measurement of NO have been published. In this study, we administrated L-arginine, a precursor of NO, and measured renal NO production and renal cortex tissue blood flow (TBF) in real time to elucidate the role of NO in renal ischemia-reperfusion injury.

MATERIALS AND METHODS

Surgical protocol.

Male Wistar rats, 10–14 weeks old with a body weight of 250–350 g, were anesthetized with pentobarbital sodium (50 mg/kg intraperitoneal injection). Venous access was established in the right internal jugular vein to infuse lactated Ringer's solution (2 ml/h), and the caudal artery was cannulated for arterial pressure measurement. In the prone position, a paramedian incision on the left side was performed to expose the left kidney and renal artery, and hemostasis was done with a forceps. The rectal temperature was continuously monitored and maintained at 37°C by means of a temperature controller (ATC-101B, Unique Medical Co., Tokyo, Japan) throughout the experiment.

Measurement of renal NO production.

The direct and real-time measurement of renal NO production was carried out with a nitric oxide monitoring device (NO-501, Inter Medical Co., Nagoya, Japan). Diffusion current was calibrated as 1000 pA = 1 μmol/ml[10]. The NO-sensitive electrode 0.2 mm in diameter was inserted into the renal cortex 2 mm deep.

Measurement of renal cortex tissue blood flow (TBF).

Renal cortex TBF (ml/min/100 g) was measured in real time with a laser Doppler flowmeter (FLO-C-1, Omegawave Co., Tokyo, Japan). This method is non-invasive and can measure even very low tissue blood flow. TBF was expressed as voltage which is well correlated with blood flow measured by the conventional hydrogen clearance method[11]. The laser electrode was wedged between the kidney surface and aluminum plate and fixed with adhesive.

Experimental protocol.

Twenty-four animals were divided into 4 groups. Group 1 and Group 1L: 30 min renal artery occlusion without and with L-arginine administration, Group 2 and Group 2L: 60 min occlusion without and with L-arginine. Baseline NO production and TBF were measured, and the left renal artery was then occluded for 30 or 60 min. Toward the end of occlusion, L-arginine (300 mg/kg) was administrated in Group 1L and Group 2L. The occlusion was released in all groups after 30 minutes or 60 minutes arterial occlusion to allow reperfusion of renal vasculature for the next 60 min. Renal NO production, renal cortex TBF, arterial blood pressure and heart rate were measured in real time throughout the study.

Statistical analysis.

Results were described as mean ± SD and non paired t-test was used for the comparison

between groups, and paired t-test was used for intragroup comparison. A P value of less than 0.05 was considered significant.

RESULTS

Vital Signs.

No significant difference was observed in mean artery pressure or heart rate among the four groups.

Measurement of renal NO production.

Time course changes in renal cortex NO production are shown in Fig. 1 (Group 1 and Group 1L) and Fig. 2 (Group 2 and Group 2L). Pre-occlusion values (baseline) were 4.2 ± 1.2 μmol/ml in Group 1, 5.0 ± 2.7 μmol/ml in Group 1L, 4.2 ± 3.5 μmol/ml in Group 2, and 5.4 ± 2.9 μmol/ml in Group 2L, respectively. There were no significant differences among these values. The renal artery occlusion caused a significant increase in NO production, and values at 15 min were 19.2 ± 7.1 μmol/ml in Group 1, 21.7 ± 8.5 μmol/ml in Group 1L, 16.8 ± 6.7 μmol/ml in Group 2, and 19.2 ± 8.2 μmol/ml in Group 2L, respectively. The NO production gradually decreased toward the end of artery occlusion. The release of renal artery occlusion caused a sudden return of NO production to baseline values in all groups within 5 minutes. Thereafter, NO production stabilized at low values until the end of study, and values at 60 min after reperfusion were 3.4 ± 1.5 μmol/ml in Group 1, 4.7 ± 2.3 μmol/ml in Group 1L, 3.0 ± 1.6μ mol/ml in Group 2, and 4.9 ± 2.8 μmol/ml in Group 2L, respectively.

Measurement of renal cortex TBF.

Time course changes in renal cortex TBF are shown in Fig. 3 (Group 1 and Group 1L), and Fig. 4 (Group 2 and Group 2L). Pre-occlusion TBF (baseline) were 35.6 ± 2.9 ml/min/100 g in Group 1, 31.3 ± 3.3 ml/min/100 g in Group 1L, 33.2 ± 3.6 ml/min/100 g in Group 2, and 32.5 ± 2.3 ml/min/100 g in Group 2L, respectively. There were no significant differences among these values. The release of occlusion caused a sudden increase in TBF in non L-arginine administered groups: 32.1 ± 3.1 ml/min/100 g in Group 1, and 26.0 ± 5.6 ml/min/100 g in Group 2 at 1 minute. On the other hand, TBF in L-arginine administered groups increased slowly: 9.9 ± 3.5 ml/min/100 g in Group 1L, and 11.1 ± 3.0 ml/min/100 g in Group 2L at 1 minute. These values were significantly lower than corresponding 1

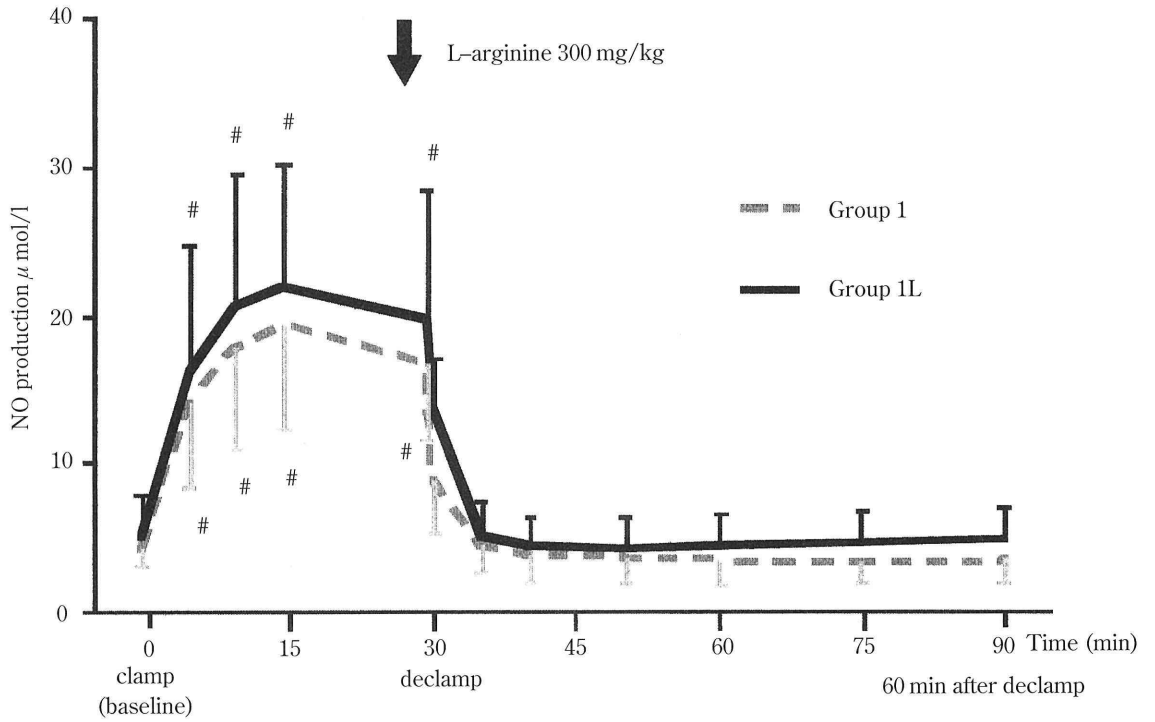

Fig. 1 Changes of renal NO production in Group 1 and Group 1L.
Group 1 = 30 min renal artery occlusion, Group 1L = 30 min renal artery occlusion with L-arginine. Data are means ± SD.
#$p < 0.05$ versus baseline.

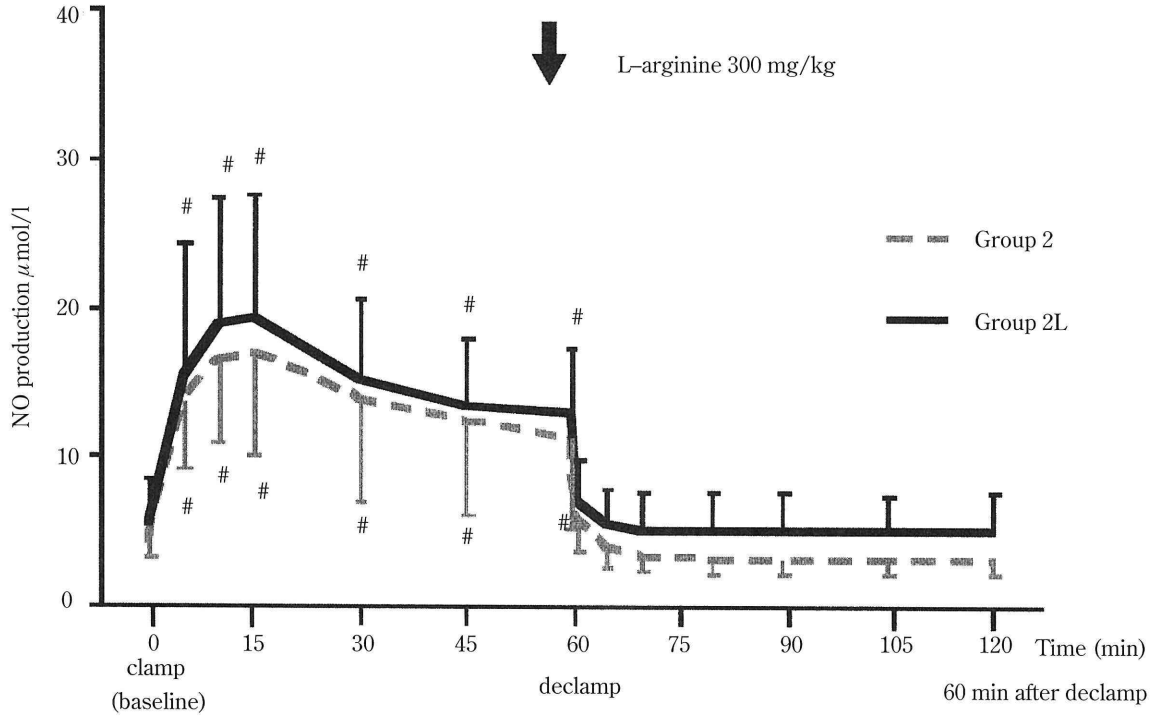

Fig. 2 Changes of renal NO production in Group 2 and Group 2L.
Group 2 = 60 min renal artery occlusion, Group 2L = 60 min renal artery occlusion with L-arginine. Data are means ± SD.
#p < 0.05 versus baseline.

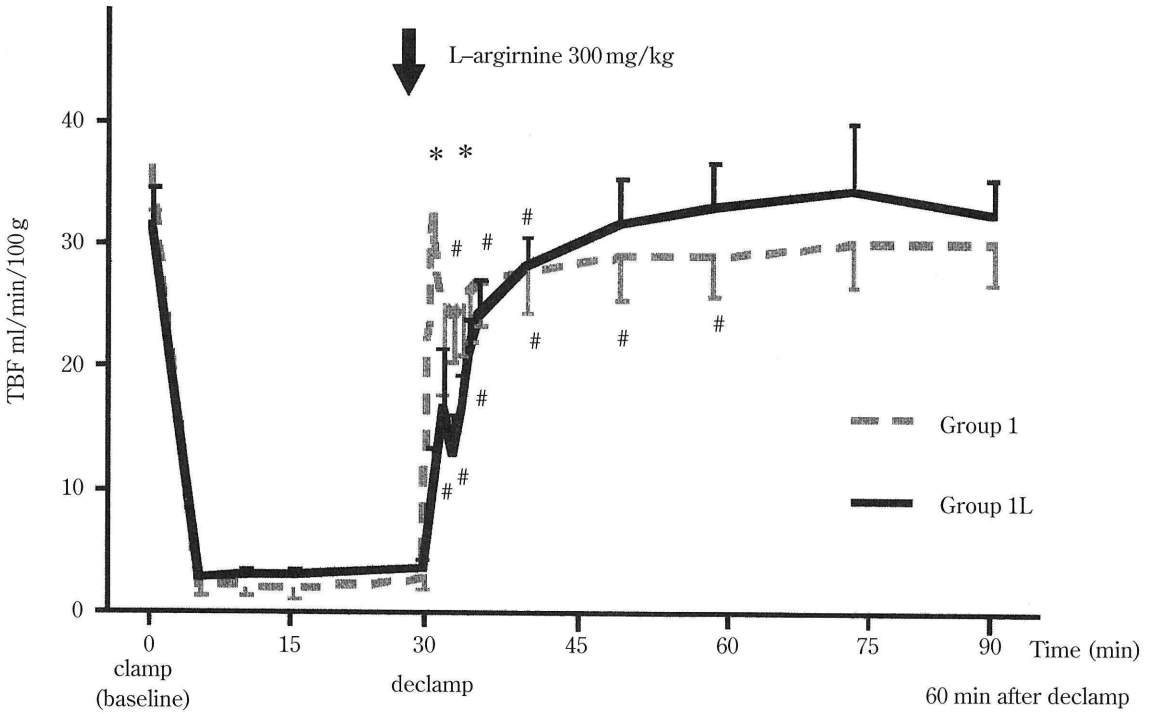

Fig. 3 Changes of renal cortex tissue blood flow in Group 1 and Group 1L.
Group 1 = 30 min renal artery occlusion, Group 1L = 30 min renal artery occlusion with L-arginine. Data are means ± SD.
* p < 0.05 compared with group 1L. #p < 0.05 versus baseline .

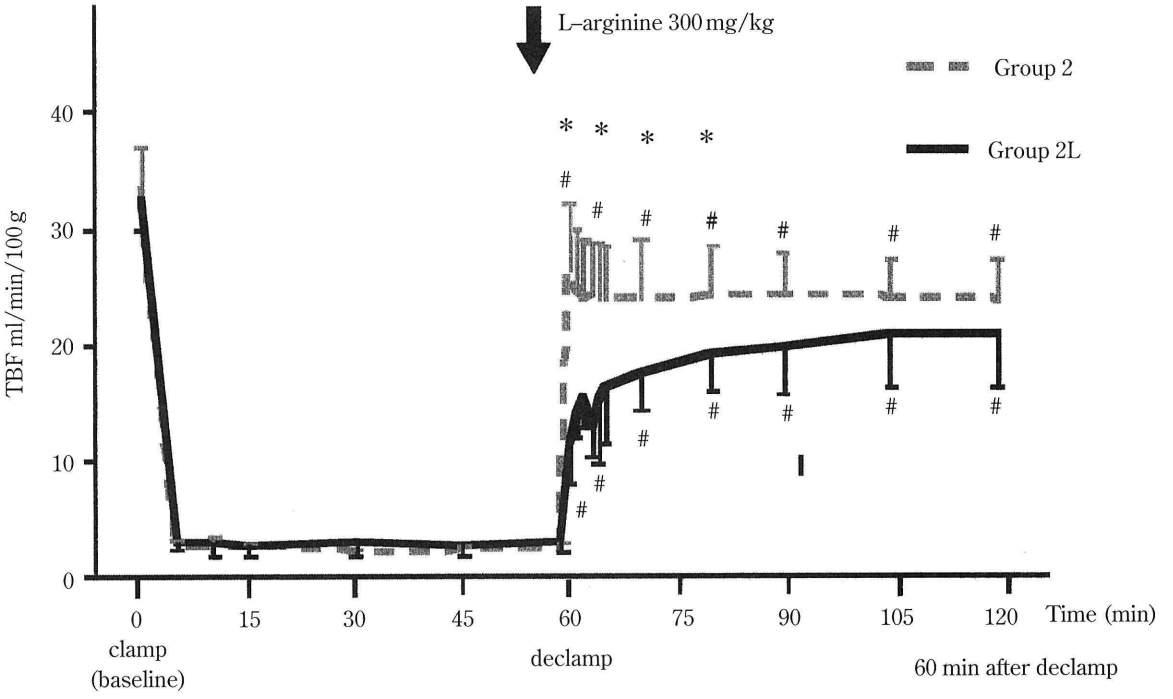

Fig. 4 Changes of renal cortex tissue blood flow in Group 2 and Group 2L.
Group 2 = 60 min renal artery occlusion, Group 2L = 60 min renal artery occlusion with L-arginine. Data are means ± SD.
*$p < 0.05$ compared with group 2L . #$p < 0.05$ versus baseline .

minute values in Group 1 and Group 2. TBF 60 minutes after release of occlusion was 30.4 ± 3.4 ml/min/100 g in Group 1, 32.3 ± 2.8 ml/min/100 g in Group 1L, 23.5 ± 3.7 ml/min/100 g in Group 2, and 21.0 ± 4.7 ml/min/100 g in Group 2L, respectively. In Group 2 and Group 2L, these values were significantly lower than baseline values.

DISCUSSION

It has been shown previously that the serum and urinary levels of nitrite (NO_2) and nitrate (NO_3) correlate with endogenous whole body NO production. However, these measurements were not obviously useful in determining real-time production of NO in particular organs such as kidneys. Direct and real-time measurement of NO production has not been possible until recently because of its extremely short half-life. In this study, we used a special device with a NO sensitive electrode to determine NO levels by measuring real-time production of NO-related electron current.

The role of NO during ischemia.

It has been reported that NO production has been found to be increased by ischemia in all organs, regardless of how NO was measured. Ischemic stress releases norepinephrine from the sympathetic nerve endings, and alpha 1-adrenoceptor (adenylyl cyclase system and protein kinase C system) activity enhances the mechanisms whereby NO is produced and released from the ischemic myocardium[12~14]. Acute hypoxia (Po_2 averaged 20–40 mmHg for 15 min) stimulates NOS activity in isolated proximal renal tubular cells to increase NO production[7]. On the other hand, it has been found that prolonged hypoxia (Po_2 averaged 20 mmHg for 24 hours) inhibits NO production because the steady-state levels of endothelium NOS mRNA are reduced under profound and prolonged hypoxic condition[15].

As shown in Fig. 1 and Fig. 2, NO production increased sharply during renal artery occlusion and peaked at 15 minutes, then gradually decreased. It has been suggested that elevation of Ca^{2+} in endothelial cells by activated alpha 1-adrenoceptor or hypoxic stress activates constitutive NOS (cNOS), and NO production increases sharply during the early phase of renal artery occlusion. On the other hand, mechanisms involved in the gradual reduction of NO levels are unknown. The

mechanisms may be impaired enzymatic function and inhibited NOS activity secondary to prolonged hypoxia or lack of oxygen during the late phase of renal artery occlusion.

The role of NO during the early phase of reperfusion.

Previous studies have shown that ischemia-reperfusion injury is caused by an increase in renal vascular resistance with a resultant decrease in renal blood flow, and L-arginine administration improved but NOS inhibitor administration worsened renal hemodynamics[16~18]. Therefore, it is generally agreed that ischemia-related impaired vascular sensitivity is due mainly to reduced EDRF/NO activity in renal ischemia-reperfusion injury.

Recent studies, however, have indicated that there is a growing ambiguity about the role of NO and its metabolic product, peroxynitrite, in early ischemia-reperfusion injury. NO itself is cytotoxic since it is a free radical. Probably the most important event for cellular injury is the rapid reaction between NO and superoxide, producing NO metabolite, peroxynitrite. Peroxynitrite decays and generates the highly toxic hydroxyl radical, thus accounting for cytotoxicity during the early phase of reperfusion. In fact, recent studies demonstrated that administration of L-arginine, a precursor of NO, resulted in more significant tubular cell injury, leading to acute renal failure. In contrast, NOS inhibitor administration protects the renal tubular cell from ischemia-reperfusion insult[19~21].

As shown in Figures 1–4, in the non-L-arginine administered groups, the release of renal artery occlusion caused the sudden return of NO production to baseline values with a sudden increase in TBF (hyperemia) within two or three minutes. On the other hand, L-arginine administered groups showed significant lower values of TBF than the non-L-arginine administered groups. There were no significant differences in NO levels regardless of whether or not L-arginine was administered. However, momentarily increased NO secondary to L-arginine administration in the very early stage of reperfusion may not be measurable because of the extremely rapid reaction with superoxide and hemoglobin. It is, however, speculated that a lack of immediate increase in renal cortical TBF in the L-arginine administrated groups might have been due to excessive peroxynitrite secondary to increased NO production.

The role of NO during the late phase of reperfusion.

Several studies showed that renal NOS levels were significantly increased during reperfusion. Conger et al indicated that NOS/NO activity is maximal at baseline at 1 week and cannot be increased further by exogenous stimuli of NOS activity in a model of noradrenaline-induced renal failure[22]. On the other hand, Schramm et al suggested that the production of NO reduced during the late phase of reperfusion[23]. Weight et al reported that the DNA damage increased with the duration of ischemia and NOS activity was inhibited during the early phase of reperfusion (20 min). But, by 80 min of reperfusion, DNA damage had returned towards control values[24]. In this regard, some different observations on the role of NO during reperfusion may be explained by different duration of ischemia or hypoxia, and the different NO measurement points.

In this study, NO production was measured in real time up to 60 minutes after reperfusion. NO production sharply decreased to pre-occlusion values after release of renal artery occlusion and stabilized at low values until the end of study. NO production did not increase from the pre-occlusion values, although TBF decreased significantly 60 min after reperfusion in Group 2 and Group 2L. It is conceivable that the longer the ischemic time (30 min vs 60 min), the more damage to the renal vascular beds so that the function of NO as EDRF appeared to be inhibited. Two biochemically different isoforms of NOS have been identified: the constitutive form (cNOS) and the inducible form (iNOS). It is unlikely that iNOS was stimulated by ischemia during the period of our experiment. The iNOS usually requires hours of stimulation, but once stimulated, it generates greater amounts of NO for a long time. On the other hand, the cNOS activity may have been inhibited possibly secondary to impaired enzymatic function, leading to decreased NO production with reduced EDRF function during the late stage of reperfusion.

In conclusion, it was indicated that low values of TBF during the late phase of reperfusion might be due to reduced NO activity.

Then, the L-arginine administration before release of occlusion did not improve TBF.

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ラット腎虚血及び再灌流障害における一酸化窒素の役割

平　林　　剛

東京医科大学麻酔学教室
（指導：一色　淳主任教授）

目的：一酸化窒素（NO）は不安定で，半減期が極度に短く，従来はNOを直接測定することは困難とされていた．従って，腎虚血再灌流障害におけるNOの変動はいまだ明らかにされていない．本研究ではラット腎虚血再灌流モデルにおける腎皮質NO産生量を直接かつ経時的に測定し，同時に腎皮質血流量を測定して，NOの役割を検討した．

方法：ペントバルビタール麻酔下の雄ウィスター系ラットを用い，レーザードップラー血流計及び電気化学法によるNO測定装置（NO-501）を使用した．対照群は左腎動脈を30分または60分間遮断し，再灌流後60分間まで動脈血圧，心拍数，腎皮質NO産生量と腎皮質血流量を経時的に測定した．実験群は再灌流前にLアルギニン（300 mg/kg）を投与した．

結果：1）腎虚血時，NO産生量は急激に増加した．約15分後に最高値に達した後，漸減した．2）再灌流直後，腎皮質血流量は対照群では30分60分虚血群ともに速やかに回復したが，実験群では両群とも対照群に対し有意に低下した．この時のNO産生量は全ての群において急激に遮断前値まで復した．3）再灌流45～60分後においては，30分間虚血Lアルギニン投与及び非投与群の腎皮質血流量及びNO産生量はともに遮断前値まで回復した．しかし，60分間虚血Lアルギニン非投与群では腎皮質血流量が遮断前値より有意に低下したにもかかわらずNO産生量は遮断前値よりも低値を示す傾向を認め，Lアルギニン遮断前投与によっても改善を認めなかった．

結論：再灌流45～60分後における血流障害はNO産生障害によるものと推測された．それに対して，Lアルギニンを再灌流前に投与しても明らかな血流改善効果は認めなかった．

キーワード：一酸化窒素，腎虚血，再灌流障害，Lアルギニン