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# Real-time monitoring of nitric oxide in ischemia-reperfusion rat kidney

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**Abstract** In this study we attempted to clarify the release of nitric oxide (NO) and its role in the ischemia-reperfusion rat kidney. After right nephrectomy, male Wistar rats were divided into four groups: one sham operated and three groups who underwent ischemia (30 min) and reperfusion of the left renal artery. Thirty minutes prior to ischemia-reperfusion, two groups were injected intraperitoneally with 10 and 30 mg/kg of $N^G$-nitro-L-arginine methylester (L-NAME). Real-time monitoring of blood flow and NO release in the rat kidney was measured with a laser Doppler flowmeter and an NO-selective electrode, respectively. Serum creatinine and blood urea nitrogen (BUN) levels were measured 1 and 7 days after the induction of ischemia-reperfusion. Clamping of the renal artery decreased blood flow to 1–5% of the basal level measured before clamping. After removal of the clip, the blood flow of the 30 mg/kg L-NAME rats was significantly lower than that of the controls. Immediately following the clipping of the renal artery, NO release rapidly increased. After removing the clip, NO release immediately returned to three-quarters of the basal level. Serum creatinine and BUN levels of the ischemia-reperfusion rats were slightly but not significantly higher and those of 30 mg L-NAME rats were significantly higher than those of the control or ischemia-reperfusion rats 1 day and 7 days after ischemia-reperfusion. Our data suggest that NO acts as a cytoprotective agent in ischemia-reperfusion injury of the rat kidney.

**Key words** Kidney · Nitric oxide · Ischemia-reperfusion injury · Rat

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## Introduction

Ischemia-reperfusion injury in the kidney is often observed in renal operations such as transplantation, partial nephrectomy and enucleation of renal cell carcinoma. Nitric oxide (NO) is reported to contribute the maintenance of renal circulation and urine formation [1]. The inhibition of NO synthase has been reported to reduce renal blood flow, urine flow rate, and urinary $Na^+$ excretion in experimental studies [5, 8, 13]. Recent studies have demonstrated that ischemia-reperfusion injury in the kidney is in some part associated with free radicals [7] and NO [12]. Several reports have suggested that the NO-L-arginine pathway is involved in most of renal defense mechanisms which act against ischemic insult and its consequences [12]. Schramm et al. reported that treatment with L-arginine produced a significant improvement in glomerular filtration rate (GFR) in acute renal failure rats [16]. On the other hand, peroxynitrite, produced from the reaction of NO and superoxide, is reported to have harmful effects on various tissues during ischemia-reperfusion [10]. Espinosa et al. reported that L-arginine significantly increased renal ischemic damage, as judged by histological data [4]. Although some reports have indicated NO's important role in ischemia-reperfusion injury in the kidney, the mechanism of NO release is still unclear. In this study, we attempted to clarify the role of NO and NO release in ischemia-reperfusion injury in rat kidney.

## Material and methods

### Production of the animal model

All animal experiments were performed in accordance with the guidelines set by the Tottori University Committee for Animal Experimentation. Male Wistar rats, 8 weeks old, weighing 250–300 g (SLC, Shizuoka, Japan), were divided into four groups: those undergoing 30 min ischemia and reperfusion (I-R), those with 30 min ischemia and reperfusion accompanied by treatment with 10 mg/kg $N^G$-nitro-L-arginine methylester (L-NAME), those

with the same amount of ischemia-reperfusion accompanied by 30 mg/kg of L-NAME (10-NA, 30-NA), and sham-operated rats (C) ($n = 8$ in each group). Right nephrectomy was performed on all rats 30 min prior to the induction of ischemia. Our preliminary experiment revealed that right nephrectomy does not affect left renal blood flow within 30 min. Rat kidneys were subjected to ischemia following reperfusion according to previous reports with minor modification [14] In short, under pentobarbital anesthesia (30 mg/kg, i.p.), the left renal artery was clamped with a small clip (Sugita standard aneurysm clip, holding force 145 g, Mizuho Ikakogyo, Tokyo, Japan) for 30 min in the I-R, 10-NA, and 30-NA groups. Thirty minutes after the removal of the clip, rats were allowed to establish reperfusion. In the 10-NA and 30-NA groups, L-NAME (10 and 30 mg/kg) was injected i.p. 30 min prior to the ischemia. One day and 7 days after the induction of ischemia-reperfusion, blood samples were collected to measure serum creatinine and serum BUN levels using creatinine kit and BUN kit (Wako, Osaka, Japan), respectively.

### Measurement of blood flow in the kidney

Blood flow in the rat kidney was measured with a laser Doppler flowmeter (BRL-100, Bioresearch Co., Nagoya, Japan) as previously reported with a minor modification [15, 19]. Briefly, under pentobarbital anesthesia, the probe was attached to the rat kidney and measured the blood flow before, during, and after ischemia. The effect of L-NAME (30 mg/kg) on blood flow in the rat kidney was also evaluated.

### In vivo real-time monitoring of NO release in the rat kidney

Real-time monitoring of NO release in the rat kidney was conducted in the experimental groups as reported previously, with minor modifications [6, 15, 20]. In brief, an anesthetized rat was placed on a heating plate (37 °C), and the kidney was accessed via a central incision of the upper abdomen. An NO-selective electrode (NOE-47, tip diameter of 0.2 mm, Inter Medical Co., Nagoya, Japan) was inserted into the cortex of the left rat kidney. The reference electrode was placed in subcutaneous tissue. NO was measured with a NO-monitor (Model NO-501, Inter Medical Co., Nagoya, Japan) and expressed in terms of a current in picoamperes (pA). Our preliminary observations indicated that the electrode current increased with the concentration of an NO donor, *S*-nitro-*N*-acetylpenicillamine (SNAP) in a linear fashion, and that the electrode was not affected by 100 mM nitrite, or nitrate, or by the surperoxide delivered from the xanthine–xanthine oxidase system in PBS buffer [15, 20].

### Histological examination of the rat kidney

Sections (5 μm) were stained with hematoxylin and eosin (H&E) and viewed to assess morphological changes. After 1 day and 7 days following the induction of ischemia, the rat kidneys of each group were immediately removed and fixed with 3.7% formaldehyde-saline. After the fixation, the tissues were embedded in paraffin.

### Data analysis

For monitoring of in vivo NO release in the rat kidney, the electrode was calibrated using an NO donor SNAP according to previous reports as follows [6, 15, 20]

$$\mathrm{NO(pA)} = 4941 \times \mathrm{SNAP\ (mM)} - 680$$

$$\mathrm{NO\ (mM)} = 1.3 \times 10^{-3} \times \mathrm{SNAP\ (M)}$$

Statistical analysis of the differences between groups was performed using analysis of variance and the multiple comparison Fisher's test. $P \leq 0.05$ was regarded as the level of significance.

### Drugs and chemicals

L-NAME, SNAP and pentobarbital were purchased from Sigma (St Louis, MO, USA). All other chemicals were of reagent grade.

## Results

### Serum BUN and serum creatinine levels in the rat

Table 1 shows serum BUN and serum creatinine levels in the experimental animals. Thirty minutes of ischemia and subsequent reperfusion did not significantly increase the serum BUN and creatinine levels in the rat 1 day or 7 days after ischemia-reperfusion induction. Treatment with 10 mg/kg of L-NAME and treatment with 30 mg/kg of L-NAME increased the serum creatinine and serum BUN levels compared to the C or I-R groups measured 1 day and 7 days after ischemia-reperfusion induction. The inhibition of NO production decreased the renal function, as indicated by serum creatinine and BUN levels.

### Measurement of blood flow in the kidney

Table 2 and Fig. 1 demonstrate the blood flow during ischemia-reperfusion in the rat kidney. Clamping of the rat left renal artery decreased blood flow to 1–5% of the

**Table 1** Serum BUN and creatinine in experimental animals. I-R, 10-NA, 30-NA and C indicate 30 min ischemia and reperfusion, 30 min ischemia and reperfusion with treatment of 10 and 30 mg/kg L-NAME, and age-matched controls, respectively. Data are mean ± S.E.M. 6–8 separated determinations in each group. $P < 0.05$ is level of significance

| | Serum BUN (mg/dl) | | Serum creatinine (mg/dl) | |
|---|---|---|---|---|
| | 1 day | 1 week | 1 day | 1 week |
| C | 20.7 ± 1.4 | 17.5 ± 0.8 | 0.68 ± 0.02 | 0.36 ± 0.05 |
| I-R | 38.3 ± 5.0 | 21.9 ± 3.1 | 0.92 ± 0.09 | 0.45 ± 0.09 |
| 10-NA | 68.6 ± 14.4[a,b] | 23.3 ± 2.6[d] | 1.48 ± 0.32 | 1.18 ± 0.34 |
| 30-NA | 93.4 ± 17.4[a,b] | 28.7 ± 4.4[a,d] | 2.57 ± 0.48[a,b] | 4.60 ± 1.03[a–d] |

[a] Significantly different from C
[b] Significantly different from I-R
[c] Significantly different from 10-NA
[d] Significantly different from corresponding values in the same group for day one

**Table 2** Blood flow and release of nitric oxide in the rat kidney. Rats pretreated with L-NAME (30 mg/kg) or without (control) were subjected to the ischemia (30 min) and reperfusion by clamping of the left renal artery. Data are mean ± S. E. M. of 6–7 separate determinations in each group. $P < 0.05$ is level of significance

| | Basal level | During ischemia (% to basal) | During reperfusion (% to basal) |
|---|---|---|---|
| Blood Flow (mV) | | | |
| L-NAME (-) | 171.4 ± 19.0 | 2.6 ± 0.2[a] (1.5) | 131.8 ± 5.9[a,c] (76.9) |
| L-NAME (30 mg/kg) | 121.8 ± 20.9 | 3.8 ± 1.9[a] (3.1) | 86.8 ± 11.4 (71.3) |
| NO release (mM) | | | |
| L-NAME (-) | 2.0 ± 0.8 | 26.6 ± 3.4[a,b] (1327) | 2.3 ± 1.3 (118) |
| L-NAME (30 mg/kg) | 3.9 ± 1.0 | 13.8 ± 4.1[a] (350) | 3.6 ± 0.9 (91.9) |

[a] Significantly different from basal level
[b] Significantly different from corresponding values in L-NAME treated group
[c] Significantly different from basal and ischemia level

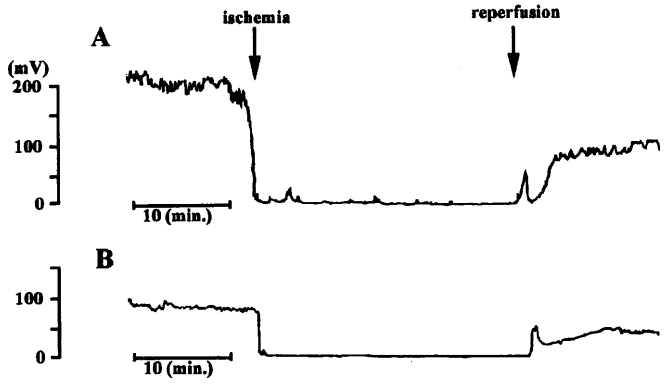


**Fig. 1A, B** Representative tracing of blood flow in rat kidney. Rats were subjected to the ischemia-reperfusion (indicated by arrows) and blood flow was monitored using a laser Doppler flowmeter **(A)**. Blood flow was also monitored in kidney of rats treated with L-NAME (30 mg/kg) 30 min before the ischemia **(B)**

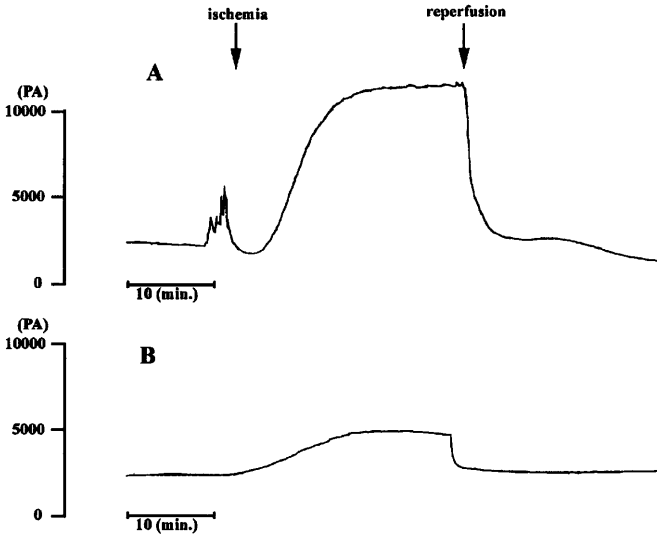


**Fig. 2A, B** Representative tracing of nitric oxide (NO) release in rat kidney cortex. Rats were subjected to the ischemia-reperfusion (indicated by arrows) and NO was monitored using the selective electrode inserted in rat kidney cortex without **(A)** or with **(B)** L-NAME (30 mg/kg) 30 min before the ischemia

basal level measured before the clamping (Table 2). After removal of the clip, the blood flow recovered to three-quarters of the basal level within 5 min. When rats were treated i.p. with L-NAME (30 mg/kg) 30 min before the ischemia, blood flow in the kidney was slightly but not significantly decreased compared to that of controls. In the recovery phase, however, blood flow in the L-NAME treated rat was significantly smaller than that in the control group (Table 2). Our data indicated that treatment with L-NAME directly or indirectly decreased the blood flow in the rat kidney, especially in the recovery phase.

## In vivo NO release in the kidney

In addition to examining the effect of L-NAME, in order to clarify the mechanisms of NO release in ischemia-reperfusion injury, we used a NO-selective electrode to monitor NO release in the rat kidney. Released NO levels in the rat renal cortex before, during, and after the induction of ischemia are summarized in Fig. 2 and Table 2. The time-course of NO release is shown in Fig. 2. Immediately following the clipping of the renal artery, NO release rapidly increased, and it reached a plateau (a 13-fold increase on the basal level) approximately 10–15 min after the clipping of the renal artery. In contrast, when a NO electrode was inserted into the rat kidney treated with 30 mg/kg L-NAME, only a slight increase in NO release was observed (Fig. 2). NO release in the L-NAME treated rat kidney was significantly inhibited compared to that in the L-NAME untreated rat kidney. After removing the clip, NO release returned to almost the basal level. There was no significant difference in NO release before or after the ischemia (Table 2). These data suggest that treatment with L-NAME (30 mg/kg) significantly inhibits the increase of NO release during ischemia, but not the basal level nor during reperfusion.

## Histological examination of the rat kidney

Figure 3 shows H&E staining of the rat kidney. Samples were taken 30 min after the induction of reperfusion and

7 days after the induction of ischemia-reperfusion. In comparison with control rat kidneys, the ischemia-reperfusion kidneys treated with or without L-NAME (30 mg/kg) demonstrated histological damage: numerous red blood cells resulting from vessel extravasation, rupture of the microcirculation and leukocyte infiltration in the cortex of the experimental rat kidneys (Fig. 3). Seven days after ischemia-reperfusion, however, significant alteration was not detected by light microscopic observation in any groups.

## Discussion

This is the first report describing the direct monitoring of NO release in the kidney during ischemia-reperfusion. Serum creatinine and BUN levels of the 30 mg L-NAME treated rats were significantly higher than those of control or ischemia-reperfusion rats without L-NAME treatment. Clamping of the rat renal artery decreased blood flow to 1–5% of the basal level measured before the clamping. During reperfusion, the blood flow of 30 mg L-NAME rats was significantly lower than that of the controls. Immediately following the clipping of the renal artery, NO release rapidly increased (a 13-fold increase on basal level). After removing the clip, NO release immediately returned to the basal level. In histological studies, the ischemia-reperfusion rat kidney with or without L-NAME treatment showed vessel extravasation, ruptures of the microcirculation, and leukocyte infiltration. Histological observation of the experimental rat kidney also supported our biochemical and physiological data. Our data suggest that NO acts as a cytoprotective agent in ischemia-reperfusion rat kidney.

NO is synthesized from L-arginine by NO synthase (NOS), which is widely distributed in the kidney. Constitutive and calmodulin dependent NOS, cNOS, is synthesized in the endothelial cells of glomerular vessels, vasa recta, and also in tubular epithelial cells. iNOS is expressed in the mesangial and tubular cells [12], while NO is an important molecule in the maintenance of normal diuresis, natriuresis, and glomerular filtration rate [18]. In this study, the response to ischemia-reperfusion of NO release is prompt and dynamic. Just after the clamp of the left renal artery, NO release in the renal cortex is rapidly increased, and within 15 min it reached a plateau. This suggests that activation of NOS in the kidney is first phase of ischemia, and that NO is an important molecule to maintaining homeostasis. In the present study, we attempted to directly monitor NO release in the rat kidney during ischemia-reperfusion. The ischemia caused an increase in NO release in the rat kidney (a 13-fold increase on basal level), and

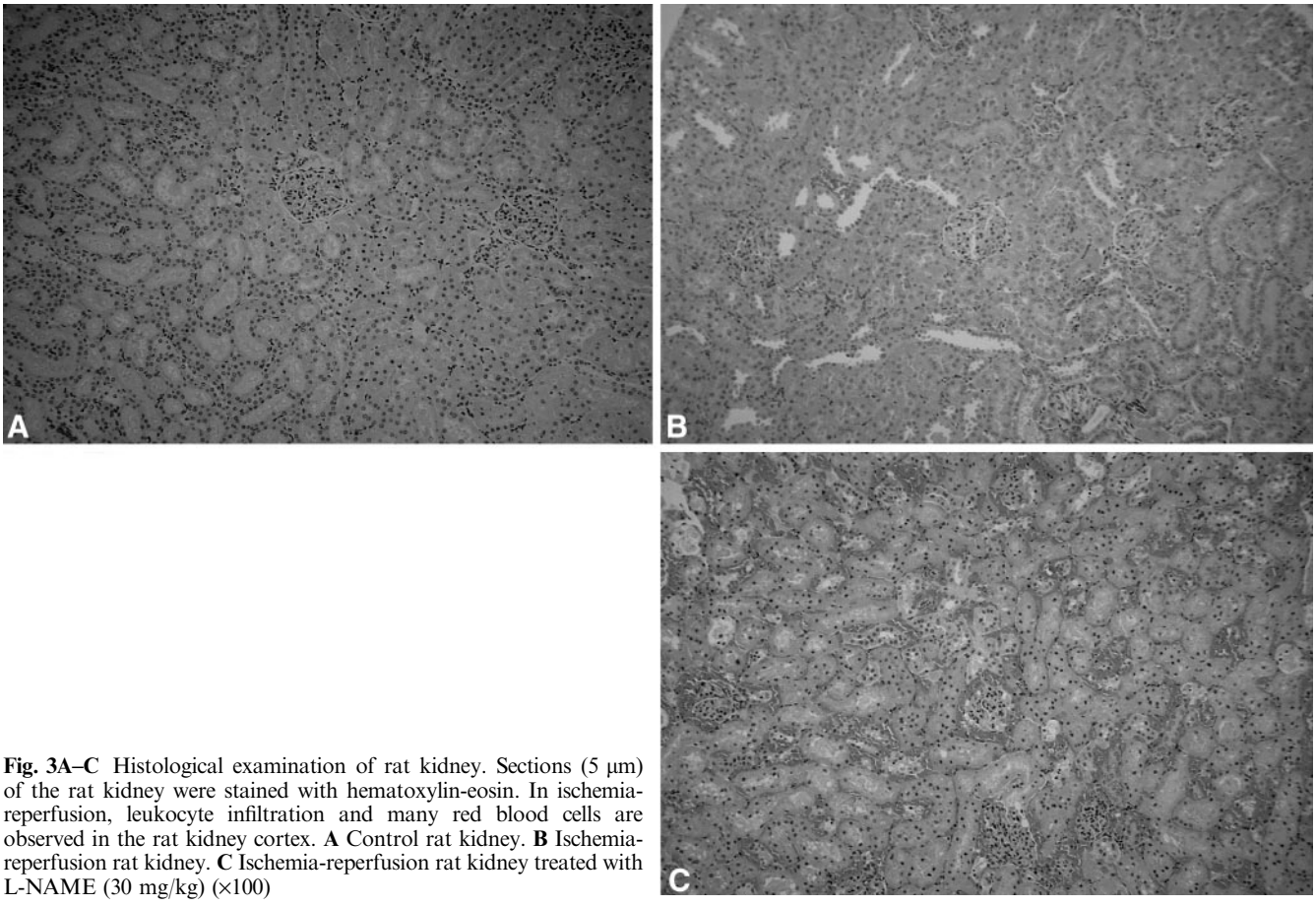


**Fig. 3A–C** Histological examination of rat kidney. Sections (5 μm) of the rat kidney were stained with hematoxylin-eosin. In ischemia-reperfusion, leukocyte infiltration and many red blood cells are observed in the rat kidney cortex. **A** Control rat kidney. **B** Ischemia-reperfusion rat kidney. **C** Ischemia-reperfusion rat kidney treated with L-NAME (30 mg/kg) (×100)

reperfusion immediately returned NO release to the basal level. The changes in NO release during ischemia-reperfusion suggest that the kidney may maintain the necessary blood supply via the circulatory system by dilating arteries with released NO during the ischemia-reperfusion rather than that accumulated NO is used as a source of peroxynitrite formation in the kidney.

The mechanisms of ischemia-reperfusion injury are complicated; during the ischemic phase, the endothelium is primed both to produce free radicals and to secrete chemokines, while in reperfusion, activated leucocyte-mediated tissue injury has been reported to be caused via these main pathways, production of free radicals via the respiratory burst, release of intrinsic enzymes, and physical obstruction of capillaries [3, 9]. NO prevents the polymorphonuclear neutrophil (PMN) component of ischemic renal injury by blocking PMN retention and the deleterious effects of activated PMN on glomerular and tubular function [9, 12]. On the other hand, reactive oxygen metabolites are formed during and following ischemic injury. Peroxynitrite, reacting from NO and superoxide radicals, is also reported to be a highly reactive compound that has harmful effects on various cells and tissues [3, 10]. Thus, NO is regarded to be cytoprotective and cytotoxic during ischemia-reperfusion in many cells and tissues.

Tome and associates reported that in the renal artery clamping model using rats, administration of L-NAME protects the recovery of GFR, functional clearance of Na (FENa), and water excretion and has the deleterious effect of NOS inhibition on those parameters [17]. Furthermore, NO is reported to be a cell protecting agent in the kidney against ischemia-reperfusion injury [2, 11]. In contrast to these findings, Espinosa et al. reported that L-arginine did not affect the impaired renal blood flow (RBF) and GFR and significantly increased renal damage as evaluated by histological data [4].

As the movement of NO release in the kidney during ischemia-reperfusion is still unclear, we attempted the direct monitoring of NO release in rat kidneys during ischemia-reperfusion in the present study. The ischemia caused an increase in NO release in the rat kidney, and reperfusion immediately returned NO release to the basal level. The changes in NO release during ischemia-reperfusion suggest that the kidney may maintain the necessary blood supply via the circulatory system by dilating arteries with released NO during the ischemia. Thus, NO directly and/or indirectly prevents renal function on ischemia-reperfusion injury. This increase in NO production may prevent adhesion and infiltration of polymorphonuclear neutrophil leukocytes (PMN). In reperfusion, NO release in the kidney was rapidly decreased. Wada and associates explained that a rapid fall in NO current just after removal of the clamp reflects the quenching of NO by the superoxide radicals generated upon reoxygenation rather than the rapid diffusion of NO by recovered blood flow in the rat stomach, and reported NO as a cytotoxic agent during ischemia-reperfusion injury [20]. Furthermore, we reported the deleterious effect of nitric oxide on the rat urinary bladder during ischemia-reperfusion [14]. In addition, Yu et al. reported that NO works as a cytotoxic agent in renal tubular ischemia-reperfusion injury [21]. NO has both cytotoxic and cytoprotective effects on ischemia-reperfusion injury in many tissues. The important question remains as to why the role of NO is contrary between organs during ischemia-reperfusion.

From this study the following conclusions can be drawn: (1) ischemia-reperfusion induced by clamping the rat renal artery caused histological damage of the renal cortex, (2) administration of L-NAME during ischemia-reperfusion reduced the blood flow to the kidney as compared to the controls , (3) increase in NO release during ischemia returned to the basal level immediately after reperfusion and (4) ischemia-reperfusion injury was prevented by the effects of NO measured by serum BUN and creatinin.

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## ANNOUNCEMENTS

# 2000

### WHO Consensus Conference: Public Health and Clinical Significance of Premalignant Alterations in the Genitourinary Tract June 8–9 2000, Stochholm, Sweden

*Information:* Prof. Lennart Andersson, WHO Collaborating Center for Urologic Tumors, Karolinska Hospital, SE-171 76 Stockholm, Sweden; Fax +46-8-32 61 13, e-mail: Lennart.Andersson@kirurgi.ki.se

### Urology 2000: State of art-looking at the future 14–15 June 2000, Lipari, Eolian Islands

*Topics:* Vaginal and abdominal prolapse repairs, rationale for urologic cancer lymphadenectomy, future perspectives in reconstructive surgery using tissue engineering, bladder overactivity, the ageing male: a challenge for urologist, how to manage male incontinence, erectile dysfunction: present and future, continent urinary diversion, prostate cancer, gene therapy for urological cancer, endourology, laparascopy, reconstructive urology, paediatric urology, urodynamics and BPH, urodynamics and female incontinence, superficial bladder cancer, invasive bladder cancer, kidney cancer.

*Information:* Prof. Carlo Aragona, Chairman and director of the Course, Unit Operativa di Urologia, Azienda Ospedallera Papardo, Contrada Papardo, 98158 Messina, Italy; Tel: ++39 090 3992491, ++39 090 3992281, Fax: ++39 090 3992284, e-mail: carlo.aragona@mail.net.it or to Organising Secretariat, Progetto GEA s.r.l., Serenella La Cavera, Via F.P. Di Blasi, 1, 90144 Palermo, Italy; Tel: ++39 091 6262660, ++39 091 6264517, Fax: ++39 091 303937, e-mail: progettogea@manol.com

### 28th Munich Endourological Symposium with Nursing Staff Seminar 5–6 October 2000, Munich, Germany

*Chairman:* Prof. Dr. R. Hartung,

*Information:* Dr. R. Paul, Department of Urology, Technische Universität München, Klinikum rechts der Isar, Ismaninger Str. 22, 81675 München; Tel: 089/41402507, Fax: 089/41402585, e-mail: mriu@lrz.tu-muenchen.de, Internet: http://www.med.tu-muenchen.de/uro/endo.html

### 4th International Symposium on Uro–Onkology: Advances in Diagnosis and Treatment of Prostate Cancer 19–21 October 2000, Marburg, Germany

*Topics:* Live Surgery (ascending, descending, nerve-sparing retropubic prostatectomy, perineal prostatectomy, laparoscopic prostatectomy, art. Sphincter implantation, penile prosthesis implantation), pathology and molecular biology of PCA, therapeutic options in organ confined PCA, treatment of locally advanced and metastatic PCA.

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