



Original Contribution

Nitric oxide and renal protection in morphine-dependent rats

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ABSTRACT

Morphine treatment for 5 days protects heart against ischemia–reperfusion (IR) injury. This study evaluated the involvement of nitric oxide (NO) in morphine-induced renal protection. Three weeks after right nephrectomy, increasing doses of morphine were administered ($20\text{--}30\text{ mg kg}^{-1}\text{ day}^{-1}$, 5 days) to develop dependence in rats. The left kidney underwent 45-min ischemia and 24-h reperfusion. Some rats were pretreated with naloxone (5 mg kg^{-1}) or L-NAME (20 mg kg^{-1}). In one group, IR was induced 24 h after the last dose of morphine during the withdrawal period. Plasma nitrite/nitrate levels and serum creatinine and BUN were measured. Creatinine clearance and fractional excretion of sodium (FE_{Na}) were calculated. Myeloperoxidase (MPO) activity, malondialdehyde (MDA) level, and inducible NO synthase (iNOS) expression were determined and histopathology was studied in the left kidney. IR increased serum creatinine and BUN, plasma NO ($p < 0.01$), FE_{Na} , iNOS expression ($p < 0.001$), MPO activity, MDA level, and tissue damage and decreased creatinine clearance. Morphine decreased plasma NO ($p < 0.05$ vs IR), serum creatinine and BUN ($p < 0.01$), FE_{Na} , MPO activity, MDA level, iNOS expression, and tissue damage ($p < 0.05$), but increased creatinine clearance ($p < 0.05$). Pretreatment with naloxone significantly increased NO production and iNOS expression in morphine-treated rats after IR ($p < 0.01$ vs morphine dependence + IR). Pretreatment with L-NAME in morphine-treated rats decreased NO production (10.7 ± 1.9 , $p < 0.01$ vs morphine dependence + IR) but could not change iNOS expression after IR. Both naloxone and L-NAME significantly abolished the protective effects of morphine dependence on functional and histological factors. The protective effect of morphine dependence on serum creatinine, BUN, FE_{Na} , and creatinine clearance persisted during the withdrawal period, whereas iNOS expression decreased. NO production was not decreased during the withdrawal period ($p > 0.1$ vs morphine dependence + IR group). Morphine dependence provided renal protection in the acute phase and during withdrawal. Excessive increase or decrease in NO production abolished the effects of morphine, which suggested a role for balanced NO production and iNOS expression.

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Nitric oxide (NO) plays an important role in cardiac ischemic preconditioning (IPC) [1]; a model in which brief periods of arterial occlusion and reperfusion could preserve tissue function [2]. Previously it had been demonstrated that protection was lost when nitric oxide synthase (NOS) inhibitors were administered [3] and, on the other hand, expression of inducible NO synthase (iNOS) increased in cardiac myocytes after IPC [4]. One step in IPC involves activation of NOS, production of NO and subsequent activation of guanylyl cyclase, production of cGMP, and activation of protein kinase G that finally

activates preconditioning pathways [5–8]. The role of NO has been shown in liver [9], retina [10], and renal preconditioning [11].

Three NOS isoforms, endothelial (eNOS; in renal vasculature), neuronal (in macula densa), and iNOS (in several segments of the renal tubule) are involved in NO production in renal tissue [12]. The effects of NO on Glomerular Filtration Rate, on the regulation of renal blood flow, and in maintaining renal functions have been demonstrated [13]. Moreover, up-regulation or down-regulation of the various NOS isoforms or alterations in their activity have been described in renal ischemia–reperfusion (IR) [14,15]. NO release and NO donors [15,16] protect the kidneys against IR injury. Also eNOS activation and iNOS expression contribute to the kidney model of IPC, especially in the late phase [17,18].

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Some studies have shown that opioid receptor activation mimics IPC and protects the heart and other organs against IR injury [19–21]. The role of NO in this pathway has been observed in the acute [22] and late phases of protection [23]. There is also evidence that morphine produces its crucial late cardioprotective effects via induction of iNOS, which was completely aborted by either selective pharmacologic inhibition of iNOS or targeted disruption of the iNOS gene [24].

In a study by Lee and Emala designed to evaluate the role of protein kinase C and Gi/o proteins in renal protection, a single dose of morphine could not prevent renal IR injury [25]. However, our recent study showed that multiple intermittent doses of morphine have a potent protective impact on the kidney [26]. This was in agreement with the work of Peart and Gross, which demonstrated profound cardioprotection in morphine-dependent rats [27]. The complex effects of NO on kidney function, its involvement in a renal IPC model, and the role of NO as a signaling molecule in morphine-induced heart preconditioning pathways encouraged us to evaluate the possible role of this molecule in the signaling pathway of morphine-dependence-induced renal protection.

Materials and methods

All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Iran University of Medical Sciences and were in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

Animals and drugs

A total of 110 male Wistar rats, weighing between 180 and 250 g, were used in this study. The rats were housed in groups of seven with food and water available, under a 12-h light/dark cycle (light 7:00 AM to 7:00 PM) and controlled temperature (22 ± 2 °C).

The following drugs were used: morphine sulfate (Temad, Tehran, Iran), naloxone (Temad), L-NAME (Sigma Chemical Co., St. Louis, MO, USA), and pentobarbital (Temad). The drugs were dissolved in saline; all drug solutions were prepared immediately before administration. L-NAME and pentobarbital were administered intraperitoneally (ip), whereas morphine and naloxone were administered subcutaneously (sc).

Morphine dependence

Morphine was injected for 5 days. The dose regimen was 20 mg $\text{kg}^{-1} \text{day}^{-1}$ on the first and second day and 30 mg $\text{kg}^{-1} \text{day}^{-1}$ on the third and fourth day; the total daily dose was administered in two equally divided injections at 9:00 AM and 5:00 PM. A final dose of

30 mg kg^{-1} was injected on the fifth day, 4.5 h before IR. Control groups received saline instead of morphine. Our last study showed that this method was reliable in producing morphine dependence and subsequent withdrawal signs after drug cessation [26].

Experimental design

The right kidney was removed, under general anesthesia with pentobarbital (45 mg kg^{-1} , ip). After 3 weeks of recovery, the rats were divided into 10 groups each with 11 rats (Table 1). Two groups of rats received a single dose of naloxone (5 mg kg^{-1}) 3 h before IR, in addition to the other treatments. L-NAME (20 mg kg^{-1} ; nonselective NOS inhibitor) was administered 3 h before sham operation or IR in three groups of rats. In one group ischemia–reperfusion was induced 24 h after the last dose of morphine (morphine dependence) to evaluate the effect of morphine withdrawal on renal IR.

To induce IR, the left renal artery was exposed through a small flank incision and occluded with a nontraumatic arterial clamp for 45 min. The clamp was released and each animal was stored in a metabolic cage for 24 h of reperfusion time. After 24 h, urine volume was recorded and stored at -20 °C until assayed for creatinine and sodium concentrations. Blood samples were drawn from the carotid artery using heparinized syringes. The first part of the blood sample was centrifuged at 2000 g for 10 min to determine blood urea nitrogen (BUN), serum creatinine, and sodium. The remaining part of the blood sample was compartmentalized by centrifugation to obtain plasma for measurement of nitrite/nitrate levels.

Histological examination

Left kidneys were cut longitudinally and processed for light-microscopic observation. Kidneys were fixed in phosphate-buffered 10% formalin and embedded in paraffin wax, cut into four sections 4–5 μm in thickness, and stained with hematoxylin and eosin (H&E). Two pathologists scored the histopathology of prepared sections in a double-blind manner. The severity of injury was graded from 1 to 4 according to the following criteria [28]:

- (0) no signs of necrosis (no damage);
- (1) necrosis of individual cells;
- (2) necrosis of all cells in adjacent proximal convoluted tubules, with survival of surrounding tubules;
- (3) necrosis confined to the distal third of the proximal convoluted tubule with a band of necrosis extending across the inner cortex; and
- (4) necrosis affecting all three segments of the proximal convoluted tubule.

Table 1

Experimental protocol and time scales used for morphine dependence and IR induction in evaluated groups

Group	4 days before IR				Day of ischemia			2 days after		N
	Day 1	Day 2	Day 3	Day 4	8:00 AM	10:00 AM	1:00 PM	Day 1, 1:00 PM	Day 2, 1:00 PM	
Sham	S	S	S	S	S	S	Sham	End	—	11
IR	S	S	S	S	S	S	IR	End	—	11
MD + Sham	M10	M10	M15	M15	M30	S	Sham	End	—	11
MD + IR	M10	M10	M15	M15	M30	S	IR	End	—	11
Nal + IR	S	S	S	S	S	Nal	IR	End	—	10
MD + Nal + IR	M10	M10	M15	M15	M30	Nal	IR	End	—	9
L-NAME + Sham	S	S	S	S	S	L-NAME	Sham	End	—	11
L-NAME + IR	S	S	S	S	S	L-NAME	IR	End	—	10
MD + L-NAME + IR	M10	M10	M15	M15	M30	L-NAME	IR	End	—	11
Withdrawal + IR	M10	M10	M15	M15	M30	—	—	IR	End	9

Morphine-dependent (MD) rats in all groups received morphine for 4 days twice daily (8:00 AM and 9:00 PM), and a final bolus dose 4.5 h before IR, whereas other groups received saline in the same manner. Sham, sham operation, S, saline (0.5 ml), M, morphine (10, 15, or 30 mg kg^{-1} , sc), Nal, naloxone (5 mg kg^{-1} , sc; one dose 3 h before IR); L-NAME, nonselective NOS inhibitor (20 mg kg^{-1} , ip; one dose 3 h before IR); IR, start of ischemia–reperfusion procedure; End, end of reperfusion period; Withdrawal + IR, in an extra group of rats (N=9), ischemia–reperfusion was induced 24 h after the last dose of morphine (morphine dependence) to evaluate the effect of morphine withdrawal on renal IR.

Analytical procedures

A Hitachi multianalyzer was used for measurement of creatinine and BUN levels. Sodium concentration was determined using a flame photometer (Hitachi, 205D; Hitachinaka, Japan) on serum and urine samples; thereafter, the creatinine clearance and fractional excretion of sodium (FE_{Na}) were calculated.

Measurement of nitrite/nitrate levels in plasma

$NO_2^- + NO_3^-$ concentrations were determined in plasma using a nitric oxide colorimetric assay kit (Roche Applied Science, Indianapolis, IN, USA). Briefly, heparinized blood samples (0.5 ml) were centrifuged at 20 °C for 45 min (4000 rpm, $r = 7$ cm). Samples were diluted with nitrite- and nitrate-free distilled water in a 1 to 3 ratio and centrifuged at 4500 g. Then, 500 μ l of plasma was deproteinized using 20 mg dry sulfosalicylic acid and stored at -80 °C until analysis. The nitrate present in the sample was reduced to nitrite by reduced NADPH in the presence of nitrate reductase. Then nitrite was reacted with Griess reagents (sulfanilamide and *N*-1-(naphthyl)ethylene diamine dihydrochloride) to give a red-violet diazo dye. Spectrophotometric measurement of the absorbance at 540 nm of this diazo dye determined the nitrite concentration in the solution. The value was expressed as the total nitrite plus nitrate.

Malondialdehyde (MDA) measurement

MDA, as an index of lipid peroxidation, was measured in renal tissue. For measurement of MDA the thiobarbituric acid method, which measures MDA-reactive products, was used. MDA, as a thiobarbituric acid-reactive substance, reacts with thiobarbituric acid (TBA) to produce a red complex that has maximal absorbance at 532 nm. Briefly, kidney tissue was weighed and homogenized in a KCl solution. Phosphoric acid (3 ml; 1%) and TBA (1 ml; 0.6%) were added to 0.5 ml of homogenate tissue and the mixture was heated for 45 min in a boiling water bath. The MDA concentration was calculated from the intensity of the pink color of the final product at 532 nm. Results are expressed as nmol MDA/g wet tissue.

Measurement of myeloperoxidase (MPO) activity as an indicator of neutrophil presence

Kidney tissue (0.2 to 0.4 g) was weighed and homogenized in a solution containing potassium phosphate buffer (50 mmol L^{-1} , pH 6.0) and hexadecyltrimethylammonium bromide (0.5%, w/v). The homogenate solution was centrifuged at 20,000 g at 4 °C, and the supernatant was used for evaluation. H_2O_2 -dependent oxidation of *o*-dianizidine 2HCl was used to measure the MPO activity. One unit of MPO activity was the amount of MPO per gram of tissue weight that

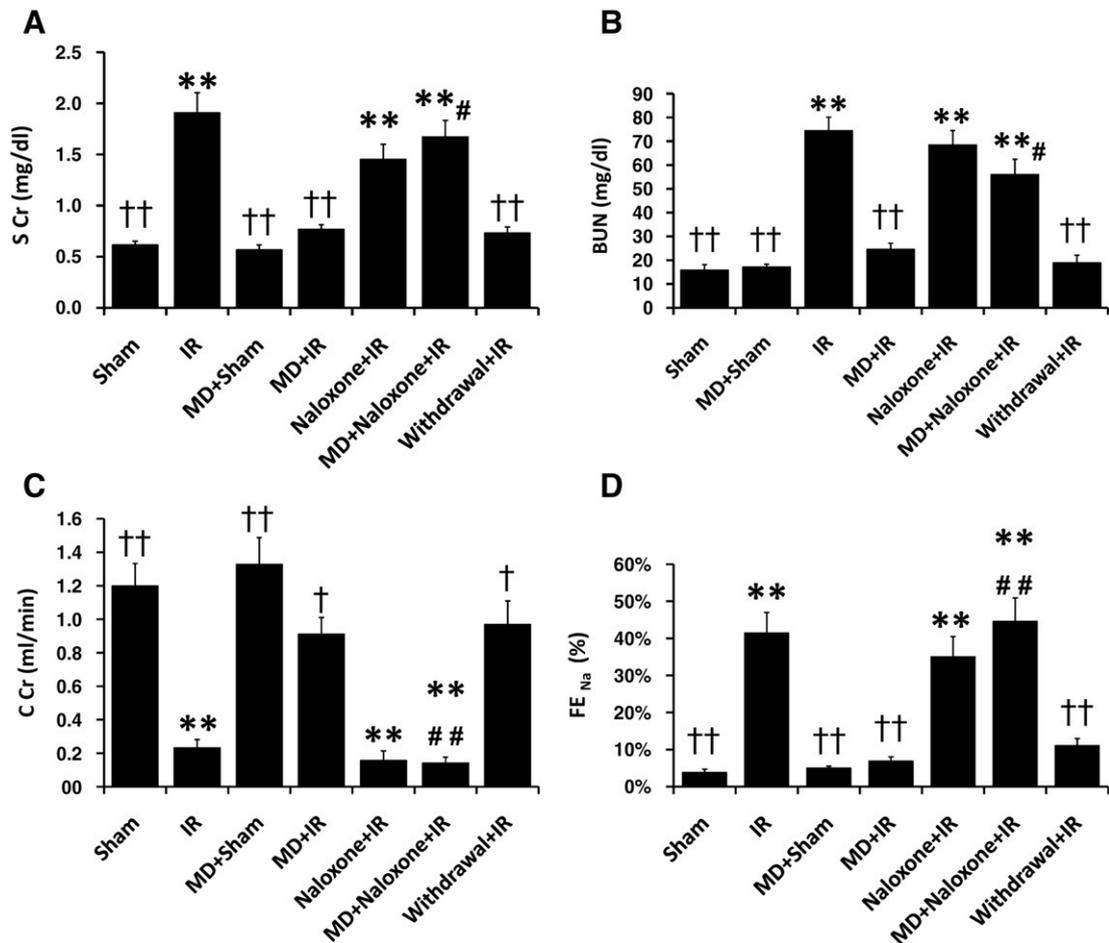


Fig. 1. Renal function assessment in morphine-dependent and naloxone-treated rats after ischemia reperfusion. (A) Serum creatinine (SCr) and (B) BUN were measured after 24 h of reperfusion in serum samples. (C) Creatinine clearance (CCr) was calculated from serum and urinary creatinine concentration and urinary volume was collected during 24 h of reperfusion. (D) Fractional excretion of sodium (FE_{Na}) was calculated from serum and urinary sodium concentration, urinary volume, and creatinine clearance. Data are given as means \pm SEM. IR, ischemia–reperfusion ($N = 11$); MD + Sham, morphine dependence and sham operation ($N = 11$); MD + IR, morphine dependence + IR ($N = 11$); Nal + IR, naloxone (5 mg kg^{-1}) before IR ($N = 10$); MD + Nal + IR, morphine dependence and naloxone (5 mg kg^{-1}) before IR ($N = 9$); Withdrawal + IR, morphine dependence and 24 h of withdrawal before IR ($N = 9$). ** $p < 0.01$ vs sham, † $p < 0.05$ and †† $p < 0.01$ vs IR, and # $p < 0.05$ and ## $p < 0.01$ in comparison with morphine dependence + IR group.

changed the rate of spectrophotometrically measured absorbance, 1 unit per minute at 460 nm.

iNOS expression in renal tissue

Western blotting was used for measurement of iNOS expression in renal tissue. Cellular protein was extracted from 100 mg of tissue in Western blot lysis buffer. Samples were centrifuged at $22,000 \times g$ for 20 min (4°C). One hundred micrograms of extracted protein was mixed with sample buffer and boiled for 5 min. Samples were separated on a 7% gel and electroblotted to a nitrocellulose membrane for 2 h. After being washed in distilled water, membranes were blocked overnight and incubated with rabbit anti-iNOS or mouse anti- β -actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were detected using a chemiluminescence-based protocol (Pierce, Rockford, IL, USA) and were visualized on light-sensitive film.

Statistical analysis

Results for evaluation of NO, MDA, MPO, serum creatinine, BUN, creatinine clearance, and FE_{Na} are presented as means \pm SEM. Means of groups were compared by one-way analysis of variance and then post hoc analysis (Tukey test) was performed for assessing specific group comparisons. The Mann-Whitney U test was used for comparison of histological data. The level of statistical significance was accepted as $p < 0.05$. Calculations were performed using the SPSS statistical package (version 14).

Results

Of the 110 rats used for this study 6 died, 5 during ischemia and 1 in the reperfusion period. The exact number of rats in each group can be found in Table 1.

Serum creatinine, BUN, and functional assessments

As shown in Figs. 1A and B, morphine dependence had no significant effect on serum creatinine or BUN after sham operation. IR significantly increased serum creatinine and BUN in comparison to the sham-operated group (1.8 ± 0.1 and 74.4 ± 5.7 vs 0.5 ± 0.1 and 15.9 ± 2.3 mg dl^{-1} , respectively; $p < 0.01$). Morphine dependence decreased serum creatinine and BUN concentrations after IR (0.7 ± 0.1 and 24.6 ± 2.6 mg dl^{-1} ; $p < 0.01$, Figs. 1A and B). L-NAME did not change serum creatinine or BUN in the sham-operated group or after IR (Figs. 2A and B). Naloxone by itself had no effect on serum creatinine and BUN concentrations after IR (Figs. 1A and B). Pretreatment with naloxone or L-NAME before IR in morphine-dependent rats increased serum creatinine and BUN concentrations compared with the morphine dependence + IR group ($p < 0.05$, Figs. 1A and B, and $p < 0.05$, Figs. 2A and B, respectively). When IR was induced 24 h after the last dose of morphine to evaluate the effect of morphine withdrawal, serum creatinine and BUN (0.7 ± 0.1 mg dl^{-1} and 18.9 ± 3.2 mg dl^{-1}) were not significantly changed in comparison to the morphine dependence + IR group, whereas the results were significantly less than those of the IR group ($p < 0.01$, Figs. 1A and B).

Creatinine clearance used as an indicator of glomerular function was normal in the sham-operated (1.2 ± 0.1 ml min^{-1}) and morphine-dependence + sham (1.3 ± 0.2 ml min^{-1} , Fig. 1C) groups. IR significantly reduced creatinine clearance (0.2 ± 0.1 ml min^{-1}). Morphine

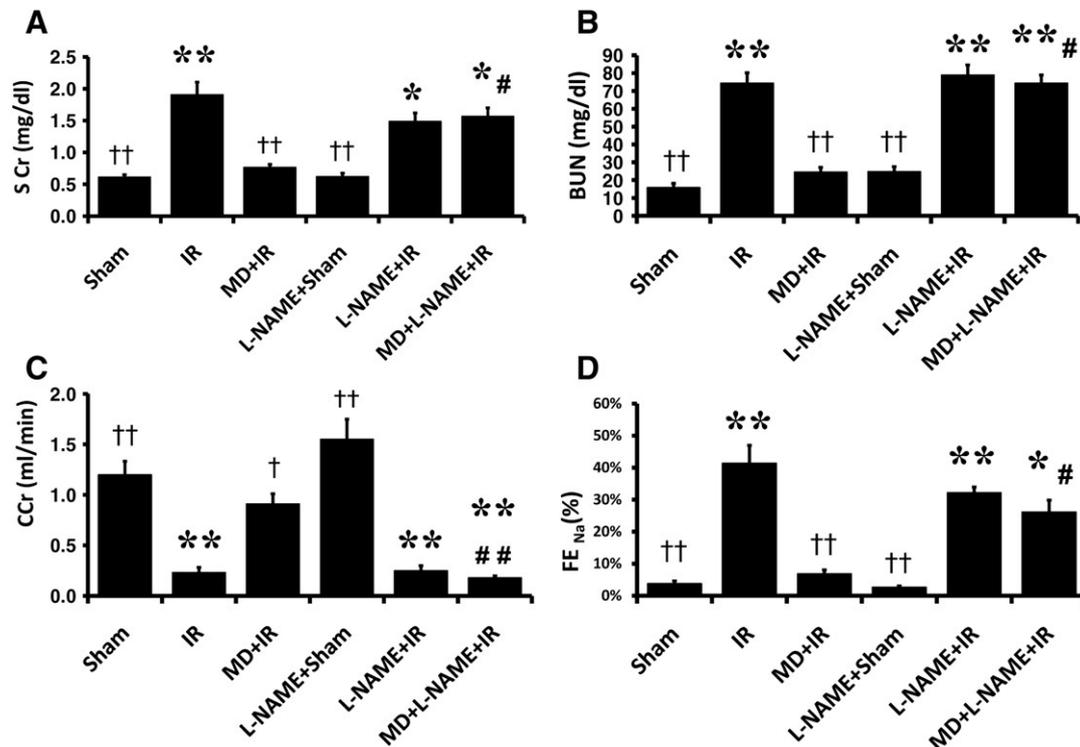


Fig. 2. Renal functional assessment in L-NAME-treated rats subjected to sham operation (Sham), ischemia reperfusion (IR), and morphine dependence (MD). (A) serum creatinine (SCr) and (B) BUN were measured after 24 h of reperfusion in serum samples. (C) Creatinine clearance (CCr) was calculated from serum and urinary creatinine concentration and urinary volume was collected during 24 h of reperfusion. (D) Fractional excretion of sodium (FE_{Na}) was calculated from serum and urinary sodium concentration, urinary volume, and creatinine clearance. L-NAME + Sham, $N = 11$; L-NAME + IR, $N = 10$. MD + L-NAME + IR, morphine dependence and L-NAME (20 mg kg^{-1}) before IR ($N = 11$); Withdrawal + IR, morphine dependence and 24 h of withdrawal before IR ($N = 9$). Data are presented as means \pm SEM. * $p < 0.05$ and ** $p < 0.01$ vs sham, † $p < 0.05$ and †† $p < 0.01$ vs IR, and # $p < 0.05$ and ## $p < 0.01$ vs. morphine dependence + IR group.

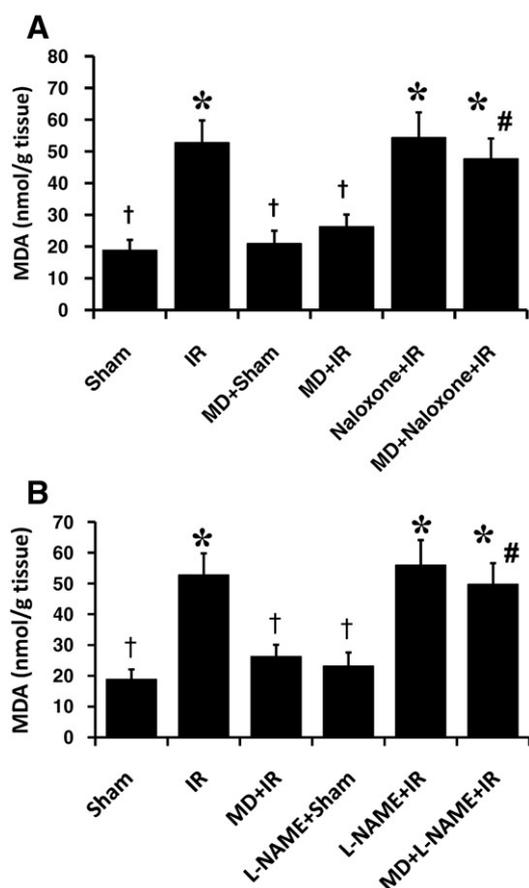


Fig. 3. Renal tissue malondialdehyde (MDA) level as an index of lipid peroxidation was measured 24 h after reperfusion in morphine-dependent (MD) rats treated with (A) naloxone and (B) L-NAME. Data are given as means \pm SEM. Naloxone (5 mg kg^{-1}) was given before IR, and L-NAME (20 mg kg^{-1}) was given before IR or sham operation. * $p < 0.01$ vs sham, † $p < 0.01$ vs IR, and # $p < 0.05$ in comparison with morphine dependence + IR.

dependence increased creatinine clearance after IR ($0.9 \pm 0.1 \text{ ml min}^{-1}$, $p < 0.05$; Fig. 1C). Although naloxone or L-NAME in sham-operated rats or after IR (Figs. 1C and 2C), pretreatment with naloxone or L-NAME reduced creatinine clearance in morphine-dependent rats after IR in comparison to the morphine dependence + IR group ($0.1 \pm 0.0 \text{ ml min}^{-1}$, $p < 0.01$, Fig. 1C, or $0.2 \pm 0.0 \text{ ml min}^{-1}$, $p < 0.01$, Fig. 2C, respectively). Induction of IR during the withdrawal period could not change creatinine clearance ($1.0 \pm 0.1 \text{ ml min}^{-1}$) in comparison to the morphine dependence + IR group ($p > 0.05$), whereas it was significantly more than in the IR group ($p < 0.05$, Fig. 1C).

FE_{Na} was used as an indicator of proximal tubule function and did not change in the sham-operated, morphine dependence + sham, and L-NAME + sham groups (4 ± 0.0 , 5 ± 0.0 , and $3 \pm 0.0\%$, respectively, Figs. 1D and 2D). IR significantly increased FE_{Na} ($41 \pm 5.5\%$, $p < 0.01$, Fig. 1D). Morphine dependence significantly decreased FE_{Na} after IR compared to the IR group ($7 \pm 1\%$, $p < 0.01$, Fig. 1D). FE_{Na} was not changed significantly by naloxone or L-NAME after IR compared with the IR group (35 ± 0.1 or $32 \pm 0.0\%$, respectively, Figs. 1D and 2D). Pretreatment with naloxone or L-NAME increased FE_{Na} in morphine-dependent rats after IR in comparison to the morphine dependence + IR group ($45 \pm 6\%$, $p < 0.01$, or $26 \pm 4\%$, $p < 0.05$, respectively). The effect of morphine dependence on the decrease in FE_{Na} after IR persisted during the 24-h withdrawal period ($p < 0.01$ vs IR, Fig. 1D).

Renal tissue MDA

The renal tissue MDA content in the sham-operated group ($18.8 \pm 3.3 \text{ nmol g}^{-1}$ tissue) was significantly increased by ischemia

reperfusion ($52.7 \pm 7.1 \text{ nmol g}^{-1}$ tissue, $p < 0.01$, Fig. 3A); however, morphine dependence significantly decreased the IR-induced elevation of renal MDA level ($26.2 \pm 3.9 \text{ nmol g}^{-1}$ tissue, $p < 0.01$; Fig. 3A). Renal MDA content increased in morphine dependence + IR rats pretreated with naloxone or L-NAME ($47.6 \pm 6.5 \text{ nmol g}^{-1}$ tissue, $p < 0.05$ vs morphine dependence + IR, Fig. 3A, or $49.7 \pm 6.9 \text{ nmol g}^{-1}$ tissue, $p < 0.05$ vs morphine dependence + IR, Fig. 3B, respectively).

MPO activity in renal tissue

In sham-operated and morphine dependence + sham-operated rats MPO activity was 6 ± 0.1 and $7.1 \pm 0.7 \text{ U g}^{-1}$ tissue, respectively. MPO activity increased significantly in the IR group ($22.7 \pm 2.0 \text{ U g}^{-1}$ tissue, $p < 0.001$, Fig. 4A). In tissue samples obtained from morphine-dependent rats subjected to IR, the activity of MPO significantly decreased ($8.3 \pm 0.9 \text{ U g}^{-1}$ tissue, $p < 0.001$, Fig. 4A). MPO activity remained unchanged in the naloxone + IR compared to the IR group. However, naloxone pretreatment in morphine dependence + IR rats increased MPO activity in renal tissue ($19.3 \pm 2.1 \text{ U g}^{-1}$, $p < 0.05$ vs morphine dependence + IR, Fig. 4A). MPO activity in L-NAME-treated sham-operated and IR groups was 7.7 ± 0.7 and $24.5 \pm 2.8 \text{ U g}^{-1}$ tissue, respectively (Fig. 4B). Pretreatment with L-NAME in morphine dependence + IR rats increased MPO activity ($28.1 \pm 2.9 \text{ U g}^{-1}$, $p < 0.001$ vs morphine dependence + IR, Fig. 4B).

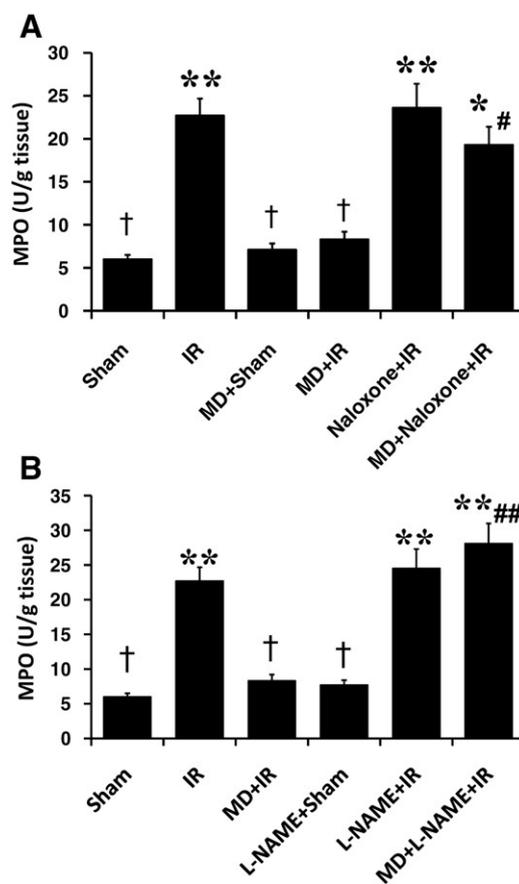


Fig. 4. Renal tissue myeloperoxidase (MPO) activity, a quantitative index of neutrophil accumulation in inflamed tissue, was measured 24 h after reperfusion in morphine-dependent (MD) rats treated with (A) naloxone and (B) L-NAME before IR or sham operation. Data are given as means \pm SEM. Naloxone (5 mg kg^{-1}) was given before IR, and L-NAME (20 mg kg^{-1}) was given before IR or sham operation. * $p < 0.01$ and ** $p < 0.001$ vs sham, † $p < 0.01$ vs IR, and # $p < 0.05$ and ## $p < 0.001$ vs. morphine dependence + IR.

Nitrite and nitrate levels in plasma

As shown in Fig. 5A, plasma nitrite/nitrate level was $15.8 \pm 1.9 \mu\text{M}$ in the sham-operated group and increased significantly after IR ($64.0 \pm 8.6 \mu\text{M}$, $p < 0.01$). Morphine dependence increased plasma nitrite/nitrate levels in sham-operated rats ($23.3 \pm 2.1 \mu\text{M}$, $p < 0.05$, Fig. 5A). In the morphine dependence + IR group, nitrite/nitrate level was higher than in the sham-operated rats ($37.8 \pm 3.1 \mu\text{M}$, $p < 0.05$), but in comparison to the IR group it decreased significantly ($p < 0.05$, Fig. 5A).

The level of nitrite/nitrate in naloxone-pretreated rats was not changed in comparison with the IR group, but in the morphine dependence + naloxone + IR group plasma nitrite/nitrate level increased compared with the morphine dependence + IR group ($61.1 \pm 7.4 \mu\text{M}$ vs $37.8 \pm 3.1 \mu\text{M}$, respectively, $p < 0.05$, Fig. 5A). L-NAME pretreatment in the sham-operated group could not reduce nitrite/nitrate levels (Fig. 5B), but caused a significant decrease in NO production after IR ($8.2 \pm 2.8 \mu\text{M}$, $p < 0.01$ vs IR, Fig. 5B). In comparison with the morphine dependence + IR group, L-NAME pretreatment in morphine-dependent rats significantly decreased plasma nitrite/nitrate levels after IR to $10.8 \pm 1.9 \mu\text{M}$ ($p < 0.05$, Fig. 5B). Although NO production after IR in the withdrawal period was also more than that of sham-operated rats ($p < 0.05$), this value was significantly less than that in the IR group ($p < 0.05$). There was no significant difference between the morphine dependence + IR and the withdrawal + IR group (Fig. 5A).

iNOS protein expression

Morphine dependence increased iNOS expression in sham-operated rats ($p < 0.05$, Fig. 6). In renal tissues prepared 24 h after

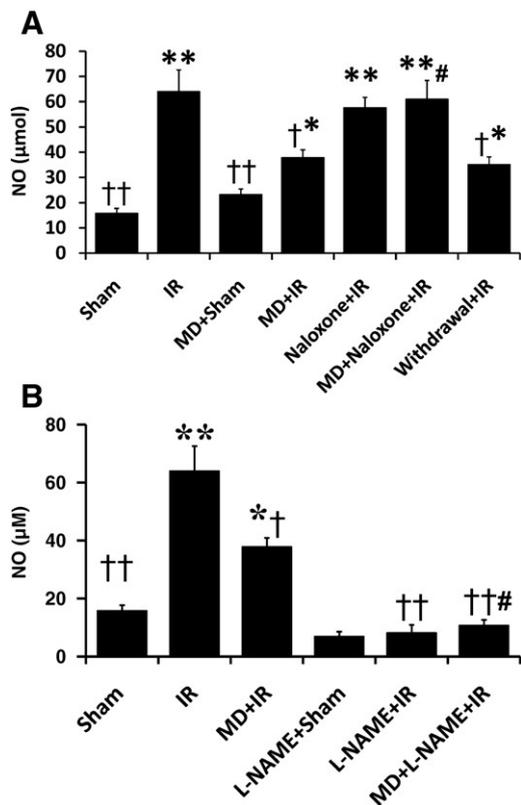


Fig. 5. Plasma nitrite/nitrate levels (NO) were measured in samples derived from morphine-dependent (MD) rats treated with (A) naloxone and (B) L-NAME 24 h after reperfusion. Data are given as means \pm SEM. Naloxone was given at 5 mg kg^{-1} and L-NAME at 20 mg kg^{-1} . Withdrawal + IR, morphine dependence and 24 h of withdrawal before IR ($N = 9$). $^{\dagger\dagger}p < 0.05$ and $^{**}p < 0.01$ vs sham, $^{\dagger}p < 0.05$ and $^{\dagger\dagger\dagger}p < 0.01$ vs IR, and $^{\#}p < 0.05$ compared to morphine dependence + IR.

IR, iNOS expression increased significantly ($p < 0.01$). In morphine-dependent rats subjected to IR, iNOS expression significantly decreased compared with the IR group ($p < 0.05$, Fig. 6). Naloxone by itself could not change iNOS expression after IR; however, in morphine-dependent rats it significantly up-regulated iNOS expression in comparison with the sham ($p < 0.01$), IR ($p < 0.05$), or morphine dependence + IR ($p < 0.01$) groups. L-NAME in sham-operated animals had no significant effect on iNOS expression, but decreased iNOS expression after IR ($p < 0.01$, Fig. 6). L-NAME pretreatment in morphine-dependent rats had no significant effect on iNOS expression after IR (Fig. 6). Expression of iNOS protein significantly decreased after IR treatment with withdrawal ($p < 0.01$ vs IR and $p < 0.05$ vs morphine dependence + IR group, Fig. 6).

Histopathology

In all of the sham-operated groups, regardless of type of treatment (i.e., saline, morphine dependence, or L-NAME), there was no sign of tubular cell necrosis or other tissue damage (Fig. 7A). Forty-five minutes of ischemia and 24 h of reperfusion induced significant glomerular and tubular cell necrosis with nuclear degradation extended to inner parts of cortex and major parts of proximal tubules in many specimens ($p < 0.001$ vs sham; Fig. 7B). Moreover, cast formation and tubular dilation with severe corticomedullary hyperedema were dominant in this group. L-NAME could not change the IR-induced damage by itself and scores of histological damage remained unchanged in L-NAME + IR rats (Table 2 and Fig. 9A). Morphine dependence significantly attenuated the rate of cell necrosis and reduced damage only to individual cells of proximal tubules (Fig. 8A) and decreased histological scores in comparison with the IR group ($p < 0.05$, Table 2). Naloxone could not change the histological damage after IR (Fig. 8B, Table 2). Pretreatment with naloxone abolished the tissue protection in morphine-dependent rats after IR and increased the amount of cellular necrosis, especially in adjacent parts of proximal tubules ($p < 0.05$ vs morphine dependence + IR, Table 2, Fig. 8C). Pretreatment with L-NAME in morphine-dependent rats caused the distribution of necrotic cells to extend to the distal parts of proximal tubules, which increased the histological score to grade 3 in major parts of the preparations ($p < 0.05$ vs morphine dependence + IR, Table 2). Tubular cast formation, hyperedema, and glomerular damage were seen in specimens prepared after ischemia-reperfusion from naloxone- or L-NAME-pretreated morphine-dependent rats (Figs. 8C and 9B).

Discussion

Morphine dependence preserved renal morphology and function. Moreover, it increased iNOS expression and NO production and also attenuated IR-induced higher iNOS expression and NO production. Naloxone caused excessive iNOS expression and increased NO production and abolished morphine dependence-induced renal protection. In this study, for the first time, we demonstrated that L-NAME eliminated all protective effects of morphine dependence after renal IR; L-NAME decreased NO production in morphine-dependent rats, but iNOS expression remained unchanged; and last but not least, the protective effects of morphine dependence on renal function were observed during the withdrawal period, 24 h after the last dose of morphine. After IR, the expression of iNOS decreased in the morphine withdrawal period, but NO production was similar to that in the morphine dependence + IR group.

Studies on the heart, intestine, skeletal muscle, and CNS demonstrated that morphine and exogenous or endogenous activation of opioid receptors reduced IR injury [19,21,29–32]. To our knowledge, there was only one study that evaluated the effects of morphine on renal IR injury and which showed that a single dose of morphine (5 mg kg^{-1}) was not able to protect kidney [25]. Our recent study in

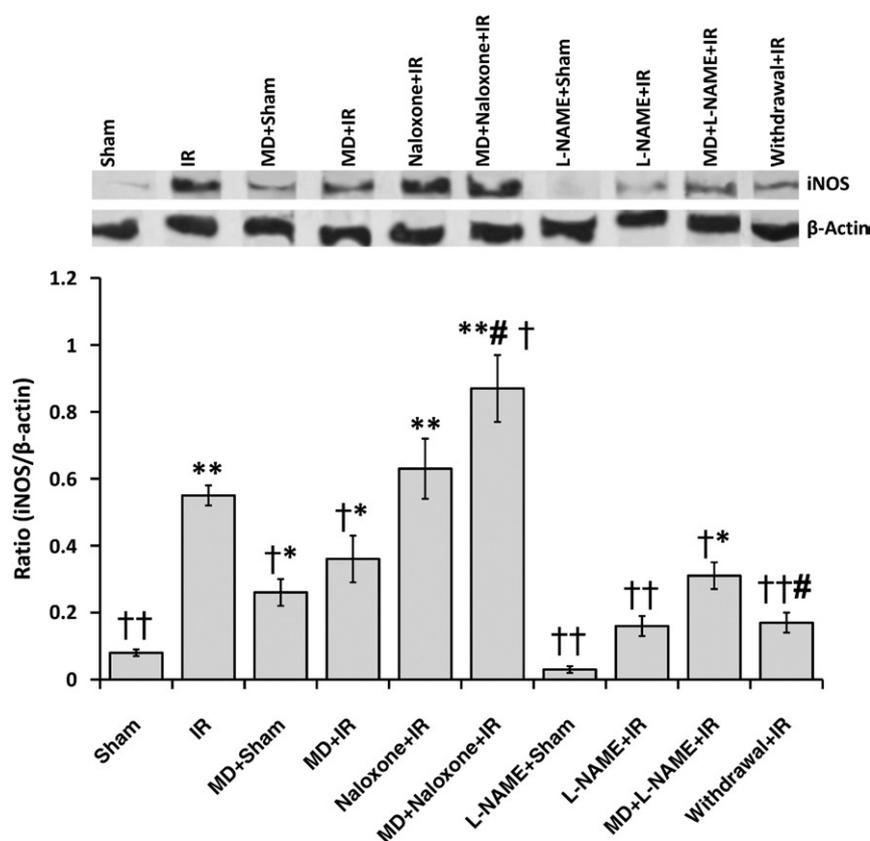


Fig. 6. iNOS protein expression was measured in renal tissue 24 h after reperfusion in the various groups. (A) The results of Western blot analysis for iNOS protein levels. (B) The ratio of iNOS expression to β -actin was normalized and the results are shown as means \pm SEM. Naloxone was given at 5 mg kg^{-1} and L-NAME at 20 mg kg^{-1} . Withdrawal + IR, morphine dependence and 24 h of withdrawal before IR ($N=9$). * $p<0.05$ and ** $p<0.01$ vs sham, † $p<0.05$ and †† $p<0.01$ vs IR, and # $p<0.01$ compared to morphine dependence + IR.

morphine-dependent rats [26] revealed that chronic morphine treatment preserved renal histology and function, which directed us to evaluate NO as a signaling molecule in this pathway.

Peart and Gross showed that heart exposure to morphine for 5 days produced a marked increase in ischemia tolerance that was significantly greater than the effect of brief morphine administration and this protection persisted after morphine withdrawal for 24 h [27]. Similarly, aged mouse heart survived more after chronic morphine administration than after a short-term administration [33]. Furthermore, a recent study suggested that cardiac protection in morphine-dependent rats occurred through a pathway independent of that described for classical acute and delayed preconditioning, although the exact mechanism remains unclear [34].

Role of NO in morphine dependence-induced renal protection

Morphine dependence increased iNOS expression and NO production in sham-operated rats but decreased them after IR. Some studies on the role of NO in renal IR injury have suggested protective effects [15,35–37], whereas others suggested harmful ones [38–40]. This suggests that NO may play a dual role in IR injury [41,42]. In this study, IR was accompanied by increased iNOS expression and elevated NO production (Fig. 5A), which may contribute to renal injury [42,43]. Some studies state that inhibition of iNOS expression or activity or scavenging of NO can ameliorate or prevent renal injury [44–46]. IR induced iNOS expression and NO production, decreased in morphine-treated rats (Fig. 5A). Initially these results seem to support the idea that morphine dependence protects the kidneys by decreasing iNOS expression and subsequent NO production during IR. However,

intervention with naloxone and L-NAME revealed that NO production is a key element in morphine dependence-induced renal protection.

Role of opioid receptors

Pretreatment with naloxone after chronic morphine administration abolished morphine's protective effects and its inhibitory effects on iNOS and NO, thus vigorously increasing NO production and iNOS expression and inducing renal tissue damage after IR. These findings suggest:

First, morphine dependence-induced renal protection seems to be receptor dependent. Fryer et al. showed that $\delta 1$ -opioid receptor antagonist, when administered either 30 min before or 48 h after receptor agonist treatment, prevented cardioprotection [47]. These data highlight the importance of the opioid receptor's reoccupation for stimulation of signal transduction pathways involved in delayed cardioprotection [47,48]. Delayed protection of kidney by morphine dependence also required activation of opioid receptors immediately before IR, similar to other renal preconditioning models [49,50].

Second, and the more important point, was NO activity modulation by morphine dependence. Five days of treatment with morphine caused a small but significant increase in iNOS expression and NO production (Figs. 5A and 6). Nevertheless in the morphine dependence + IR group, not only was such increase not superimposed on the IR-induced NO production, but also the morphine dependence conversely inhibited further iNOS expression and NO production. Naloxone created a new scenario in which morphine-induced iNOS expression was accessible for IR to induce further NO formation, whereas the opioid receptors were blocked.

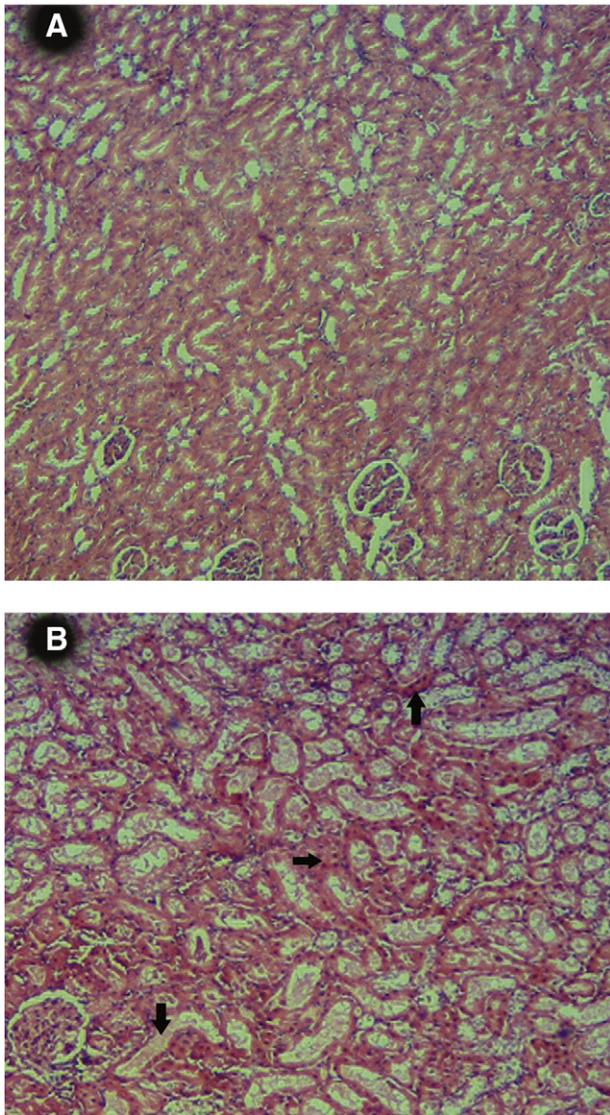


Fig. 7. Representative H&E-stained photomicrographs of the kidney of (A) sham-operated (original magnification $\times 4$) and (B) IR-treated rats (original magnification $\times 10$). Tubular lumen swelling, cast formation, and extended interstitial hemorrhage are shown by black arrows. Necrosis of tubular cells is not shown in this photomicrograph (B).

Contribution of iNOS expression and activity

The results of treatment with L-NAME were helpful in understanding the current controversy over the production of NO in morphine-treated sham-operated or IR rats. Although pretreatment with L-NAME effectively decreased NO production in the morphine dependence + IR rats, their kidneys experienced severe functional and histological damage, suggesting that the presence of some level of NO activity is required for morphine dependence to induce renal protection. Although iNOS expression in the L-NAME-treated morphine-dependent rats was the same as in the morphine dependence + IR group, NO production decreased significantly in this group (Figs. 5B and 6), suggesting that in addition to its expression, iNOS activity is required for morphine dependence to induce protection. It has been reported that NO release increased secondary to elevated NOS expression after four short episodes of IR [15]. In a heart model, brief periods of IR before the main IR propagated iNOS expression leading to NO production. The authors postulated that the early production of NO stimulates iNOS expression through intracellular signal pathways, and the production of iNOS protein mediates the late-phase protection by producing new NO [4]. Although 5 days of treatment with morphine increased iNOS expression and NO production, L-NAME administration before IR inhibited iNOS activity and probably other NOS isoforms, which attenuated NO production, leading to the presence of higher iNOS in renal tissue without increased NO production (Figs. 5B and 6).

Opioid receptor, modulation of iNOS–NO system, and renal protection

Previous studies have shown that morphine increases NO release [22] and activates NOS subtypes including iNOS, leading to myocardial tissue protection in the late phase after 24 h of preconditioning [51,52]. The role of iNOS activation and NO release in delayed renal protection against IR injury has also been demonstrated [17,35,53].

Goligorsky et al. showed that low levels of NO may be protective, whereas higher NO content may be harmful [54]. In the present study, overproduction or inhibition of NO eliminated the protective effects of morphine. Decreased NO production and subsequent tissue damage by L-NAME treatment on one hand, and increased iNOS expression and NO production with tissue damage in naloxone-treated morphine-dependent rats on the other hand, suggested that morphine dependence balances the expression of iNOS and production of NO by activation of opioid receptors, which protects the kidney against IR injury.

Another finding of this study was that the protection persisted during the withdrawal period (24 h after the last dose of morphine), suggesting that morphine dependency not only produced short-term effects, but also induced long-term modulation and protection, a

Table 2

Histological scores obtained from each group and comparison of the severity of tissue damage between groups

Group	Number (%) of observations in each grade					Total (%)
	0	1	2	3	4	
(1) Sham	97 (88.18)	11 (10)	2 (1.82)	—	—	110 (100%)
(2) IR*	2 (1.82)	21 (19.09)	34 (30.91)	51 (46.36)	2 (1.82)	110 (100%)
(3) MD + sham	93 (84.55)	14 (12.73)	3 (2.73)	0	0	110 (100%)
(4) MD + IR*,#	17 (15.45)	57 (51.82)	22 (20)	12 (10.91)	2 (1.82)	110 (100%)
(5) Nal + IR*	4 (4)	11 (11)	25 (25)	59 (59)	1 (1)	100 (100%)
(6) MD + Nal + IR* ,†	1 (1.11)	10 (11.11)	29 (32.22)	47 (52.22)	3 (3.33)	90 (100%)
(7) L-NAME + sham	103 (93.64)	6 (5.45)	1 (0.91)	0	0	110 (100%)
(8) L-NAME + IR*	0	5 (5)	31 (31)	64 (64)	0	100 (100%)
(9) MD + L-NAME + IR* ,†	4 (3.64)	16 (14.55)	37 (33.64)	53 (48.18)	0	110 (100%)

Number of observations in each grade (percentage of each grade observation) in each group is shown. Five slices prepared from each kidney were scored by two blinded pathologists. Total observation is the number of rats in each group \times the number of slices from each kidney \times the two pathologists. IR, ischemia reperfusion; MD, morphine dependence; Nal, naloxone (5 mg kg^{-1}). Mann-Whitney *U* test.

* $p < 0.001$ in comparison with sham group.

$p < 0.05$ compared each group that has IR treatment with the IR group.

† $p < 0.05$ comparing morphine dependence + naloxone + IR or morphine dependence + L-NAME + IR group with morphine dependence + IR group.

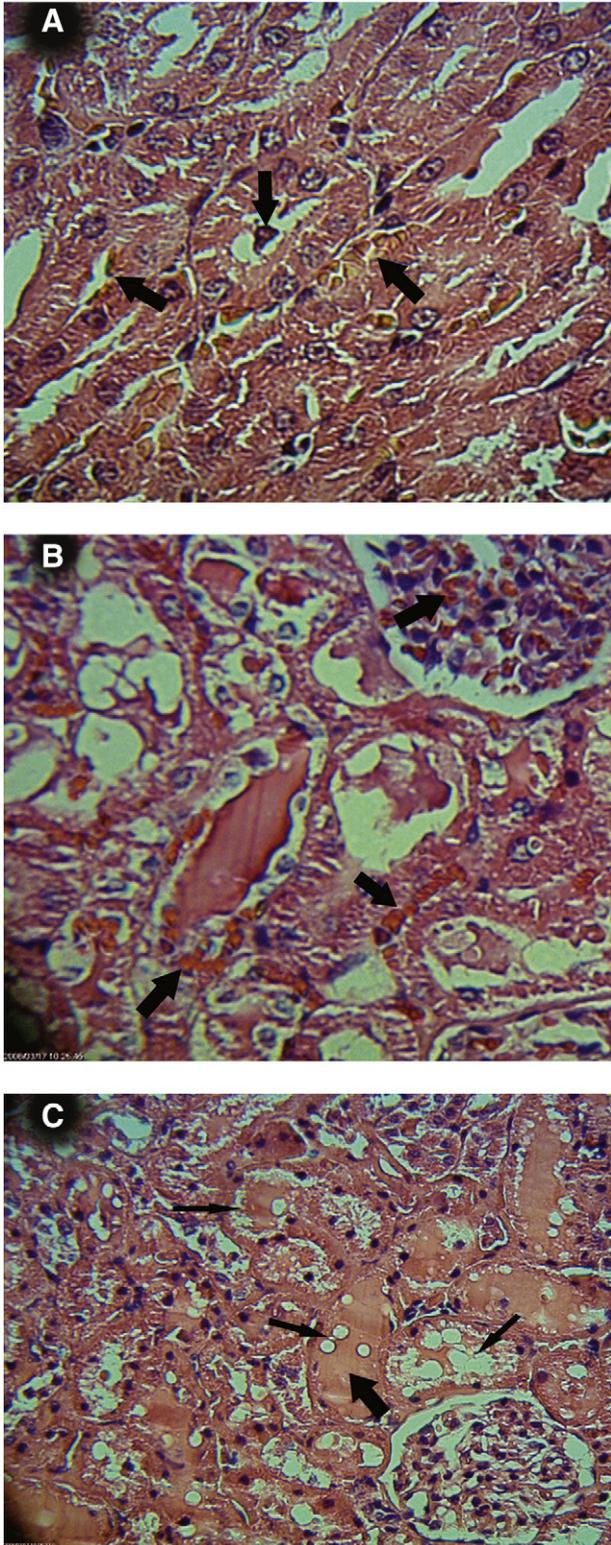


Fig. 8. Photomicrographs of kidneys from the morphine dependence + IR and morphine dependence + naloxone + IR groups. (A) Morphine-dependent rat after IR (H&E; original magnification $\times 40$), grade 1 histological score with individual cell necrosis (arrows) and tubular lumen expansion (stars). (B) Naloxone pretreatment (H&E; original magnification $\times 40$) induced extended glomerular and tubular cell necrosis (arrows). (C) Naloxone pretreatment in morphine-dependent rats (H&E; original magnification $\times 4$) increased histological damage by extending necrotic cells to inner cortical areas and apparent congestion (thick arrows), tubular epithelium degradation, and cast formation (thin arrows).

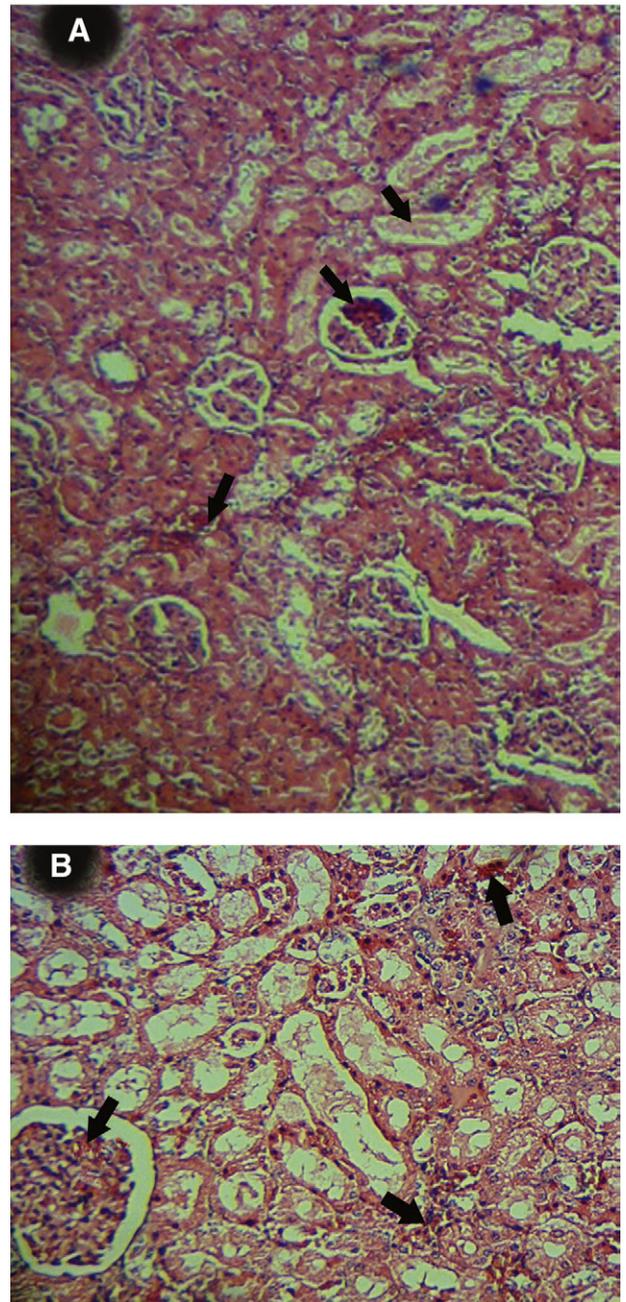


Fig. 9. Effects of pretreatment with L-NAME on histological changes. (A) L-NAME + IR group shows a histological profile similar to that of the IR group; tubular lumen cessation with casts and distributed interstitial inflammation along with tubular and glomerular cell necrosis (H&E; original magnification $\times 4$). (B) L-NAME pretreatment completely reversed the protective effects of morphine dependence on renal tissue by increasing the necrosis and damage to glomerular structure and extending the tubule-interstitial destruction to corticomedullary structures (H&E; original magnification $\times 4$).

phenomenon identical to the heart model of preconditioning by chronic morphine treatment [27,35]. Park et al. demonstrated that the protective effects of renal preconditioning via 30 min of bilateral kidney ischemia lasted for 12 weeks [17]. Interestingly, 30 min ischemia increased iNOS and eNOS, but NO production at the withdrawal period was not decreased (compared with the morphine dependence + IR group), which suggested the involvement of other NOS isoforms in the late phase of protection by morphine dependence. The precise mechanisms responsible for the late phase of protection require further evaluation.

In conclusion, morphine dependence preserved renal function and histology against IR injury by activation of opioid receptors and

subsequent production of NO. Morphine dependence produced both short- and long-term renal protection in rats that seems to require not only NO production but also its modulation. Further studies are needed to determine the potential subcellular mechanisms responsible for this marked renal protection achieved by chronic morphine exposure.

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