

Full Length Research Paper

Role of heat shock protein in renal ischemic reperfusion injury in rats

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The current study was designed to address the question of whether inducing heat shock proteins (HSPs) by herbimycin A might have a renoprotective effect in ischemic reperfusion (I/R) injury to rat kidney. The study was conducted on 56 male Wistar albino rats that were divided into three groups: Group I was subdivided into group Ia in which sham operation was done, Group Ib in which sham operation was done also received KNK437 (an inhibitor of heat HSP) prior to sham operation as well as after sham operation and Group Ic that received herbimycin A (1 h prior to sham operation as well as 1 and 24 h after sham operation, group II was subdivided into Group IIa, in which I/R to the kidney was induced, and group IIb, in which I/R was induced and also received KNK437 prior to induction of I/R as well as after induction of I/R; Group III consisted of rats with I/R that received herbimycin A without KNK437 (Group IIIa) or with KNK437 (Group IIIb) prior to induction of I/R as well as after the induction of I/R to the kidney. Administration of herbimycin A resulted in a significant renoprotective effect assessed histologically and biochemically, where there was a significant decrease in serum urea nitrogen as well as in creatinine concentration in herbimycin-treated rats as compared to non-treated rats. Herbimycin also resulted in antiapoptotic effect evidenced by a significant decrease in renal caspase-3 activity, which is possibly mediated by a significant decrease in measured renal mitogen activated protein kinase (MAPK-p38). These renoprotective actions are mostly mediated by herbimycin-induced renal HSP70, since the protective effect of the drug was markedly attenuated by the HSP inhibitor, KNK437.

Key words: Heat shock protein, herbimycin, ischemia reperfusion injury, mitogen activated protein kinase.

INTRODUCTION

Ischemia reperfusion (I/R) injury is a serious concern in a variety of clinical circumstances including organ transplantation, infarction and stroke (Kosaka et al., 2003). Renal I/R injury is associated with significant morbidity and mortality (Godwin et al., 2010). Renal I/R injury can occur during renal transplantation (especially during organ retrieval and storage) and reperfusion-injury in the early transplant period is associated with late allograft failure (Bonegio and Lieberthal, 2002).

Exposure of the kidney to sub-lethal ischemic, hypoxic, thermal or chemical insults stimulates intrinsic mechanisms that can protect kidneys against a subsequent insult, a phenomenon termed "preconditioning" (Jo et al., 2006).

Heat shock proteins (HSPs) are thought to be one of the mediators of heat-shock preconditioning. HSPs, also known as stress proteins and molecular chaperones, play a central role in protecting cellular homeostatic processes from environmental and physiologic insult by preserving the structure of normal proteins and repairing or removing damaged ones (Stankiewicz et al., 2005). HSPs synthesis is controlled by a specific family of transcription factors; heat-shock factors (HSFs) (Wang et al., 2002). HSP induction occurs after HSF translocates to the nucleus and binds to the heat shock element (HSE), a specific region in the promoter of heat shock inducible genes (Cotto et al., 1996).

Evidence indicates that over expression of HSPs diminishes the intracellular calcium response to stress. It down regulates the basal enzymatic activities of protein kinase C, protein kinase A as well as P-38 mitogen activated protein kinase (P-38 MAPK). These

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observations suggest that the cytoprotection induced by HSP is related to decreased signal transduction activity (Kiang, 2003).

One major question is whether it is possible to induce HSPs through pharmacological agents or gene therapy without stress to the organism, so that it can be beneficial for the treatment of disease. Several HSP inducers have been reported in the literature. However, most agents induce HSP concomitant with toxicity. On the other hand, herbimycin A has been reported to induce HSPs and provide protection against insult at nontoxic concentrations (Hegde et al., 1995).

Herbimycin A is a benzoquinoid ansamycin antibiotic that acts as a tyrosine kinase inhibitor and also induces some HSPs in various cell lines (Hamel et al., 2000). HSPs induction by herbimycin A is thought to protect rat cardiomyocytes and myogenic cells against simulated ischemia (Conde et al., 1997; Morris et al., 1996). Heat-shock transcription factor (HSF) activation was observed in herbimycin A-treated primate cells suggesting that herbimycin A could bypass regulatory pathways of stress protein expression (Hegde et al., 1995).

KNK437, a novel benzylidene lactam compound, was found to inhibit the synthesis of HSPs at the mRNA level, possibly due to inhibition of the activation of HSF1 (Voyer and Heikkila, 2008; Yokota et al., 2000).

The aim of the present study was to assess the possible renoprotective effect of inducing HSPs, e.g. HSP70, by herbimycin A in I/R injury in rat kidney.

MATERIALS AND METHODS

Animals and grouping

The present study was conducted on 56 male Wistar albino rats weighing 200 to 250 g each. The rats were housed under the same controlled environmental conditions, fed normal laboratory diet and they had free access to water. All experiments were performed in accordance with national animal care guidelines and were pre-approved by the Faculty of Medicine, Alexandria University Ethics Committee. Rats were divided into the following groups:

- Group I (n = 24): Sham operated control rats, this group was further divided into:

- Group Ia (n = 8): Rats that received 1 ml physiological saline intraperitoneally (i.p.), 1 h prior to as well as 1 and 24 h after sham operation.

- Group Ib (n = 8): Rats received the same regimen of group Ia, together with administering KNK437, dissolved in olive oil (Sigma Chemical Co., St. Louis, MO), at a dose of 200 mg/kg (Koishi et al., 2001) i.p. half an hour before physiological saline administration.

- Group Ic (n = 8): Rats that received herbimycin A (Sigma Chemical Co., St. Louis, MO) at a dose of 3 mg/kg i.p., 1 h prior to as well as 1 and 24 h after sham operation.

- Group II (n = 16): I/R control rats, this group was further divided into:

- Group IIa (n = 8): Rats that received 1 ml physiological saline i.p., 1 h prior to as well as 1 and 24 h after the induction of I/R.

- Group IIb (n = 8): Rats received the same regimen of group IIa, together with administering KNK437, dissolved in olive oil, at a dose of 200 mg/kg i.p. half an hour before physiological saline administration.

- Group III (n = 16): Herbimycin A-treated rats, this group was further divided into:

- Group IIIa (n = 8): Rats that received herbimycin A (Sigma Chemical Co., St. Louis, MO) at a dose of 3 mg/kg i.p., 1 h prior to as well as 1 and 24 h after the induction of I/R.

- Group IIIb (n = 8): Rats received the same regimen of group IIIa, together with administering KNK437, dissolved in olive oil, at a dose of 200 mg/kg i.p. half an hour before herbimycin administration.

Doses of herbimycin A used in the present study were chosen based on stated references reporting documented HSP, inducing effect of the drug at this dose (Inushima et al., 2001; Sachidhanandam et al., 2003).

Induction of I/R to the kidney

According to Melnikov et al. (2001), the I/R to the kidney was induced. In brief, animals were anesthetized with pentobarbitone (40 mg/kg body weight (bw) i.p.) and were placed under a warm lamp to maintain body temperature. A midline incision was made and the renal pedicles were bilaterally clamped for 20 min with non-traumatic vascular clamps. The time of ischemia was chosen to obtain a reversible model of ischemic acute renal failure and to avoid animal mortality. After 20 min, the clamps were removed. The kidneys were observed for restoration of blood flow by returning to their original color. The abdomen was closed in two layers and the animals were allowed to recover. Sham surgery consisted of the same surgical procedure except that clamps were not applied.

Biochemical measurements

48 h following I/R to the kidney, blood was collected from retro-orbital venous plexuses, rats were sacrificed and kidneys isolated. Excised kidneys were carefully blotted from any external blood on the surface. Part of each kidney was taken for histological evaluation and the rest was divided into 3 segments and immediately frozen in liquid nitrogen and then stored at -80°C until later biochemical analysis. Blood samples were centrifuged; serum was separated and stored at -80°C until being analyzed.

The following parameters were determined.

Renal function tests

Serum urea nitrogen concentration (Richards et al., 1984) and serum creatinine concentration (Zilva et al., 1987) by colorimetric determination using kits supplied by Sigma Chemical Co., St. Louis, MO.

Renal HSP70 level

Renal HSP70 level was measured by enzyme linked immunosorbent assay (ELISA) (Lowry et al., 1951; Gray et al., 1999). Approximately 0.5 cm³ piece of tissue was homogenized in 1 ml of extraction buffer provided by the kit (Assay Designs Stressgen HSP70 ELISA kit), supplemented with 1 µg/ml aprotinin (a protease inhibitor). After centrifugation at 21,000 g for 10 min, the supernatant was used to estimate proteins by Lowry's method,

the rest was diluted by the sample diluent provided by the kit in ratio 1:4 and was used in the assay procedure. HSP70 values were expressed in ng/mg protein.

Renal caspase-3 activity (as a marker of apoptosis)

A specimen was taken from each frozen kidney homogenized with caspase 3 reaction buffer supplied with the kit. The supernatants obtained after centrifugation were used to determine enzyme activity by caspase 3 colorimetric assay kit (Assay Design, Inc. Michigan USA) (Kaushal et al., 1998). The standard protocol was followed as detailed by the manufacturer. The caspase 3 activity was expressed as unit/mg tissue weight. Unit is defined as the amount of enzyme needed to convert one picomole of substrate per minute at 30°C.

Renal mitogen activated protein kinase

The renal mitogen activated protein kinase (phospho-p38 MAPK) concentration was measured by phospho-p38 TiterZyme enzyme immunometric assay (EIA) kit. Harper and LoGrasso (2001) renal tissue was lysed in a radioimmunoprecipitation assay (RIPA) lysis buffer modified by the addition of phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (PIC) immediately prior to use. Samples lysed in RIPA cell lysis buffer plus inhibitors must be diluted at least 1:80 with (N-morpholino)-2-hydroxypropanesulfonic acid (MOPSO) buffered saline plus inhibitors prior to running in the assay.

Histological examination

Four-micrometer sections were stained with periodic acid-Schiff (PAS) reagents. Tubular injury was scored semi-quantitatively using a scoring system ranging from 0 to 4 (Ysebaert et al., 1997). Tubular injury was defined as a tubular dilatation, sloughing of tubular epithelial cells, or naked tubular basement membrane. Only tubules in the outer strip of the outer medulla (most sensitive zone for ischemic injury) were included. The scoring system was as follows: 0, no tubular injury; 1, <20% of tubules injured; 2, 21 to 50% tubules injured; 3, >50% damage of tubule cells; 4, total destruction of all epithelial cells.

Immunohistochemistry

Immunohistochemistry was performed to localize the expression of HSP70 in renal cortex and medulla by using antibodies against HSP70.

Statistical analysis

All data were expressed as mean \pm standard error of the mean (S.E.M) for eight rats per experimental group. Statistical group analysis was performed with SPSS 17.0 statistical software. One-way analysis of variance (ANOVA) was used to compare the mean values of quantitative variable among the groups. Least significant difference (LSD) was used to identify the significance of pair wise comparison of mean values among the groups. Pearson's correlation coefficient (r) was used to measure the mutual relationship between two quantitative variables. Statistically significant differences were assumed at P less than or equal 0.05 (Winer, 1971).

RESULTS

Biochemical results

Significant increase in serum urea nitrogen and creatinine concentrations, renal phospho-p38 MAPK concentration and in caspase-3 activity, with no significant change in renal HSP70 concentration, have been observed in non-treated rats killed 48 h after I/R as compared to sham operated rats.

Administration of herbimycin A resulted in a significant increase in HSP70, significant renoprotective effect was evidenced by a significant decrease in serum urea nitrogen and creatinine concentrations. Herbimycin A administration also resulted in antiapoptotic effect as evidenced by a significant decrease in renal caspase-3 activity, and a significant decrease in renal MAPK-p38 in rats killed 48 h following I/R as compared to non-treated I/R injury control rats.

To define the role of HSP70 in herbimycin-induced renal protection, herbimycin-treated group was administered KNK437. KNK437 significantly decreased HSP70-induction, renoprotective and antiapoptotic effects of the drug. In sham operated and I/R rats, KNK437 administration alone did not result in a significant change in measured parameters as compared to rats that did not receive KNK437 (Table 1).

Histological results

Histological examination of PAS-stained kidney sections indicated much less necrosis of proximal tubule cells and less obstruction and red cell trapping in the outer medulla of herbimycin A-treated animals when compared with kidneys of rats with I/R (Figure 1). Quantitative analysis of tubular necrosis showed a significant decrease in necrosis score in herbimycin A-treated rat kidneys as compared to non-treated rats with I/R injury. When KNK437 was administered prior to herbimycin A, the drug lost its renoprotective effect (Table 1).

Immunohistochemical results

Immunohistochemistry

In non-treated I/R injury rats, HSP70 immunopositivity was detected in minimal amounts in the cortex and in the medulla. Following treatment with herbimycin A, HSP70 immunopositivity was increased in the cortex and in the medulla. When KNK437 was administered prior to herbimycin A, the drug lost its HSP70-inducing effect (Figure 2).

Correlation results

Correlation values (r) between HSP70 and the renal

Table 1. Effect of herbimycin A (with or without KNK437) on serum urea nitrogen and creatinine concentrations, renal heat shock protein70 (HSP70), mitogen activated protein kinase (phosphor-p38 MAPK) concentrations and caspase-3 activity as well as renal necrosis score in studied groups, 48 h following ischemia reperfusion (I/R) injury to rat kidney (mean \pm S.E.M).

Group	Parameter					
	Serum urea nitrogen (mg/dl)	Serum creatinine (mg/dl)	Renal HSP70 (ng/mg protein)	Renal phospho-p38 MAPK (pg/g tissue weight)	Renal caspase-3 activity (U/mg tissue weight)	Renal necrosis score
Normal control (Ia) n = 8	28.17 \pm 2.17	0.2 \pm 0.08	3.65 \pm 0.23	126 \pm 24.10	3.87 \pm 0.64	0
Normal control administered KNK437 (Ib) n = 8	29.24 \pm 2.78	0.24 \pm 0.07	3.35 \pm 0.16	129 \pm 19.65	3.79 \pm 0.37	0
Normal control administered herbimycin A (Ic) n = 8	30.11 \pm 3.29	0.28 \pm 0.05	3.79 \pm 0.23	127 \pm 21.67	3.49 \pm 0.24	0
I/R (IIa) n = 8	89.45 \pm 4.38*	2.80 \pm 0.05*	3.89 \pm 0.45	613 \pm 54.18*	10.98 \pm 2.11*	3.5 \pm 0.2*
I/R administered KNK437 (IIb) n = 8	82.17 \pm 2.87 [#]	2.78 \pm 0.03 [#]	3.58 \pm 0.54	599 \pm 38.21 [#]	11.35 \pm 1.70 [#]	3.2 \pm 0.4 [#]
I/R treated with herbimycin A (3 mg/kg i.p) (III a) n = 8	52.52 \pm 3.15* ^{&}	1.03 \pm 0.06* ^{&}	9.76 \pm 0.65* ^{&}	368 \pm 49.34* ^{&}	5.98 \pm 0.62* ^{&}	1.6 \pm 0.1* ^{&}
I/R treated with herbimycin A and administered KNK437 (IIIb) n = 8	78.69 \pm 6.67 ^{#^}	2.77 \pm 0.27 ^{#^}	4.86 \pm 0.66 ^{#^}	591 \pm 65.87 ^{#^}	8.91 \pm 0.82 ^{#^}	3.1 \pm 0.3 ^{#^}
F value	47.32	67.19	72.91	76.54	55.21	49.33
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

N = number of rats in each group. *Significant compared to normal control group (Ia). [#]Significant compared to normal control group (Ib). [&]Significant compared to I/R group (IIa). [^]Significant compared to I/R treated with herbimycin A (III a).

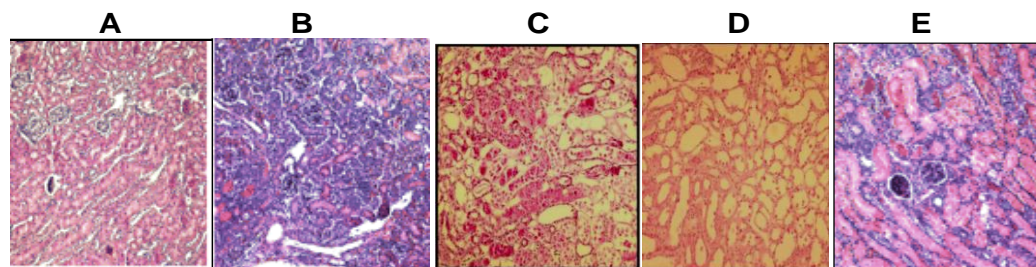


Figure 1. Light microscopic examination: PAS \times 200. (A) Sham operated rat kidney and herbimycin A treated rat kidney, (B) normal structure, (C) I/R demonstrates severe renal tubular necrosis, (D) treatment with herbimycin or (E) lack of effect when KNK437 was given prior to herbimycin.

function parameters: Serum urea nitrogen and creatinine and the apoptosis marker, caspase-3, were measured. A significant negative correlation between HSP70 and urea nitrogen, creatinine and caspase-3 was detected (Table 2).

DISCUSSION

In the present study, we provide evidence that herbimycin A has a protective effect against I/R in rat kidneys. Herbimycin A-treated kidneys showed

functional protection following I/R. Furthermore, histological examination revealed less necrosis of proximal tubule and less obstruction in the outer medulla in herbimycin A-treated rat kidneys as compared to non-treated rat kidneys.

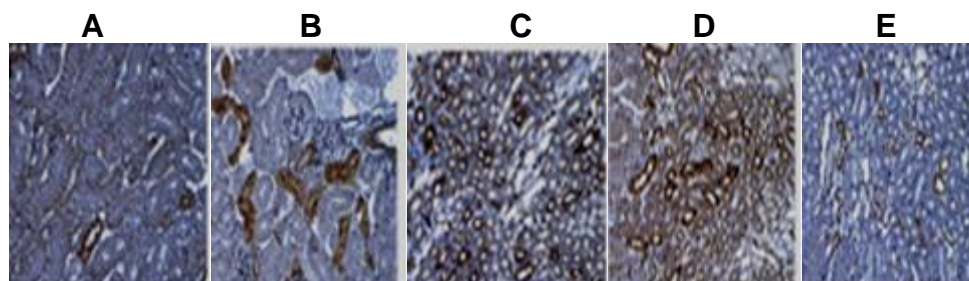


Figure 2. Immunohistochemical expression of HSP70 in: Non-treated renal ischemia reperfusion injury rat kidney cortex (A) and medulla (C) and in herbimycin A treated renal ischemia reperfusion injury rat kidney cortex (B) and medulla (D) and in KNK437 pre-treated rat renal medulla (E).

Table 2. Correlation values (R) between heat shock protein70 (HSP70) and serum urea nitrogen, serum creatinine and renal caspase-3 activity.

Parameter	Urea nitrogen	Creatinine	Caspase-3
HSP-70	-0.65*	-0.39*	-0.43*

*P < 0.01.

Apoptosis has been proposed as the mechanism that produces cellular demise in ischemic rat kidneys (Dagher, 2004), and this might be the target of actions of herbimycin. In the present study, I/R increased caspase-3 activity 48 h after I/R. However, herbimycin A treatment was accompanied by decreased activity of caspase-3.

The results of our study clearly demonstrate that herbimycin A may exert such a protective effect by inducing the expression of some HSPs, e.g. HSP70, which in turn inhibits apoptosis as evidenced by a significant decrease in caspase-3 activity and as has been reported by previous data indicating that HSP70 can inhibit apoptosis by interfering with target proteins (Ravagnan et al., 2001).

Several lines of evidence in the present study support the previous statement. First, treatment of rats with herbimycin A markedly induced the levels of HSP70 and increased immunohistochemical expression of HSP70 in renal cortex and medulla in I/R injury rats. Correlated with these results, administration of herbimycin A produced functional protection against I/R, and furthermore, KNK437, an inhibitor of HSP, when administered prior to herbimycin A, markedly attenuated the protective actions of the drug in I/R rats, suggesting that the protective effect of herbimycin A on I/R is probably through the induction of HSP70. A nephrotoxic effect of KNK437 or herbimycin A was excluded in the current study by the finding of lack of significant difference in assessed parameters between sham operated that received KNK437 or herbimycin A and that did not receive KNK437 or herbimycin A. Moreover, herbimycin A failed to induce HSP70 in sham operated rats, denoting that

ischemic stress is a prerequisite for herbimycin A-induced HSP70 expression.

The induced HSP70 by herbimycin A may help renal tubular cells adapt to I/R. In general, HSP70 inhibits a signal transduction pathway leading to apoptosis by preventing stress-induced activation of JNK and p38-MAPK activation (Gabai et al., 1997). In addition, HSP70 strongly reduces the activation of stress-induced kinases in response to tumor necrosis factor- α and interleukin-1 (Jaattela et al., 1992) and decreases apoptotic cell death and proapoptotic genes that appear following I/R injury (Yeh et al., 2010). Indeed, previous studies have reported that p38-MAPK activation was markedly reduced in renal ischemic preconditioning (Park et al., 2001) as well as in hepatic ischemic preconditioning (Massip-Salcedo et al., 2006).

Therefore, we propose that induction of some HSPs by herbimycin A inhibits apoptotic cell death in rat kidneys with I/R injury by inhibiting MAPK activity, as was observed in this study.

Through our study, we demonstrated that herbimycin A induces some HSPs, e.g. HSP70, but it is still unknown how the drug induces these HSPs. It has been suggested that herbimycin A may directly modify the transcription factor nuclear factor kappa B (NF- κ B) (Ogino et al., 2004). As a possible link between NF- κ B and stress, protein transcription has been suggested (Petrof et al., 2004), thus herbimycin A might possibly induce some HSPs via inhibitory action on NF- κ B.

The results of our study are in accordance with that of a study demonstrating the ability of herbimycin A to induce HSP70 and protect against apoptosis in hepatocytes (Kaushal et al., 1998).

Conclusions

This study demonstrates a role for some HSPs in protection against I/R injury to the kidney. The ability of herbimycin A to induce ischemic tolerance suggests that pharmacological strategies to increase stress protein expression might have potential merit to prevent ischemic injury to the kidney and other organs. Clinical studies will be necessary to evaluate the therapeutic properties of herbimycin A in preventing I/R injury not only in kidneys but also in other solid organs.

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