Claudia Akemi Shibuya Teshima[1], Mirian Watanabe[1], Sandra Hideko Nakamura[1], Maria de Fátima Fernandes Vattimo[2]

Renoprotective effect of statin: a ischemia-reperfusion animal model

Efeito renoprotetor da estatina: modelo animal de isquemia-reperfusão

1. Researcher of the Experimental Animal Models Laboratory (LEMA) of the Escola de Enfermagem da Universidade de São Paulo – USP - São Paulo (SP), Brazil.
2. Full Professor, Professor for the Escola de Enfermagem and Experimental Animal Models Laboratory (LEMA) of the Escola de Enfermagem da Universidade de São Paulo – USP - São Paulo (SP), Brazil.

Work developed at Experimental Animal Models Laboratory (LEMA) of the Escola de Enfermagem da Universidade de São Paulo – USP - São Paulo (SP), Brazil.

Submitted on July 26, 2010
Accepted on September 8, 2010

Author for correspondence:
Maria de Fátima Fernandes Vattimo
Av. Dr. Enéas de Carvalho Aguiar, 419 - Cerqueira César
ZIP CODE: 05403-000 - São Paulo (SP), Brazil.
Phone: +55 11 3061-8837 / Fax: + 55 11 3061-7546
E-mail: nephron@usp.br

ABSTRACT

Objective: Ischemic acute kidney injury comes from multifactor causes and has alarming morbidity and mortality. Statins, HMG-CoA reductase inhibitors, have shown renoprotective effects, with antioxidant, anti-inflammatory and vascular actions. The heme oxygenase-1 can be involved in those statin pleitropic effects on the renal function. This study was performed to evaluate if the statin renoprotective effect in rats comes from a heme protective mechanism.

Methods: The ischemia model was produced by bilateral clamping of the renal pedicles for 30 minutes, followed by reperfusion. Adult Wistar male rats, weighting between 250-300g were divided into the following groups: SHAM (control); Ischemia (30 minutes renal ischemia); Ischemia+Statin (sinvastatin 0.5mg/kg,orally for 3 days); Ischemia+Hemin (Hemin, 1.0mg/100g, intraperitoneal, 24 hours before surgery); Ischemia+SnPP (SnPP 2µmol/kg, intraperitoneal, 24 hours before surgery); Ischemia+Statin+Hemin; Ischemia+Statin+SnPP. Renal function (creatinine clearance by the Jaffé method), urinary peroxides, urinary osmolarity and immunohistochemical for ED-1 were evaluated.

Results: The results showed that sinvastatin ameliorated renal function, urinary osmolarity, reduced urinary peroxides excretion and the macrophage infiltration in rats submitted to renal ischemia. The heme oxigenase-1 inducer and its association with sinvastatin induced similar patterns of renal function improvements.

Conclusion: This study confirmed the renal function statins protective effect, with antioxidant and anti-inflammatory actions, and suggests that this effect can have an interface with the heme renal protection system.

Keywords: Kidney/injuries; Simvastatin; Hydroxymethylglutaryl-CoA reductase inhibitors; Heme oxygenase-1; Rats, Wistar

INTRODUCTION

Hypotension and/or sepsis ischemia are among the most frequent causes of ischemic acute kidney injury (iAKI) in critically ill patients. In the post-transplantation period, it may lead to graft loss. iAKI involves a cascade of events with glomerular hemodynamic changes, tubular injury, inflammatory response activation and oxygen reactive species (ORS) release.[1] *In vivo* renal ischemia/reperfusion (I/R) models are used to elucidate the pathophysiologic mechanisms or implement renoprotective pharmacologic interventions. In this context, simvastatin has been considered, for its pleiotropic renal actions as anti-inflammatory, anti-oxidant and vascular protection. It should be emphasized that the pleiotropic actions are characterized by independent primary li-

polytic action. Recent evidence confirms the prevention of inflammatory vascular reactions both in clinical trials and animal models following statin administration.[2]

Cell protection and adaptation mechanisms, as the heme oxygenase-1 (HO-1) enzyme, are induced after ischemic insult. Heme oxygenase is a limiting enzyme degrading heme, facilitating its conversion to biliverdin. This requiring nicotinamide adenine dinucleotide phosphate (NADPH) conversion releases ferrous metal and produces carbon monoxide which, by biliverdin reductase action, transforms biliverdin into bilirubin. All this reaction resulting molecules are released in equimolar amounts and have antioxidative, anti-inflammatory, anti-apoptosis, and possibly immune system modulatory actions. Nephrotoxicity and renal ischemia animal models studies confirmed that this enzyme'S protective effect may result from by-products release.[3]

The lack of clinical possibilities to rescue or prevent acute kidney injury (AKI) in severely ill patients causes both discomfort and indignation for its unfavorable statistics. This scenario stimulates the development of studies aimed to elucidate the precise injury mechanisms, where pharmacological interventions, as statins and the heme oxygenase system, may represent therapeutic alternatives. Indeed, abrupt diseases, such as AKI, may limit the preventive procedures. However, statins have demonstrated significant results also after one single dose given 30 minutes before the insult, stressing its promising performance even after acute situations.[4] This study was aimed to evaluate the sinvastatin renal protective effects in the I/R animal model, and to evaluate if the heme oxygenase-1 mediation may be involved.

METHODS

All procedures in this study complied with the Brazilian College of Animal Experimentation (COBEA) Ethical Principles, and were approved by the Animal Experimentation Ethics Committee of the Biological Sciences Institute of the Universidade de São Paulo. All animals were provided free access to water and food, and were kept in thermal conditions with night and day cycles during the entire experiment.

Wistar male adult rats weighting between 250 and 300 gram were divided into the groups: SHAM – surgery simulation; Ischemia – bilateral renal pedicles clamping for 30 minutes; Ischemia+Statin – renal ischemia in animals pre-conditioned for 3 days with Sivascor® (Baldacci) 0.5 mg/kg given by oral gavage (p.o.); Ischemia+Hemin – renal ischemia and pre-conditioning with Hemin-Sigma, HO-1 inducer, 1 mg/100 g, intraperitoneal (i.p.) 24 hours before the surgery; Ischemia+Tin Protoporfirin (SnPP) – renal ischemia and pre-conditioning with SnPP-Sigma, selective HO-1 inhibitor, 2 µmol/kg, intraperitoneal (i.p.), 24 hours before the surgery; Ischemia+Statin+Hemin – renal ischemia and pre-conditioning with sinvastatin and Hemin; Ischemia+Statin+SnPP – renal ischemia and pre-conditioning with sinvastatin and SnPP.

The animals were anesthetized with thiopental sodium 40-50 mg/kg, i.p. and laparotomized for bilateral renal pedicles clamping for 30 minutes, and renal reperfusion. The animals were kept in metabolic cages to collect 24 hours urine for renal function (RF) and oxidative stress study. After this period, the animals were again anesthetized with thiopental sodium 60 mg/kg for a new laparotomy and blood drawn by abdominal aorta puncture for renal function study. Each animal's left kidney was excised for immunohistochemical evaluation.

Renal function

Was evaluated by the creatinine clearance by measuring the plasma and urinary creatinine using the Jaffé method.[5]

Tubular function

Urine osmolarity was measured with the Advanced Osmometer® model 3D3.

Oxidative stress

Urinary peroxides (UP) measurement using the FOX-2 method. The UP levels measurement is considered a biomarker of hydrogen peroxide generation and predictive of the oxidative stress extension for *in vivo* experimental models.[6,7]

Immunohistochemistry

Immunohistochemistry stained slides using anti ED-1 monoclonal antibody (macrophage and monocytes) – Serotec were evaluated.

Statistical analysis

The GLM (ANOVA univariate) method and multiple comparisons adjusted for Bonferrone tests were used. $P < 0.05$ values were considered significant.

RESULTS

Table 1 presents the significant glomerular filtration reduction (Ischemia 0.20 ± 0.02 versus SHAM 0.60 ± 0.07;

P < 0.05) and tubular function (Ischemia 766 ± 188 versus SHAM 1793 ± 191; P < 0,05) in the 30 minutes ischemia animals versus the SHAM control group, confirming the iAKI model, maintaining urinary flow. Pretreatment with sinvastatin, HO-1 inducer and the sinvastatin-inducer association showed improved RF with increased creatinine clearance values (Ischemia+Statin 0.49 ± 0.04; Ischemia+Hemin 0.46 ± 0.03; Ischemia+Statin+Hemin 0.54 ± 0.07, versus Ischemia 0.20 ± 0.02; P < 0.05) and tubular function (Ischemia+Statin 1153 ± 404; Ischemia+Hemin 1013 ± 211; Ischemia +Statin+Hemin 1314 ± 394 versus Ischemia 766 ± 188; P < 0.05). The HO-1 inhibitor administration and the sinvastatin+HO-1 inhibitor association induced creatinine clearance increase, however when the treatments were compared, the statistical difference favored Hemin, HO-1 inducer (Ischemia+Statin+Hemin 0.54 ± 0.07 versus Ischemia + statin + SnPP 0.36 ± 0.07; P < 0.05).

Table 2 presents the urinary peroxidase (UP) values. The animals submitted to ischemia were observed to have higher UP values versus control, confirming the oxidative mechanism involvement in this injury model (Ischemia 13.5 ± 0.8 versus SHAM 5.6 ± 0.9; P < 0.05). The animals submitted to ischemia and pretreated with either sinvastatin or HO-1 inducer responded with UP values reduction versus the Ischemia group (Ischemia+Statin 7.9 ± 1.0; Ischemia+Hemin 7.6 ± 1.0 versus Ischemia 13.5 ± 0.8; P < 0.05). The groups receiving SnPP or the drug association inducer or inhibitor with sinvastatin showed UP levels higher than the SHAM group (P < 0.05), however lower than the Ischemia group (Ischemia+SnPP 11.9 ± 1.1; Ischemia+Statin+Hemin 11.3 ± 1.1; Ischemia+Statin+SnPP 11.3 ± 1.1 versus SHAM 5.6 ± 0.9 versus Ischemia 13.5 ± 0.8).

Table 3 shows the kidneys macrophage infiltrate quantification for the different pharmacological interventions. An infiltrate values reduction was found for the groups given sinvastatin, Hemin, SnPP and Statin+Hemin, however not statistical significant.

Table 2 – Urinary peroxides values for the different groups

Groups	N	Urinary peroxides (nmol/g creatinine)
SHAM	8	5.6±0.9
Ischemia	8	13.5±0.8[a]
Ischemia+Statin	8	7.9±1.0[b]
Ischemia+Hemin	5	7.6±1.0[b]
Ischemia+SnPP	5	11.9±1.1[a]
Ischemia+Statin+Hemin	6	11.3±1.1[a]
Ischemia+Statin+SnPP	8	11.3±1.1[a]

[a]P < 0.05 versus SHAM; [b]P < 0.05 versus Ischemia. Data expressed as mean ± standard deviation.

Table 3 – Imunohistochemical macrophage infiltration quantification for the different groups

Groups N=4	Macrophages per field
Ischemia	2.90±0.60
Ischemia+Statin	0.50±0.06
Ischemia+Hemin	0.60±0.05
Ischemia+SnPP	0.80±0.05
Ischemia+Statin+Hemin	1.60±0.05
Ischemia+Statin+SnPP	2.80±0.05

Data expressed as mean ± standard deviation.

DISCUSSION

Although countless advances, both in research and clinical practice, ischemic AKI remains one of the main poor functional prognosis indicators, both for native and transplanted kidneys. Ischemic kidney injury mechanism is known to determine glomerular antigen-antibody complex deposition and induce neutrophil infiltration, which determines inflammatory response.

Table 1 – Global renal function of the different groups

Groups	N	UF (ml/min)	ClCr 100g (ml/min)	Urinary Osmolarity (mOsm)
SHAM	6	0.006±0.001	0.60±0.07	1793±191
Ischemia	7	0.013±0.008	0.20±0.02[a]	766±188[a]
Ischemia+Statin	10	0.016±0.011	0.49±0.04[b]	1153±404[a]
Ischemia+Hemin	8	0.013±0.003	0.46±0.03[b]	1013±211[a]
Ischemia+SnPP	9	0.010±0.005	0.33±0.04[a]	977±125[a]
Ischemia+Statin+Hemin	6	0.008±0.004	0.54±0.07[b]	1314±394[b]
Ischemia+Statin+SnPP	8	0.012±0.009	0.36±0.07[abc]	1218±274[b]

UF – urinary flow. [a]P < 0.05 versus SHAM; [b]P < 0.05 versus Ischemia; [c]P < 0.05 versus Ischemia+Statin+Hemin. Data expressed as mean ± standard deviation.

Mesangial cells respond to this irritant mechanism with eicosanoids, proteases and ORS secretion.[8] Several studies have shown beneficial effects of vascular protectors, vasodilators and anti-oxidants, with emphasis for statins, because their pleiotropic, anti-inflammatory and anti-oxidant effects.[9]

This study confirmed the beneficial renoprotective effect of sinvastatin pre-conditioning in the iAKI renal model, demonstrated by increased glomerular filtration, reduced tubular injury and reduced UP levels in animals subsequently submitted to 30 minutes ischemia. I/R animal model studies have shown sinvastatin renoprotective effects with improved acute tubular necrosis identified by histological examination and reduced sodium fraction excretion.[2] Pravastatin use in I/R rats model has also confirmed inflammatory response reduction as measured by IL-6 levels.[10] In clinical practice, studies evidenced lower AKI frequency after heart surgery when preoperative statins were given for two days.[11]

Different cell protection mechanisms are induced during renal I/R, including heart shock protein 32, also known as the inducible HO-1 isoform. HO-1 enzyme plays an important role in renal homeostasis.[12] Preconditioning with Hemin, HO-1 inducer, confirmed antioxidant renal protection in this I/R model, characterized by improved RF and reduced UP levels. The beneficial HO-1 inducer effect appears to be mainly related to the formation of products such as bilirubin, ferritin and carbon monoxide. Equimolar amounts bilirubin generation provide antioxidant protection, as well as ferritin formation, which inactivates iron in the oxidative injury cascade. Carbon monoxide acts as a potent vasodilator.[13]

The sinvastatin plus HO-1 inducer association failed to show superior results versus each therapy alone. The drug association strategy aimed to check if the renal function, tubular and peroxidation results would be better, worse, or would remain similar to each therapy alone. The lack of difference between the therapies either alone or associated leads to a likely catalytic similarity between sinvastatin and HO-1 which, once saturated, the common receptors couldn't trigger additional or different response.

Previous studies confirmed that statin is involved in HO-1 induction in several organs at transcription, translation and catalization levels, underscoring the interaction results observed in this study.[14]

Also it should be considered that the eNOS expression and NO levels have been related to sinvastatin renoprotection. Being highly relevant, it should be remarked that evidences show that HO-1 protective effects, or those from heme degradation, enhance the endothelial function by interfering with NO bioavailability. Three cell mechanisms illustrating this movement were described: HO and the heme products modulate the NO expression and activity, prevent vascular NO inactivation and compensate the vascular NO loss. Therefore, this study reinforces that the HO-1 effect as mediator of the sinvastatin renal function protective effect in I/R kidney injury should be considered, and implies that NO action may be involved in this context. Other studies evaluating these associated enzymes activity shall bring additional information on this cell interface.

The immunohistochemical studies results suggested that the anti-inflammatory effect of both, statin and HO-1, was in place in this I/R model. However, the minimal inflammatory infiltrate found even in the Ischemia group presumes that the ischemic time may have been insufficiently aggressive to provide more exuberant findings, which would underscore the histological injury and emphasize these agents anti-inflammatory effects.

In summary, the results presented the sinvastatin protective effects in this 30 minutes I/R renal model with improved RF, reduced oxidative injury mediators and inflammatory response. The sinvastatin pleiotropic effect was shown to have a possible interface with the heme oxigenase-1 enzyme, supposing that this is a heme renoprotection model. Studies on NO action and its interference with the HO system in the presence of sinvastatin will be providential to elucidate these still poorly understood pathways.

RESUMO

Objetivo: A lesão renal aguda isquêmica, de causa multifatorial, apresenta morbidade e mortalidade alarmantes. A estatina, inibidor de HMG-CoA redutase, tem demonstrado papel renoprotetor, com componente antioxidante, antiinflamatório e vascular. A atividade de heme oxigenase-1 pode ser mediadora desses efeitos pleitrópicos da estatina sobre o rim, ou seja, independente da ação de redução de lipídio. Esse estudo visou avaliar se o efeito renoprotetor da estatina pode ter mecanismo heme de proteção em ratos.

Métodos: O modelo isquêmico foi obtido por meio do clampeamento dos pedículos renais bilaterais por 30 minutos, seguido de reperfusão. Foram utilizados ratos Wistar, machos, pesando entre 250-300g, distribuídos nos seguintes grupos: SHAM (controle, sem clampeamento renal); Isquemia; Iquemia+Estatina (sinvastatina 0,5 mg/kg, via oral por 3 dias); Isquemia+Hemin (indutor de HO-1, 1 mg/100g, intraperitoneal 24h antes da cirurgia); Isquemia+SnPP (inibidor de HO-1, 2µmol/kg intra-

peritoneal 24h antes da cirurgia); Isquemia+Estatina+Hemin e Isquemia+Estatina+SnPP. Foram avaliados a função renal (*clearance* de creatinina, Jaffé), osmolalidade urinária, peróxidos urinários e a imunohistoquímica para ED-1.

Resultados: Os resultados mostraram que a estatina melhorou a função renal, a osmolalidade urinária, reduziu a excreção de peróxidos urinários e a infiltração de macrófagos em rins de animais submetidos à isquemia renal. O indutor da heme oxigenase-1 e a sua associação com sinvastatina reproduziram o padrão de melhora determinado pela sinvastatina.

Conclusão: O estudo confirmou o efeito renoprotetor da estatina, com ação antioxidante e antiinflamatória, e sugere que esse efeito tenha interface com o sistema heme de proteção renal.

Descritores: Rim/lesões; Sinvastatina; Inibidores de hidroximetilglutaril-CoA redutases; Heme oxigenase-1; Ratos Wistar

REFERENCES

1. Ikeda M, Prachasilchai W, Burney-Taney MJ, Rabb H, Yokota-Ikeda N. Ischemic acute tubular necrosis models and drug discovery; a focus on cellular inflammation. Drug Discov Today. 2006;11(7-8):364-70.
2. Todorovic Z, Nesic Z, Stojanovic R, Basta-Jovanovic G, Radojevic-Skodric S, Velickovic R, et al. Acute protective effects of simvastatin in the rat model of renal ischemia-reperfusion injury: it is never too late for the pretreatment. J Pharmacol Sci. 2008;107(4):465-70.
3. Hill-Kapturczak N, Chang SH, Agarwal A. Heme oxygenase and the kidney. DNA Cell Biol. 2002;21(4):307-21.
4. Nesic Z, Todorivic Z, Stojanoviic R, Basta-Jovanovic G, Radojevic-Skodric S, Velickovic R, et al. Single-dose intravenous simvastatin treatment attenuates renal injury in an experimental model of ischemia-reperfusion in the rat. J Pharmacol Sci. 2006;102(4):413-17.
5. Dórea EL, Yu L, De Castro I, Campos SB, Ori M, Vaccari EM, et al. Nephrotoxicity of amphotericin B is attenuated by solubilizing with lipid emulsion. J Am Soc Nephrol. 1997;8(9):1415-22.
6. Long LH, Evans PJ, Halliwell B. Hydrogen peroxide in human urine: implications for antioxidant defense and redox regulation. Biochem Biophys Res Commun. 1999;262(3):605-9.
7. Wolff SP. Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. Methods Enzymol. 1994;233:182-9.
8. Heyman SN, Rosenberger C, Rosen S. Experimental ischemia-reperfusion: biases and myths - the proximal vs. distal hypoxic tubular injury debate revisited. Kidney Int. 2010;77(1): 9-16.
9. Chatterjee PK. Novel pharmacological approaches to the treatment of renal ischemia-reperfusion injury: a comprehensive review. Naunyn Schmiedebergs Arch Pharmacol. 2007;376(1-2):1-43.
10. Sharyo S, Yokota Ikeda N, Mori M, Kumagai K, Uchida K, Ito K, et al. Pravastatin improves renal ischemia-reperfusion injury by inhibiting the mevalonate pathway. Kidney Int. 2008;74(5):577-84.
11. Billings FT 4th, Pretorius M, Siew ED, Yu C, Brow NJ. Early postoperative statin therapy is associated with a lower incidence of acute kidney injury after cardiac surgery. J Cardiothorac Vasc Anesth. 2010. [Epub ahead of print].
12. Sikorski EM, Hock T, Hill-Kapturczak N, Agarwal A. The story so far: Molecular regulation of the heme oxygenase-1 gene in renal injury. Am J Physiol Renal Physiol. 2004;286(3):F425-41.
13. Miyazano M, Garat C, Morris KG Jr, Carter EP. Decreased renal heme oxygenase-1 expression contributes to decreased renal function during cirrhosis. Am J Physiol Renal Physiol. 2002;283(5):F1123-31.
14. Hsu M, Muchova L, Morioka I, Wong RJ, Schröder H, Stevenson DK. Tissue-specific effects of statins on the expression of heme oxygenase-1 in vivo. Biochem Biophys Res Commun. 2006;343(3):738-44.