Protective Effect of HMG-CoA Reductase Inhibitor on Experimental Renal Ischemia-Reperfusion Injury

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Abstract

Background: Increasing evidence supports an important role for inflammation in the pathogenesis of renal ischemia-reperfusion injury (IRI). Recently, HMG-CoA reductase inhibitors, 'statins', have demonstrated anti-inflammatory effects independent of cholesterol-lowering. Hypothesis: We tested the hypothesis that a statin would improve outcome in a murine model of renal IRI. Upon finding a protective effect, we tested the hypothesis that the mechanisms by which statins protected in renal IRI was by reducing neutrophil and macrophage infiltration and upregulating the anti-inflammatory cytokine IL-6. Methods: Cervinastatin at various dosing regimens was administered to NIH Swiss mice to evaluate the effects on renal IRI. Analysis of renal structure, function, neutrophil and macrophage infiltration, cytokine production, as well as mortality was performed in cervinastatin- and saline-treated groups. Results: Primary: Cervinastatin pretreatment for 3 days led to a significant improvement in renal function, tubular injury as well as survival after IRI compared to saline-treated mice. Secondary: Neutrophil and macrophage infiltration into kidney tissue was similar in both groups. IL-6 was markedly upregulated early in the kidneys of statin-treated compared to saline-treated mice. Conclusion: These data demonstrate that a statin compound can improve the course of ischemic acute renal failure. Induction of protective molecules such as IL-6 may underlie this effect.

Introduction

HMG-CoA reductase inhibitors, 'statins', are commonly used to lower serum cholesterol levels. However, recent studies have demonstrated that statins can decrease vascular injury induced by angiotensin II as well as act as potent immunomodulators by inhibition of MHC-II-mediated T-cell activation [1, 2]. Renal ischemia-reperfusion injury (IRI) is the major cause of intrinsic acute renal failure, associated with high mortality in native kidneys, and delayed graft function in transplanted kidneys, associated with an increased rate of acute and chronic rejection [3–6]. Renal IRI is characterized by tubular and vascular injury, inflammation, and more recently, T-cell involvement [7, 8]. Though there have been abundant
pathophysiologic studies on renal IRI, there is still no specific treatment in humans. We hypothesized that statins, commonly used in hyperlipidemic patients, could also be protective in renal IRI. Using an established mouse model of renal IRI, we tested this hypothesis and found that cerivastatin pretreatment improved renal function, kidney structure and overall mortality during experimental renal IRI. Phagocyte infiltration into postischemic kidneys was evaluated as a potential underlying mechanism for protection, but this was unchanged. However, statin therapy led to a marked early increase in renal expression of the anti-inflammatory cytokine IL-6, which may have contributed to the tissue protection.

Methods

Animals

Seven to 8-week-old male NIH Swiss mice, weighing 20–30 g, were purchased from Harlan (Indianapolis, Ind., USA). Animals were housed in a pathogen-free environment under veterinarian’s observation.

Renal IRI

An established model of renal IRI in mice was used [9]. Briefly, mice were anesthetized with intraperitoneal pentobarbital (75 mg/kg), had abdominal incisions, and bilateral renal pedicles were bluntly dissected. Microvascular clamps were placed on both renal pedicles while the animal was kept at a constant temperature and well hydrated. After 30 min, the clamps were removed, the wounds were sutured, and animals were allowed to recover. Animals were sacrificed and kidneys harvested at 24 or 72 h postischemia for following analysis.

Schedule of Cerivastatin Administration

Cerivastatin powder was donated by Bayer (Leverkusen, Germany) and reconstituted with sterile saline. Mice were given 1 µg/g weight cerivastatin by intraperitoneal injection for 3 consecutive days prior to renal IRI. Control mice were given saline as vehicle. The animals were administered drug or saline in a blinded fashion. Pilot experiments were conducted with higher dose cerivastatin (100 µg/g weight for 3 days) which led to high mortality even prior to renal IRI surgery. Lower doses (0.1 µg/g weight) did not lead to protection, nor a single dose at 1 µg/g weight pre-IRI. Post-IRI treatment was also not protective.

Measurement of Renal Function

Serum creatinines were assessed at 0, 24, 48 and 72 h postischemia as a marker of renal function. Serum samples were obtained from the tail vein at each time point. An autoanalyzer (Roche Diagnostics Corp., Indianapolis, Ind., USA) was used for the measurement using a serum creatinine kit (Sigma, St. Louis, Mo., USA).

Histological Findings

At 24 and 72 h postischemia, harvested kidneys were sliced and fixed with 10% formaldehyde. After paraffin embedding, these tissues were stained with hematoxylin and eosin (HE).

Myeloperoxidase (MPO) Assay

To evaluate neutrophil and macrophage infiltration in the postischemic kidneys, a MPO assay was performed at 0 h (n = 3) and 24 h (n = 4) postischemia. Kidney samples were homogenized in ice-cold KPO4 buffer (1:20 w/v). Samples were spun at 17,000 g for 30 min at 4 °C, and pellets were washed and spun an additional two times. Then 0.5% hexadecyltrimethylammonium bromide-10 mM EDTA was added to the remaining pellet (6:1). Suspensions were sonicated and freeze thawed three times, then incubated at 4 °C for 20 min. After final centrifugation at 17,000 g at 4 °C for 15 min and addition of assay buffer (4:1), supernatants were measured for MPO. Changes in absorbance over 3.5 min were recorded at 460 nm. One unit of MPO was defined as a change of absorbance of one per minute. Results were expressed as units MPO per gram of protein which was detected using a bicinchoninic assay (Pierce Chemical Co., Rockport, Ill., USA).

Ribonuclease Protection Assay (RPA)

In order to evaluate effects on the production of a potent anti-inflammatory cytokine, IL-6, mRNA of postischemic kidneys was analyzed. Briefly, mRNA from mice kidneys 0, 2 and 24 h postischemia were isolated using TRIzol reagent (Invitrogen, Carlsbad, Calif., USA); 20 µg of each mRNA sample was hybridized with [α-32P]UTP-labeled multi-probe (mCK-1; BD Pharmingen, San Diego, Calif., USA), then digested with ribonuclease. After stopping the ribonuclease reaction by adding proteinase K, samples were purified and loaded on 5% acrylamide gel. Radioisotope signals from the gel were detected by Phosphorimager (Amersham Biosciences, Piscataway, N.J., USA) and analyzed using ImageQuaNT software (Amersham Biosciences).
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Fig. 2. HE staining for kidney tissue 24 and 72 h postischemia (400×): a saline-treated mouse 24 h postischemia, b cerivastatin-treated mouse 24 h postischemia, c saline-treated mouse 72 h postischemia, and d cerivastatin-treated mouse 72 h postischemia.

Statistics
Data are expressed as means ± SE. Student t test analysis was used for comparison of serum creatinine and MPO assay data. Mortality up to 72 h postischemia between the statin- and saline-treated groups was compared using the Kaplan-Meier method. Statistical significance was determined as p < 0.05.

Results

Effect of Cerivastatin Pretreatment on the Course of Renal IRI
Renal function, assessed by serum creatinine level, of the cerivastatin-treated group (n = 20) was significantly improved compared to the control group (n = 16) postischemia (p < 0.05) (fig. 1). HE staining revealed that saline-treated controls had more severe tubular injury than cerivastatin-treated animals, particularly at the cortico-medullary junction (fig. 2). Cerivastatin treatment also significantly improved mortality (fig. 3). At 72 h postischemia, 3 out of 20 animals died in the cerivastatin-treated group, whereas 9 out of 16 animals died in the control group (p < 0.05).

Dose Response to Cerivastatin Pretreatment
We administered various doses and regimens of cerivastatin (data not shown) prior to arriving on the dose we found to be protective. We found that a single dose pre- or

Fig. 3. Effects of statin treatment on mortality. Cerivastatin-treated animals had significantly reduced mortality compared to saline-treated animals. Straight line: saline-treated; dash line: cerivastatin-treated. * p < 0.05.
Leukocyte Infiltration and Cytokine mRNA Expression

In order to elucidate if reduced neutrophil and macrophage infiltration was the mechanism by which statin protected the kidneys, leukocyte infiltration was analyzed by MPO assay in kidney samples. No significant difference was observed between the cerivastatin- and saline-treated groups at 0 and 24 h postischemia. However, MPO activity increased in both groups with time (fig. 4). We then explored if induction of a ‘protective’ cytokine could have accounted for the protection in the statin group. The mRNA expression of IL-6 was significantly upregulated in the kidneys from cerivastatin-treated animals at 2 h postischemia compared to the saline-treated group (fig. 5).

Discussion

These findings demonstrate that a HMG-CoA reductase inhibitor has a marked protective effect on renal function and tubular injury during renal IRI. In addition, mortality rates were decreased with statin pretreatment. The experiments that we conducted were acute in nature and performed in wild-type outbred mice. The results, though novel, are consistent with the lipid-lowering independent actions that have been attributed to the statins. Statins have been shown to reduce NF-κB and AP-1 transcription, which upregulate numerous inflammatory cascades, including leukocyte adhesion molecules and cytokines which mediate IRI [10]. Statins have also been demonstrated to upregulate endothelial nitric oxide synthase, which may exert a major tissue-protective role after ischemia [11]. It has been questioned if the plethora of non-lipid-lowering effects is due to HMG-CoA inhibition or another potential pathway. Administering mevalonate in statin studies circumvents the vascular protective effect, indicating that the protective action does indeed involve HMG-CoA reductase inhibition [10]. MHC class II expression is upregulated with renal IRI [12], and may participate in the immunopathogenesis of renal injury. Lipopolysaccharide-induced upregulation of MHC class II can be decreased by statins [2].

The infiltration of neutrophils and macrophages into the postischemic kidneys did not correlate with the severi-
ty of renal dysfunction postsischemia, thus the statin effect was appeared independent of leukocyte migration. Neutrophils have been implicated in the pathogenesis of renal IRI, and MPO is a useful quantitative measure of neutrophil infiltration [13]. However, MPO also detects macrophages [14]. IL-6 is an established anti-inflammatory cytokine with tissue-protective effects. IL-6+/− mice have been shown to be more susceptible to increased systemic inflammatory response during endotoxemia compared to IL-6+/+ mice [15]. We therefore evaluated IL-6 upregulation as a potential protective mechanism underlying cerivastatin’s action. Thus finding of increased IL-6 mRNA expression early postischemia suggests that IL-6 induction may participate in the protective effect of cerivastatin treatment.

Our findings in respect to the dose response to cerivastatin demonstrate a rather narrow therapeutic/safety window. It is important to note that coinciding with the completion of these murine studies, cerivastatin was removed from the human market due to fatal rhabdomyolysis in some patients. However, though the current results are novel in the kidney, there is recent data supporting a class effect of statins in protecting from ischemic injury in other organs independent of cholesterol-lowering. Simvastatin pretreatment has been shown to attenuate myocardial IRI in mice and rats [16, 17], and atorvastatin protected from cerebral ischemia in mice [18].

Statins are widely used, though not for the prevention of IRI. Based on our current data, further studies are required for exploring the mechanisms by which statins, including other specific agents in this category, can be protective in renal IRI. In addition, clinical studies should be considered to evaluate the therapeutic potential of statins to improve the course of ischemic acute renal failure.

Acknowledgments

This work was supported by an unrestricted grant from Bayer Pharmaceuticals. The authors would like to thank Valentine Abanum for his technical assistance.

References

7 Dragun D, Tullius SG, Park JK, Maasch C, Lukitsch I, Lippoldt A, Gross V, Luft FC, Haller H: ICAM-1 antiseNSE oligodeoxynucleo-
9 Rabb H, Daniels F, O’Donnell M, Haq M, Saba S, Keane W, Tang W: Pathophysiological role of T lymphocytes in renal ischemia reper-
11 Amin-Hanjani S, Stagliano NE, Yamada M, Huang PL, Liao JK, Moskowitz MS: Mevasta-
tin, an HMG-CoA reductase inhibitor, reduces stroke damage and upregulates endothelial nit-
13 Bradley PP, Prieber DA, Christensen RD, Rohrstein G: Measurement of cutaneous in-
15 Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, Achong MK: IL-6 is an anti-inflammatory cytokine required for controlling local or systemic acute inflammatory re-
17 Lefer AM, Campbell B, Shin YK, Scalia R, Hayward R, Lefer DJ: Simvastatin preserves the ischemic-reperfusion myocardium in nor-
18 Laufs U, Gertz K, Huang P, Nickenig G, Bohn M, Dirnagl U, Endres M: Atorvastatin upregu-
lates type III nitric oxide synthase in thrombocy-
tocytes, decreases platelet activation, and pro-
tects from cerebral ischemia in normocholeste-