Rosuvastatin Ameliorates Inflammation, Renal Fat Accumulation, and Kidney Injury in Transgenic Spontaneously Hypertensive Rats Expressing Human C-Reactive Protein

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Short title: Rosuvastatin renoprotective effects

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Abstract

Recently, we derived “humanized” spontaneously hypertensive rats (SHR-CRP) in which transgenic expression of human CRP induces inflammation, oxidative stress, several features of metabolic syndrome and target organ injury. In addition, we found that rosuvastatin treatment of SHR-CRP transgenic rats can protect against pro-inflammatory effects of human CRP and also reduce cardiac inflammation and oxidative damage. In the current study, we tested the effects of rosuvastatin (5 mg/kg) on kidney injury in SHR-CRP males versus untreated SHR-CRP and SHR controls. All rats were fed a high sucrose diet. In SHR-CRP transgenic rats, treatment with rosuvastatin for 10 weeks, compared to untreated transgenic rats and SHR controls, was associated with significantly reduced systemic inflammation which was accompanied with activation of antioxidative enzymes in the kidney, lower renal fat accumulation, and with amelioration of histopathological changes in the kidney. These findings provide evidence that, in the presence of high CRP levels, rosuvastatin exhibits significant anti-inflammatory, anti-oxidative, and renoprotective effects.

Key words: rosuvastatin, kidney damage, CRP, transgenic, spontaneously hypertensive rat

Introduction

C-reactive protein (CRP) is a widely used biomarker of acute systemic inflammation. In addition, CRP levels are used as a predictor for overall mortality in patients with chronic kidney disease or end stage renal disease (Zhang *et al*. 2013). However, the extent to which CRP itself promotes inflammation and contributes to the pathogenesis of kidney disease is highly controversial (Scirica *et al*. 2006). Recently, we derived "humanized" transgenic strain of spontaneously hypertensive rat (SHR-CRP transgenic) in which expression of human CRP in the liver is associated with increased levels of circulating human CRP, systemic inflammation, metabolic and hemodynamic disturbances, and target organ injury, including increases in albuminuria and histopathological changes such as fibrosis and inflammatory cellular infiltrates in the interstitium of the kidney (Pravenec *et al*. 2011). These findings suggest that expression of transgenic CRP in the SHR and associated inflammation might be causally related to kidney injury. Statins are recommended for treatment of patients with stage 1-3 of chronic kidney disease and with increased levels of LDL cholesterol because of possible role of dyslipidemia in the pathogenesis of kidney disease (Qaseem *et al*. 2013). However, results of several meta-analyses showed that renoprotective role of statins in patients with chronic kidney disease is controversial, depending on stage of the disease, presence of diabetes, specific statin used, ethnicity, etc. (Nicolic *et al*. 2013; Hou *et al*. 2013; Palmer *et al*. 2014). In addition to decreasing LDL cholesterol levels, pleiotropic effects of statins include reduction of inflammation and oxidative stress (Yagi *et al*. 2012). Recently, we found that rosuvastatin treatment of SHR-CRP transgenic rats decreased circulating levels of inflammatory response markers IL6 and TNFα and reduced cardiac inflammation and oxidative damage (Šilhavý et al. 2014). In the current study in SHR-CRP transgenic rats, we

investigated whether rosuvastatin could protect kidneys against inflammation, oxidative stress, ectopic fat accumulation, and tissue injury.

Methods

Animals. Transgenic SHR (hereafter referred to as SHR-CRP transgenic) were derived by microinjections of SHR ova with a previously described construct containing the cDNA for human CRP under control of the apoE promoter (Koike *et al*. 2009) with the objective of driving expression of the CRP transgene in liver where CRP is normally produced (Pravenec *et al*. 2011). To investigate effects of rosuvastatin on kidney injury associated with human CRP, we randomized 12 month old male SHR-CRP transgenic to groups with or without rosuvastatin treatment and also included age matched untreated control group of male non-transgenic SHR. SHR-CRP transgenic rats were treated with rosuvastatin (5 mg/kg/day) in the drinking water for 10 weeks. In each group, we studied 8 animals. All rats were fed standard rat chow for the first 12 months and then switched to a high sucrose diet (60% sucrose) to increase risk of developing metabolic disturbances during the 10 week study period. The rats were housed in an air-conditioned animal facility and allowed free access to sucrose diet and water. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic and were approved by the Ethics Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic, Prague.

Tissue triglyceride measurements. The kidney tissue was powdered under liquid N_2 and extracted for 16 hours in chloroform:methanol, after which 2% KH_2PO_4 was added and the solution was centrifuged. The organic phase was removed and evaporated under N_2. The

resulting pellet was dissolved in isopropyl alcohol, and triglyceride content was determined by enzymatic assay (Erba-Lachema, Brno, Czech Republic).

Urine collection, microalbuminuria, creatinine, urine cGMP, and glomerular filtration rate. Rats were placed into metabolic cages for 16 hours to obtain urine samples for analysis of urinary excretion of albumin. The level of albumin in urine was analyzed by HPLC method with UV-VIS detection according to Contois *et al*. (2006). Urine albumin was adjusted for creatinine concentration (mg/g creatinine). Urine and serum creatinine was measured by Jaffe rate assay (Erba-Lachema, Brno, Czech Republic). Urine cGMP was determined by immunoassay (Immunotech, France). Glomerular filtration rate (GFR) was calculated according to the formula GFR = Urine creatinine concentration x Urine flow/Plasma creatinine concentrations.

Parameters of oxidative stress. The activity of antioxidative enzymes and concentrations of lipoperoxidation products were measured as previously described (Malínská *et al*. 2010). The activity of superoxide dismutase (SOD) was analyzed using the reaction of blocking nitrotetrazolium blue reduction and nitroformazan formation. Catalase (CAT) activity measurement was based on the ability of H_2O_2 to produce with ammonium molybdate a color complex detected spectrophotometrically. The activity of seleno-dependent glutathione peroxidase (GSH-Px) was monitored by oxidation of gluthathione by Ellman reagent (0.01M solution of 5,5'-dythiobis-2 nitrobenzoic acid). The level of reduced glutathione (GSH) was determined in the reaction of SH-groups using Ellman reagent. Glutathione reductase (GR) activity was measured by the decrease of absorbance at 340 nm using a millimolar extinction coefficient of 6220 $M^{-1}cm^{-1}$ for NADPH (using Sigma assay kit). The levels of conjugated dienes (CD) were analyzed by extraction in the media (heptane:isopropanol = 2:1) and

measured spectrophotometrically in the heptane layer. The levels of thiobarbituric acid reactive substances (TBARS) were determined by the reaction with thiobarbituric acid.

Histolgy. Kidneys (N=5) from each group (SHR-CRP, SHR-CRP + rosuvastatin and SHR wild type) were cut along long axis and processed for paraffin embedding. Multiple 4 μm thick section were cut and stained with Hematoxylin-Eosin, PAS and Azan-Mallory trichrome stain for observation under light microscopy. Slides were observed and picture acquired with a digitalized camera by an experienced pathologist blinded by the groups.

Statistical analysis. All data are expressed as means ± S.E.M. Differences between experimental groups were analyzed by one away ANOVA with adjustments for multiple comparisons by Holm Sidak testing. Statistical significance was defined as $P<0.05$.

Results

Effects of rosuvastatin on body weight, relative heart and kidney weight, microalbuminuria, urine cGMP concentration, glomerular filtration rate, and renal lipids. Table 1 shows body and organ weights in SHR-CRP rats treated with rosuvastatin or placebo and in SHR controls. As can be seen, there were no significant effects of treatment of relative heart and kidney weight. Treatment of SHR-CRP transgenic rats with rosuvastatin was associated with significantly reduced microalbuminuria when compared to untreated SHR-CRP rats, however, both untreated SHR-CRP and rosuvastatin treated SHR-CRP rats exhibited significantly higher microalbuminuria when compared to nontransgenic SHR controls (Figure 1A). In addition, we have found that levels of the major nitric oxide second messenger cyclic GMP (cGMP) are significantly increased in urine collected from SHR-CRP transgenic rats treated

with rosuvastatin when compared to untreated SHR-CRP rats and are similar to those found in nontransgenic SHR (Figure 1B). There were no significant differences in GFR among the 3 experimental groups (data not shown). Furthermore, SHR-CRP transgenic rats treated with rosuvastatin had significantly reduced ectopic fat accumulation in their kidneys when compared to untreated SHR-CRP and SHR controls (Figure 1C).

Effects of rosuvastatin on CRP-induced oxidative stress in the kidney. Table 2 shows parameters of oxidative stress in renal cortex in SHR-CRP transgenic rats treated with placebo or rosuvastatin versus SHR placebo controls. The activity of antioxidative enzyme SOD was increased in SHR-CRP rats treated with rosuvastatin when compared to untreated transgenic rats and SHR controls. The activity of GSH-dependent enzyme, GSH-Px, in SHR-CRP rosuvastatin treated rats was similar to SHR controls and significantly higher when compared to untreated SHR-CRP rats. The activity of GSH-regenerating enzyme GR and the activity of catalase were similar among the 3 groups. Levels of GSH in SHR-CRP rosuvastatin treated rats were similar to those in SHR controls and were significantly higher than in untreated SHR-CRP rats. There were no significant differences in levels of lipoperoxidation products, conjugated dienes and TBARS (Table 2).

Effects of rousvastatin on amelioration of CRP-induced kidney inflammatory injury. Qualitative assessment of SHR-CRP animals on PAS and trichrome stained sections showed glomerular fibrosis with thickening of tubular basal membrane, focal inflammatory infiltrate and occasional protein casts in tubular spaces, both these features were absent in treated SHR-CRP rats and nontransgenic SHR controls (Figure 2A). We also observed reduction of glomerular density in SHR-CRP rats compared to treated SHR-CRP transgenic and control

SHR (Figure 2B). In addition, we observed an expansion of mesangial spaces accompanied by increased cellularity which was reverted by rosuvastatin treatment (Figure 2C).

Discussion

The causal role of CRP in the pathogenesis of atherosclerosis, cardiovascular disease, and kidney injury is highly controversial (Rietzschel *et al*. 2012). There is evidence from animal models of chronic kidney disease suggesting that CRP actively increases inflammation in the kidney (Li *et al*. 2011; Liu *et al*. 2011). In our original study, we also observed that transgenic overexpression of human CRP was associated with partial and total sclerosis of glomeruli and with fibrosis and inflammatory cellular infiltrates in the interstitium which provided compelling evidence for causal role of CRP induced inflammation in target organ injury (Pravenec *et al*. 2011).

Obesity is associated with ectopic fat accumulation and lipotoxicity which may lead to kidney dysfunction (reviewed in Guebre-Egziabher *et al.* 2013). The mechanisms connecting ectopic fat accumulation with chronic kidney disease remain to be determined. In our previous study (Šilhavý et *al*. 2014), rosuvastatin treatment in SHR-CRP rats was asociated with significant amelioration of insulin resistance in adipose tissue and increase lipolysis. Thus it is possible that resistance of adipose tissue to insulin action in SHR-CRP rats is associated with reduced uptake of fatty acids and glucose, ectopic fat accumulation and lipotoxicity in the kidney. In addition, rosuvastatin treatment was associated with increased levels of the major nitric oxide second messenger cGMP in SHR-CRP transgenic rats. Similar observation was reported in mice expressing human CRP transgene (Grad *et al*. 2007). It is therefore possible that

rosuvastatin ameliorates endothelial dysfuction in the kidney that is exposed to increased levels of human CRP (Mather 2013).

Regarding histologic results, the presence of mixed features of both hypertensive nephroangiosclerosis (Olson 1998) and mesangial expansion typical of diabetic nephropathy in its early stages (Tervaert *et al*. 2010; Valk *et al*. 2011) should be noted in the SHR-CRP group. These changes were reverted by rosuvastatin treatment and were extremely mild or absent in the SHR control group. These findings are in agreement with clinical data regarding kidney oxidative stress, microalbuminuria and glomerular filtration rate, suggesting the role of both inflammation and oxidative stress in glomerular injury of hypertensive dysmetabolic patients (Adler 2004; Kopp 2013; Wolf 2004).

In the current studies, we did not measure the effects of rosuvastatin on blood pressure. Because increased blood pressure can promote oxidative stress and inflammation, it should be recognized that the anti-oxidant and anti-inflammatory effects of rosuvastatin *in vivo* may also be secondary to the ability of rosuvastatin to limit increases in blood pressure otherwise induced by human CRP. On the other hand, putative blood pressure lowering effects of rosuvastatin might be secondary to its renoprotective effects. Thus it is difficult to distinguish between effects of rosuvastatin on blood pressure and renal protection.

The mechanisms by which rosuvastatin protects kidney against inflammation, oxidative stress, and ectopic fat accumulation are not fully understood. In our recent study (Šilhavý *et al*. 2014), we determined gene expression profiles in livers isolated from SHR-CRP and SHR rats treated with rosuvastatin or with placebo and identified Neurotrophin signaling and Influenza A as the most significant KEGG pathways that play important role in therapeutic

effects of rosuvastatin. These KEGG pathways include genes involved in Toll-like receptor signaling, c-Jun N-terminal MAPK signaling, the extracellular signal-regulated Raf/Mek/Erk signaling, and nuclear factor kappa B signaling as well as some additional genes involved in innate immune and antiviral defensive mechanisms. Thus it is likely that rosuvastatin treatment affects these inflammation regulatory pathways and at the same time decreases kidney fat accumulation by its hypolipidemic effects.

The use of statins in therapy of patients with chronic kidney disease is controversial. Some studies showed that statins might protect renal function while other found no benefits or even increased proteinuria, especially in statins with high cholesterol-lowering efficacy such as rosuvastatin (for review see Kalaitzidis *et al*. 2011). Results of the current study demonstrate important role of human CRP in the pathogenesis of kidney injury and significant anti-inflammatory, anti-oxidative, and renoprotective effects of rosuvastation in the SHR-CRP model. The current study thus raises the possibility that rosuvastatin treatment might be effective especially in humans with renal disease that is accompanied with increased CRP levels.

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Acknowledgements

This work was supported by grant LH11049 to VZ and LL1204 (within the ERC CZ program) to MP from the Ministry of Education, Youth and Sports of the Czech Republic; grant NT/14325 to MP from the Ministry of Health of the Czech Republic; grant MH CZ - DRO ("Institute for Clinical and Experimental Medicine - IKEM, IN 0002301") to LK.

Figure legends

Figure 1 Levels of microalbuminuria, urine cGMP (mg)/creatinine (g) ratio, and kidney fat content in SHR nontransgenic and SHR-CRP transgenic untreated rats and SHR-CRP transgenic rats treated with rosuvastatin. Significant differences between the groups are indicated by symbols and were determined by a one-way ANOVA and post hoc Holm-Sidak test: [a] denotes $P<0.05$ SHR vs. SHR-CRP, [b] denotes SHR-CRP vs. SHR-CRP+rosu, [c] denotes SHR vs. SHR-CRP+rosu.

Figure 2 Histological analysis of kidneys from untreated SHR-CRP transgenic, SHR-CRP transgenic rats treated with rosuvastatin and SHR controls. A. Glomerular fibrosis. Untreated SHR-CRP rats showed extensive glomerular fibrosis and global sclerosis while glomeruli from both rosuvastatin treated group and SHR controls showed no significative change of glomerular morphology. Azan-Mallory stain 40x original magnification. B. Glomerular density. SHR-CRP and SHR controls showed decreased glomerular density which was ameliorated in rosuvastatin treated group. Azan-Mallory stain 10x original magnification. C. Mesangial expansion. Glomeruli from SHR-CRP group are characterized by increased cellularity of the glomerular tuft and mesangial axis expansion characterized by both an increase in the number of cells and in deposition of extracellular matrix (arrow). This alterations were ameliorated in rosuvastatin treated animals and SHR controls. PAS stain 40X original magnification.

Table 1 Body and organ weights in SHR control rats treated with placebo versus SHR-CRP transgenic rats treated with placebo or with rosuvastatin

Trait / Strain	**SHR + placebo**	**SHR-CRP + placebo**	**SHR-CRP + rosuvastatin**
Body weight (g) *	470±10	443±7	420±8[c]
Heart weight (g/100 g BW) *	0.35±0.01[a]	0.39±0.01	0.40±0.01[c]
Kidney weight (g/100 g BW)	0.35±0.01	0.38±0.01	0.37±0.01

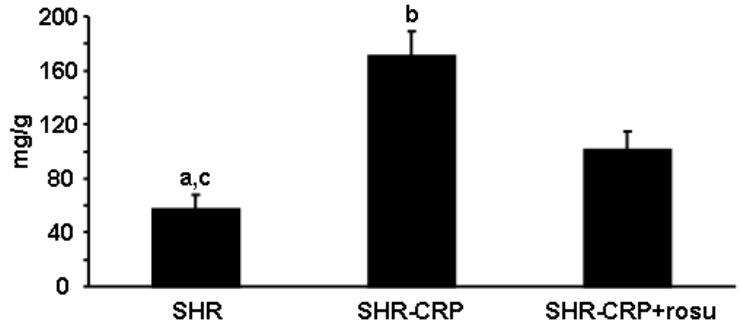
* denotes statistically significant differences in the mean values among groups as detected by one-way ANOVA; [a] denotes $P<0.05$ SHR vs. SHR-CRP, [b] denotes SHR-CRP vs. SHR-CRP+rosu, [c] denotes SHR vs. SHR-CRP+rosu.

Table 2 Parameters of oxidative stress in renal cortex in SHR control rats treated with placebo versus SHR-CRP transgenic rats treated with placebo or with rosuvastatin

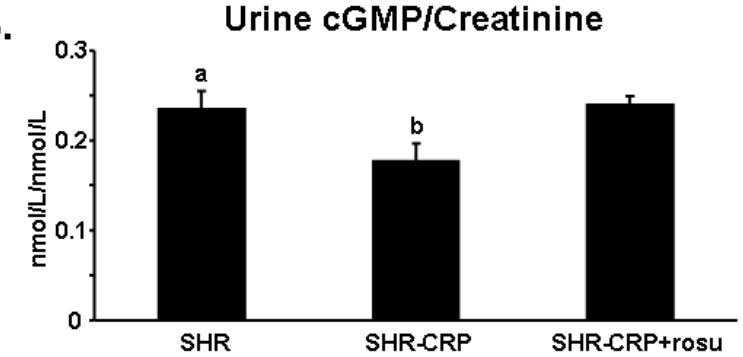
Trait / Strain	**SHR + placebo**	**SHR-CRP + placebo**	**SHR-CRP + rosuvastatin**
SOD (U/mg) *	0.040±0.003	0.034±0.003[b]	0.056±0.005[c]
GSH-Px (μM GSH/min/mg) *	127±9[a]	96±6[b]	131±10
GR (μM NADPH/min/mg)	58±6	68±4	54±5
CAT (mM H_2O_2/min/mg)	687±38	643±41	730±36
GSH (mM/g) *	9.3±0.8[a]	5.9±0.3[b]	9.5±0.8
CD (nM/mg)	13.1±1.0	14.0±0.8	13.8±1.1
TBARS (nM/mg)	0.74±0.06	0.83±0.06	0.92±0.09

* denotes statistically significant differences in the mean values among groups as detected by one-way ANOVA; [a] denotes $P<0.05$ SHR vs. SHR-CRP, [b] denotes SHR-CRP vs. SHR-CRP+rosu, [c] denotes SHR vs. SHR-CRP+rosu.

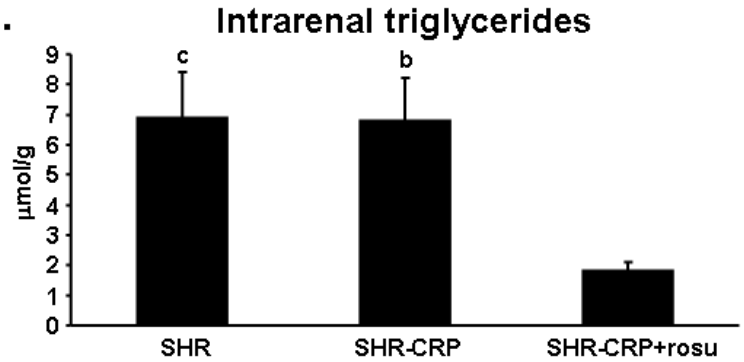
A.


Microalbuminuria
200
160
120
80
40
0
mg/g
a,c
b
SHR
SHR-CRP
SHR-CRP+rosu

B.


Urine cGMP/Creatinine
0.3
0.2
0.1
0
nmol/L/nmol/L
a
b
SHR
SHR-CRP
SHR-CRP+rosu

C.


Intrarenal triglycerides
9
8
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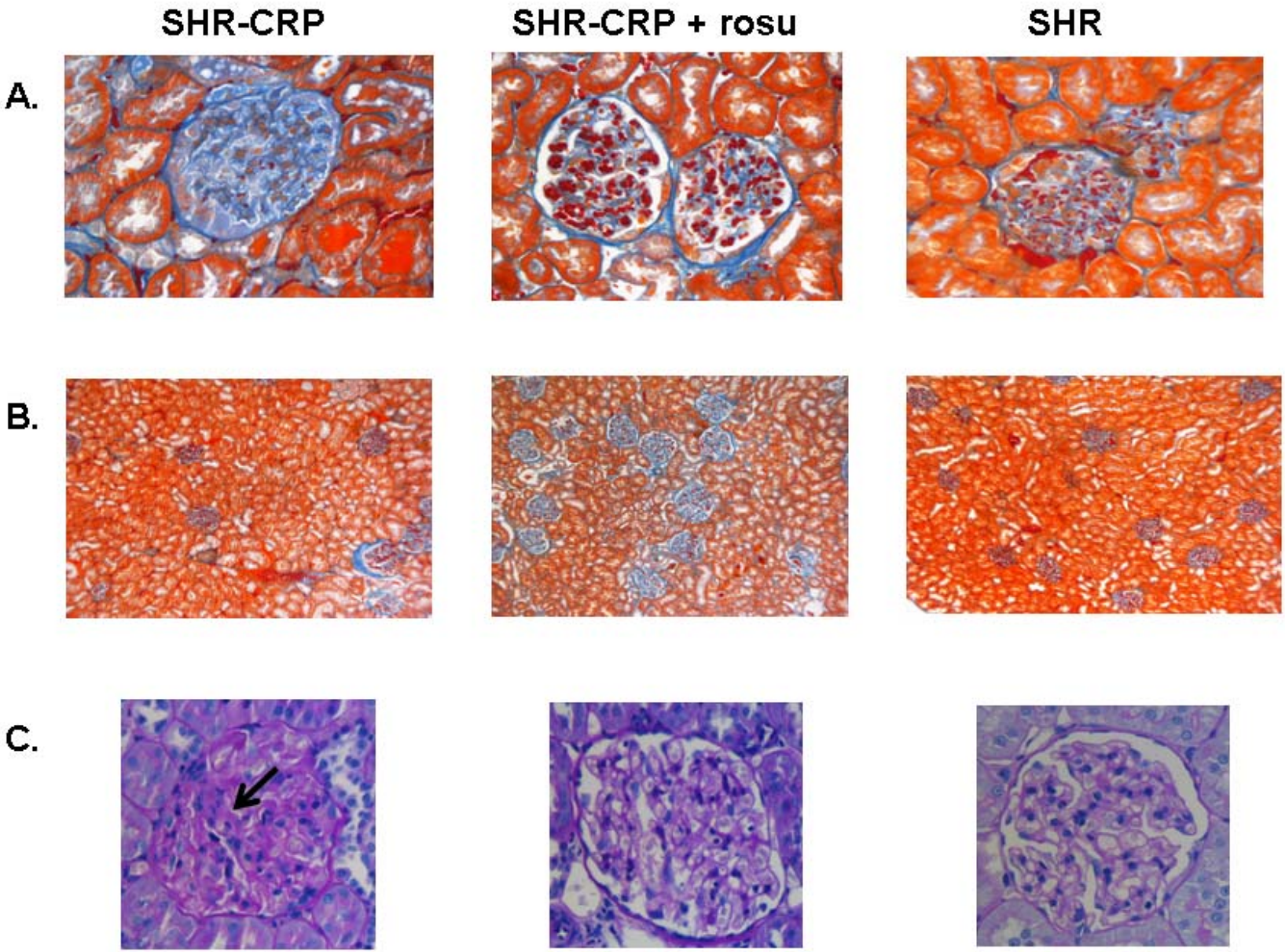
SHR-CRP
SHR-CRP + rosu
SHR
A.
B.
C.