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1 Statins reverse renal inflammation and endothelial dysfunction

2 induced by chronic high salt intake

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29 Running Head: High sodium diet induced proteinuria

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30 Abstract.

31 High salt intake (HS) is a risk factor for cardiovascular and kidney disease. Indeed, HS
32 may promote blood-pressure-independent tissue injury via inflammatory factors.
33 The lipid lowering 3-hydroxy 3-methylglutaryl-coenzyme A (HMG CoA) reductase
34 inhibitors exert beneficial lipid-independent effects, reducing the expression and
35 synthesis of inflammatory factors. We hypothesized that HS impairs kidney structure
36 and function in the absence of hypertension and these changes are reversed by
37 atorvastatin.
38 Four groups of rats were treated for 6 week in metabolic cages with their diets: Normal
39 salt (NS); HS, NS plus Atorvastatin and HS plus Atorvastatin. We measured basal and
40 final body weight, urinary sodium and protein excretion ($U_{Prot}V$) and systolic blood
41 pressure (SBP). At the end of the experimental period, cholesterolemia, creatinine
42 clearance, renal vascular reactivity, glomerular volume, cortical and glomerular eNOS
43 and TGF-β1 expression were measured. We found no differences in SBP, body weight,
44 and cholesterolemia. HS had increased creatinine clearence, $U_{Prot}V$, glomerular volume
45 at the end of the study. Acetylcholine-induced vasodilatation decreased by 40.4 % in the
46 HS ($p < 0.05$). The HS decreased cortical and glomerular eNOS and caused mild
47 glomerular sclerosis, interstitial mononuclear cell infiltration and increased cortical
48 expression of TGF-β1. All of these salt-induced changes were reversed by Atorvastatin.
49 We conclude that long term HS induces inflammatory and hemodynamic changes in the
50 kidney that are independent from SBP. Atorvastatin corrected all suggesting that the
51 NO-oxidative stress balance plays a significant role in the earlier stages of salt induced
52 kidney damage.

53 Introduction

54 High salt (sodium chloride) intake, an established habit in industrialized nations,
55 is an important risk factor for cardiovascular (27) and kidney disease (42). Indeed,
56 clinical and experimental studies have shown an independent association between
57 increased dietary salt intake and left ventricular hypertrophy, urine protein excretion,
58 and renal fibrosis and disease progression (9, 23). These associations could be of great
59 significance as proteinuria and renal disease have been implicated as independent risk
60 factors for cardiovascular mortality in hypertensives, diabetics, and in the elderly (18).
61 Although, high salt diet increases blood pressure in susceptible humans and in
62 experimental animals (19, 34, 40), it could also cause tissue injury independently from
63 blood pressure. For instance, salt promotes kidney and myocardial fibrosis along with
64 increased TGF-$ß_1$ expression and abnormal microvascular function; all of these in the
65 absence of changes in blood pressure (55). In addition, high salt diet impairs the
66 vasodilator response to acetylcholine (Ach), a marker of endothelial dysfunction. Under
67 normal conditions, ACh-induced vasodilatation entails the expression of endothelial
68 nitric oxide synthase (eNOS) and thereby nitric oxide (NO) activity. Acute high-salt
69 intake causes abnormal vascular smooth muscle relaxation in aorta and in skeletal,
70 cerebral, and resistance arteries (56). Whether chronic high salt intake, in the absence of
71 blood pressure changes, causes similar effects on endothelial function in the kidney is
72 unknown.

73 Interestingly, the lipid lowering 3-hydroxy 3-methylglutaryl-coenzyme A
74 (HMGCoA) reductase inhibitors (statins) exert beneficial lipid-independent effects on
75 endothelial function, increasing NO up-regulation, and reducing oxidative stress and
76 vascular inflammation (11, 21, 46, 58). Thus, in the kidney, statins could also
77 counteract inflammation by rising eNOS activity and hence NO bioavailability.

78 Consequently, we hypothesized that HS intake impairs kidney structure and
79 function in the absence of significant blood pressure changes and that these changes are
80 reversed by HMG CoA reductase inhibition. Accordingly, the aim of this study was to
81 assess whether high-salt intake induces renal endothelial dysfunction and inflammation
82 as reported in other vascular territories in the absence of hypertension and to evaluate
83 the effects of statins on these salt-induced changes.

84

85 Methods

86 *Animals*. Male Wistar Hokkaido rats (≈185g, from Faculty of Veterinary Science,
87 Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina) were housed
88 individually and maintained on ad libitum standard rat chow and tap water in 12
89 hours/12 hours light/dark cycle. After a 1 week acclimation period, the rats were divided
90 into 4 groups of 10 animals each: 1) untreated on normal salt (NS) diet, 2) untreated on
91 high salt (HS) diet, 3) Atorvastatin-treated on NS diet (NS+Ator), and 4) Atorvastatin-
92 treated on HS diet (HS+Ator). The NS rats were fed a diet containing 0.8 % NaCl. The
93 HS groups were fed a diet containing 4% NaCl. Atorvastatin was mixed into the rat
94 chow to receive a dose of 30 mg/kg per day (58). After one week of acclimation in the
95 metabolic cages and after six weeks on their respective regimens, we measured body
96 weight, urinary sodium excretion and systolic blood pressure (SBP) by the tail cuff
97 method (16). Briefly, for 7 days, the rats were acclimated to restraint and tail-cuff
98 inflation. Each rat was placed in a plastic restraint maintained at 33–36°C with its tail
99 passing through the optical sensor and the compression cuff, and then taped to the
100 platform. The cuff was connected to a blood pressure monitor. On inflation, the cuff
101 stopped the blood flow through the tail and upon deflation; the sensor detected the
102 reappearance of the blood flow. The pressure lecture at this point was used as measure
103 of SBP. Five to eight readings were performed and averaged for a single session value.
104 Then blood was drawn from the tail to measure serum creatinine and cholesterol. Body
105 weights were measured once a week. Twenty-four-hour urine was collected for the
106 determination of urinary excretion of protein, creatinine and sodium as described
107 previously (16).

108 All procedures were conducted on accordance with the *Guiding Principles in*
109 *the Care and Use of Animal,* as approved by the Council of the American Physiological
110 Society.

111 Renal vascular reactivity. At the end of the experimental period, animals were
112 anesthetized with sodium thiopental (40 mg/kg ip) and placed on a heating pad to
113 maintain rectal temperature at 37°C throughout the study. After tracheal intubation,
114 mean blood pressure was measured through a polyethylene catheter (PE-50) inserted
115 into the right carotid artery and recorded via a transducer connected to a multichannel
116 polygraph (Tekmar Co). The right jugular vein was cannulated with PE-50 tubing to
117 infuse saline during the surgery at a rate of 0.4 ml·100 $g^{-1} \cdot h^{-1}$. Then, through a midline
118 abdominal incision an electromagnetic flowmeter probe was positioned around the left
119 renal artery to measure renal blood flow (RBF) (model T106, Transonic Systems). The
120 left femoral artery was also cannulated with PE-50 tubing and advanced through the

121 abdominal aorta up into the renal artery, being careful not to disturb renal blood flow
122 according to previous readings. Through this tubing we injected first increasing doses
123 (10^{-10} through 10^{-5} mol/L) of Acethylcholine (Ach), then Sodium Nitroprusside (SNP),
124 and finally Angiotensin II (AngII) while continuously measuring RBF. The left ureter
125 was catheterized with PE-10 tubing for urine collection.

126 Glomerular Volumen. Upon completion of the renal vascular reactivity studies,
127 the left kidney was excised to measure glomerular volume. For this, we followed a
128 sieving technique modified from the procedure described by Beierwaltes et al (3). After
129 removing and mincing the cortices, glomeruli were harvested by successive sieving
130 through a 60-mesh (250 µm) stainless steel sieve and then through two Nitex
131 monofilament screens, 48 mesh (390 µm) and 72 mesh (250 µm). The filtered
132 suspension contained glomeruli and remnants of severed tubuli that were then filtered
133 through a 200 mesh (60 µm) silk bolting cloth. The recovered glomeruli were again
134 suspended in the modified Krebs solution, centrifuged at 750g for 5 minutes and the
135 supernatants decanted. This material was transferred to an observation/incubation
136 chamber on an inverted microscope (Diaphot Nikon) containing the same solution used
137 during the sieving procedure. Glomeruli were photographed and then a grid placed on a
138 similar chamber containing the same solution and photographed at the same power
139 previously used to photograph glomeruli (x140). This photographed grid contains pre-
140 measured squares that allow calibration of a digital image system Summa Sketch II.
141 Glomerular diameters were measured in no less than 30 glomeruli per kidney and
142 volumes calculated by the Weibel Gomez method (47).

143 Determination of the expression of eNOS and TGF-β1 in kidney. After six weeks on
144 their respective regimens, the kidneys were perfused with cold heparinized PBS, and
145 then cortices were dissected and placed in RIPA-DOC buffer with protease inhibitors
146 (PMSF 100 µg/ml leupeptin 5 µg/ml, isoleucine 2 µg/ml and orthovanadate 2 µg/ml). To
147 obtain total cortex protein samples an aliquot of each cortical sample was homogenized
148 with 10 strokes with a Kontes homogenizer. Glomeruli were isolated as described
149 before (Glomerular Volumen Section). Samples were clarified by centrifugation at
150 13,000 rpm for 5 minutes at 4°C, and the supernatant was stored at -70ºC until use.
151 Protein concentration in each sample were determined by the Bradford method, further
152 diluted in Laemmli sample buffer, 100 µg protein per sample were electrophoresed in a
153 15% polyacrylamide gel, and electrotransferred to a polyvinyliden difluoride membrane
154 (Millipore). The membrane was blocked with 5% BSA for 1 hour, then incubated
155 overnight at 4ºC with primary antibody followed with appropriate HRP-conjugated
156 secondary antibody (Jackson Immunoresarch), visualized by chemiluminescence (ICN),

157 and quantified by densitometry with the Scion Image 4.0 software. The following
158 antibodies were used, anti-eNOS (1:500 dilution; Santa Cruz Biotecnology), anti-TGF-
159 β1 (1:500; Chemicon) and anti-β actin (1:1000; Santa Cruz).

160 *Histological Study.*

161 The right kidney was fixed before removal by a 4% paraformaldehyde infusion.
162 The tissue was then embedded in paraffin for assessment by light microscopy and
163 immunohistochemistry. Two to three micron sections were cut and stained with
164 hematoxylin-eosin and Schiff periodic acid. Glomerular damage (as revealed by
165 fibrosis, adherence to the capsule, and mesangial expansion and proliferation),
166 interstitial mononuclear cell infiltration and arteriolar thickening were assessed. A
167 minimum of 100 glomeruli were evaluated in each kidney. The pathologist was blind to
168 the sample and used a 40X resolution to grade the severity in a scale from 0 to 3 as
169 follows: 0 = absent, 1 = mild, 2 = moderate, 3= severe. An average score was obtained
170 for both glomerular and interstitial changes.

171 Statistical Analysis: All values are expressed as means ± 1 standard deviation.
172 The level of significance for difference between means was evaluated by One Way
173 Analysis of Variance followed by Tuckeys pos hoc tests. Basal vs. final blood pressure,
174 urinary protein excretion and eNOS expression were compared using Kruskal Wallis
175 One Way Analysis of Variance by ranks.

176

177 Results:

178 *Physiological parameters and renal function.* Mean body weight (bw) was
179 similar in all groups at baseline and throughout the experimental period (Table 1).
180 Atorvastatin had no effect on water or food ingestion (data not shown). The expected
181 increase in body weigh along the 6 week study period was similar in all groups. The
182 systolic blood pressures at baseline and at the end of the study were not different
183 between groups (Table 1). All animals remained normotensive during the 6 week of
184 treatment.

185 As expected, rats on a HS diet increased their urinary sodium excretion rate (table 1).
186 Atorvastatin caused no changes in total serum cholesterol levels irrespective of the
187 amount of salt in the diet (total serum cholesterol was 85.7±10.4 mg/dl in NS group,
188 85.2±8.3 mg/dl in NS+Ator, 80.0±11.1 mg/dl in HS and 89.1±14.9 mg/dl in HS+Ator).
189 Similarly, Atorvastatin did not change cholesterol in any group (data not shown). This
190 data is consistent with other reports (7, 17, 30, 32, 35, 44, and 45) and is thought to
191 result from a statins-induced compensatory increase in the hepatic 3-hydroxy-3-
192 methylgutaryl-CoA reductase synthesis.

193 At the end of the six week experimental period the groups receiving a HS diet
194 had higher mean creatinine clearence than NS groups (NS 0.32 ± 0.09 ml/min/100 g
195 bw, NS+Ator 0.31 ± 0.14 ml/min/100g bw, HS 0.47 ± 0.13 ml/min/100 g bw *,
196 HS+Ator 0.49 ± 0.07 ml/min/100 g bw *, * p <0.014 vs control). At six weeks, the HS
197 diet increased the urinary protein excretion rate 4.8 fold compared to basal levels (p <
198 0.05). This increase was prevented by Atorvastatin (Figure 1). In rats on NS intake, the
199 protein excretion rate at six weeks was unchanged from basal values.

200 *Renal Hemodynamic changes:* Because proteinuria is often associated to
201 endothelial dysfunction, we evaluated the effect of HS diet on endothelium-dependent
202 vasodilatation. Basal mean intra-arterial blood pressure was not different between the
203 groups (NS 86±5, NS + Ator 73±4, HS 77±3 and HS + Ator 77±5 mmHg, p > 0.20).
204 Figure 2 shows that Acetylcholine-induced vasodilatation decreased by 40 % in the HS
205 group compared to the NS group (p < 0.05). This indicates that long-term high-salt
206 intake causes endothelial dysfunction. Atorvastatin administration prevented this effect.
207 Sodium nitroprusside-induced vasodilatation and angiotensin II-induced
208 vasoconstriction were unchanged by salt intake and Atorvastatin treatment (data not
209 shown). Because Acetylcholine–induced vasodilatation implies NO release, we
210 evaluated whether the beneficial effects of Atorvastatin on endothelial dysfunction
211 were associated to changes in cortical and glomerular eNOS expression in the kidney.
212 Figure 3 shows cortical eNOS expression as measured by Western blot. The HS diet

213 decreased cortical eNOS expression by 62% ($p < 0.05$). These effects were prevented
214 by atorvastatin. In animals on a NS diet, Atorvastatin had no effect on cortical eNOS
215 expression. Figure 4 shows glomerular eNOS expression. HS diet decreased glomerular
216 eNOS expression by 48% ($p < 0.05$) while Atorvastatin prevented this fall in eNOS
217 expression. These findings demonstrate that Atorvastatin prevents the endothelial
218 dysfunction induced by a HS diet at least in part, by increasing both cortical and
219 glomerular eNOS expression.
220 *Glomerular Volume and Renal Histological changes.* Because glomerular
221 changes are often associated to increased protein excretion rate, we evaluated the HS
222 diet-induced changes on glomerular volume and histology.
223 HS intake increased glomerular volume by 33.33 % ($p<0.001$) (Table 2 and
224 Figure 5) while treatment with Atorvastatin prevented the development of glomerular
225 hypertrophy in this HS diet group. Atorvastatin did not decrease the glomerular volume
226 in NS diet rats. The increase in glomerular volume observed in animals with HS diet
227 was positively correlated with the increase protein excretion rate (r = 0.83).
228 Likewise, HS diet caused a mild glomerular sclerosis together with interstitial
229 mononuclear cell infiltration, all of which were prevented by Atorvastatin (Table 2 and
230 Figure 6). These findings suggests that HS intake causes renal inflammation and that
231 Atorvastatin can prevent it even in the absence of changes in blood pressure or serum
232 cholesterol levels.
233 The glomerular changes in the HS intake group were accompanied by a
234 significant increase in TGF-β1 expression in renal cortex. TGF-β1 expression as
235 arbitrary units (au) were: HS 1.06 ±0.39, and NS 0.52±0.11, $p <0.05$, (Figure 7). This
236 change was reversed by Atorvastatin (HS 1.06 ±0.39 vs HS+Ator 0.61±0.09, $p <0.05$).
237

238 Discussion.

239 Several lines of investigation suggest that salt plays an important role in the
240 genesis of Hypertension and in cardiovascular injury (55). However, harmful effects
241 from high salt intake have been also recognized in the absence of high blood pressure.
242 Indeed, current evidence supports a direct effect of salt intake on vascular structures
243 and although the mechanisms involved are largely unknown, it has been proposed that
244 an anomalous endothelial response may bring about inadequate nitric oxide release and
245 excessive TGFβ1 synthesis (50, 51). Indeed, in our study, after 6 weeks of high salt
246 intake, we found higher urinary protein excretion, decreased renal endothelium-
247 dependent vasodilatation, decreased glomerular eNOS expression, and increased renal
248 TGFβ-1 synthesis, all in the absence of hypertension. These changes were accompanied
249 by increased glomerular volume and incipient renal interstitial inflammation. While
250 changing neither serum cholesterol (30) nor blood pressure, Atorvastatin prevented all
251 of the salt-induced renal changes.

252 Endothelial function and glomerular eNOS expression.

253 Our results must be viewed as taking place on a background of normal blood pressure.
254 Indeed, hypertension did not follow high salt intake and yet, endothelial dependent
255 vasodilatation in the kidney decreased in a dose dependent fashion varying from 42 to
256 57% when compared to rats on normal salt intake. These findings were associated to
257 diminished cortical and glomerular eNOS expression in the kidney, suggesting this
258 could be in part responsible for the impaired endothelium dependent vasodilatation.
259 Indeed, glomerular eNOS deficiency hastens the progression of kidney disease (31) and
260 correlates with vascular injury severity (22, 38). Thus, our findings seem very relevant
261 from a pathophysiological standpoint, as they could signal the onset of vascular lesions.
262 Our results are in line with studies showing that long term HS intake leads to structural
263 and functional changes in the microcirculation that are independent from changes in
264 arterial pressure (4, 5, 25). Indeed, normotensive rats on a HS diet show impaired
265 endothelium-dependent dilation in response to acetylcholine or increased shear stress in
266 several vascular territories. They also show that this impairment is due to selective loss
267 of NO activity (4, 5). For instance, Li et al (28) measured eNOS activity in living bovine
268 aortic endothelial cells and showed that a 5-mmol/L increase in salt concentration (from
269 137 to 142 mmol/L) causes a 25% decrease in eNOS activity in a salt concentration-
270 dependent manner. In fact, NOS activity decreased by 25, 45, and 70%, with respective
271 increments of 5, 10, and 20 mmol/L in NaCl concentration. They also showed that salt
272 attenuated the nitric oxide (NO)-dependent proliferation of endothelial cells. Likewise,
273 Banday et al (1) found that HS diet decreases in vivo the eNOS protein expression in

274 thoracic aortic tissue, while Zhu et al, (56) using aortic strips isolated from
275 normotensive rats on normal or HS diet reported that methacholine-induced relaxation
276 was significantly reduced in the HS group. Other investigators find that HS diet impairs
277 endothelium dependent relaxation (1) only when combined with a pro-oxidant like L-
278 buthionine sulfoximine. In this case, the impaired endothelial dysfunction is prevented
279 by maneuvers that decrease oxidative stress.
280 These studies demonstrating salt-induced endothelial dysfunction contrast with reports
281 that show increased, not decreased NO availability in animals on HS diet. For instance,
282 Wei-Zhong et al (52) reported that HS intake (8.0%) for 7 days did not affect blood
283 pressure but increased steady-state mRNA and protein levels of eNOS in the arterial
284 wall. Also, Ortiz et al (33) showed that HS diet for 7 days increases eNOS in the thick
285 ascending limb of Henle suggesting a compensatory mechanism to prevent volume
286 overload. Other investigators have reported that increased salt intake enhances NO
287 production in salt-resistant rats (8) and in healthy humans (2). In these settings, renal
288 plasma flow, glomerular filtration rate and pressure-natriuresis improve while blood
289 pressure returns to basal values (36). In addition, enhanced NO synthesis limits the
290 production of TGF-β1. Other studies also report increased eNOS expression and
291 preserved endothelial function in short term HS diet experiments (37). A distinctive
292 feature in these studies is the acute setting (few days of oral salt loading) and the
293 measurements taken at a time when regulatory mechanisms are still fully active laboring
294 to excrete the ingested sodium. Viewed in this context, increased eNOS activity after an
295 acute sodium chloride load seems a coherent response considering the well known
296 diuretic effects of NO. Nonetheless, because eNOS undergoes post-translational
297 changes that regulate its activity, decreased eNOS expression not necessarily entails
298 decreased NO production. Because we did not measure NO production in these animals,
299 this might be a potential limitation of our study.
300 The apparent discrepancy between the latter findings and our results may reflect
301 different experimental designs; namely, time of exposure to HS intake and the amount
302 of salt in the diet. Specifically, studies failing to show endothelial dysfunction used diets
303 containing 1-4% salt and for less than 16 days (37). We believe eNOS expression may
304 not hold during long term HS intake, particular in the kidney. Precisely, solving this
305 question was our goal and for this we assessed the long term effects of HS diet on renal
306 vascular reactivity and cortical and glomerular eNOS expression. Six weeks treatment is
307 a rather extended period in the lifespan of a rat and thus, the impairment in renal
308 vascular reactivity observed after 6 weeks of HS intake could be the forerunner to
309 further hemodynamic and structural changes.

310 HS intake increases TGFβ-1 expression and proteinuria and causes tissue changes
311 We found that HS intake enhances TGFβ-1 expression. This is in agreement with
312 studies by Ying et al (50) showing that dietary salt increases TGFβ-1 expression in a
313 dose-dependent fashion. Volume expansion during HS intake may increase shear stress
314 and intracellular calcium and so TGFβ-1 expression via Protein Tyrosine kinase 2 (26,
315 43, and 54). Moreover, HS intake increases TGFβ-1 expression by enhancing the gene
316 expression of phosphorylated p38 MAP kinase, and p42/44 MAP kinase. Inhibition of
317 these kinases decreases TGF–β1 production (53). Be that as it may, the higher TGFβ-1
318 expression during HS intake may have played a role in the development of glomerular
319 hypertrophy and sclerosis and interstitial mononuclear cell infiltration (58). All of these
320 conditions are known to increase urinary protein excretion rate.
321 We canot exclude increased glomerular pressure (via efferent arteriole constriction) as
322 the cause of increased proteinuria. This is suggested by the higher creatinine clearance
323 in the HS intake group while blood pressure remained unchanged.
324 Finally, HS diet may have induced proteinuria by disturbing podocyte permeability via
325 increased TGFβ-1 expression. Indeed, podocyte TGFβ-1 receptor activation increases
326 podocyte albumin permeability (50). Thus, HS intake may increase proteinuria by
327 hemodynamic and humoral mechanisms.
328 Statins decrease HS diet-induced proteinuria and endothelial dysfunction.
329 Because HMG-CoA reductase inhibition have been shown to exert serum cholesterol-
330 independent beneficial effects on endothelial dysfunction and proteinuria in several
331 experimental models (39), we investigated whether the HMG CoA reductase inhibitor
332 Atorvastatin, could reverse the effects of HS salt diet on these parameters. In effect, 6-
333 week treatment with atorvastatin fully corrected the renal vascular response to
334 Acethylcholine and the increased protein excretion rate in rats on HS intake. These
335 effects were independent from serum cholesterol or blood pressure levels.
336 As shown in several experimental models of renal injury, statins may attain their
337 beneficial effects in more than one manner. For instance, statins block the synthesis of
338 mevalonate a precursor of isoprenoids-farnesylpyrophosphate (F-PP), and geranylpyro-
339 phosphate (G-PP) which normally attach post-translationally to intracellular signaling
340 proteins. Thus, by blocking the synthesis of F-PP and G-PP, statins prevent the
341 anchoring of growth factors to the cell membrane and cytoskeleton, hence hindering
342 signal transductions to the nucleus, activation of transcription factors, and cell
343 proliferation in the vascular endothelium (10). These mevalonate-dependent effects of
344 statins seem unrelated to eNOS. Indeed, Yagi et al showed that Pivastatin prevents
345 Angiotensin II-enhanced proteinuria in eNOS-/- mice (30).

346 Atorvastatin preserves eNOS expression during salt loading

347 The second relevant aspect of Atorvastatin treatment is the reversion of endothelial
348 dysfunction and the correction of eNOS expression in animals on HS intake. In this
349 particular, Atorvastatin could have reversed harmful HS diet effects on a) eNOS
350 synthesis and NO bioavailability, b) increased levels of oxygen free radicals leading to
351 enhanced breakdown of NO (13); or c) alterations in signal transduction pathways such
352 as receptor G protein coupling, as shown for cAMP-mediated vasodilator responses in
353 animals on HS diet (15, 29).

354 Atorvastatin could have reversed the HS-induced eNOS inhibition by several means.
355 First, Statins prevent Rac1 from migrating to the cell membrane to activate NADH-
356 oxidase and generate Reactive Oxygen Species (12, 20, 48). Second, **S**tatins inhibit pro-
357 oxidant enzyme systems (NADPH oxidase, xanthine oxidase, etc) by blocking the
358 expression of protein subunits of G-proteins (p22phox and NOX2) (20). Third, statins
359 prolong eNOS mRNA half life and up-regulate eNOS expression (49). Fourth, by
360 blocking geranylgeranylation of Rho GTPase, statins also decrease the levels of the
361 surface protein endothelin-1, a potent vasoconstrictor and mitogen (6). All these effects
362 lead to decrease superoxide anion generation and enhance eNOS activity and half life.
363 Thus, anyone of these mechanisms could have prevented the decreased eNOS
364 expression during HS diet.

365 In addition, Atorvastatin reversed the increase in TGFβ-1 expression induced by HS
366 intake. This effect could be related to Atorvastatin's ability to inhibit p38MAPK, JNK,
367 and ROCK (40).

368 In conclusion, long term high salt intake induces injurious effects on the kidney as
369 evidenced by the increase protein excretion rate, glomerular hypertrophy, interstitial cell
370 infiltration and endothelium dysfunction. These inflammatory and hemodynamic
371 changes were not related to hypertension. Atorvastatin corrected all of the changes,
372 suggesting that NO-oxidative stress balance plays a significant role in the early stages of
373 salt induced kidney damage.

374

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565

Figure and Table Legends

Figure 1. Effect of atorvastatin on urinary protein excretion during high sodium diet. NS = normal sodium diet, NS+Ator = normal sodium diet plus atorvastatin, HS = high sodium diet, HS+Ator = high sodium diet plus atorvastatin. * = $p < 0.05$ vs HS+Ator.

Figure 2. Effect of atorvastatin on the acetylcholine induced vasodilation during a high sodium diet. NS = normal sodium diet, NS+Ator = normal sodium diet plus atorvastatin, HS = high sodium diet, HS+Ator = high sodium diet plus atorvastatin. * = $p < 0.05$ vs other groups.

Figure 3. eNOS expression in renal cortex. A. Bars graph representing cortical eNOS expression, AU arbitrary units (* $p < 0.05$ vs HS+Ator and vs NS). B. Representative Western blot analysis.

Figure 4. Effect of atorvastatin on Glomerular eNOS expression during high sodium diet. A. Bars graph representing glomerular eNOS expression, AU arbitrary units (* p < 0.05 vs HS+Ator and vs NS). B. Representative Western blot analysis.

Figure 5: Representatives images of glomerular volume (40x). NS = normal sodium diet, NS+Ator = normal sodium diet plus atorvastatin, HS = high sodium diet, HS+Ator = high sodium diet plus atorvastatin.

Figure 6: A) Normal glomeruli and tubules from a rat on NS diet. The juxtaglomerular apparatus, the urinary space and the capillary lumen are preserved. B) Two glomeruli from a rat on HS intake showing mesangial matrix expansion with mild increase in cellularity. The capillary lumens are obturated. C) Outer region of renal medulla in a rat on a NS diet. D) Outer region of renal medulla in a rat on a HS diet. An enlarged interstitium surrounds the tubuli with areas of mononuclear cell infiltration. (H&E 400X).

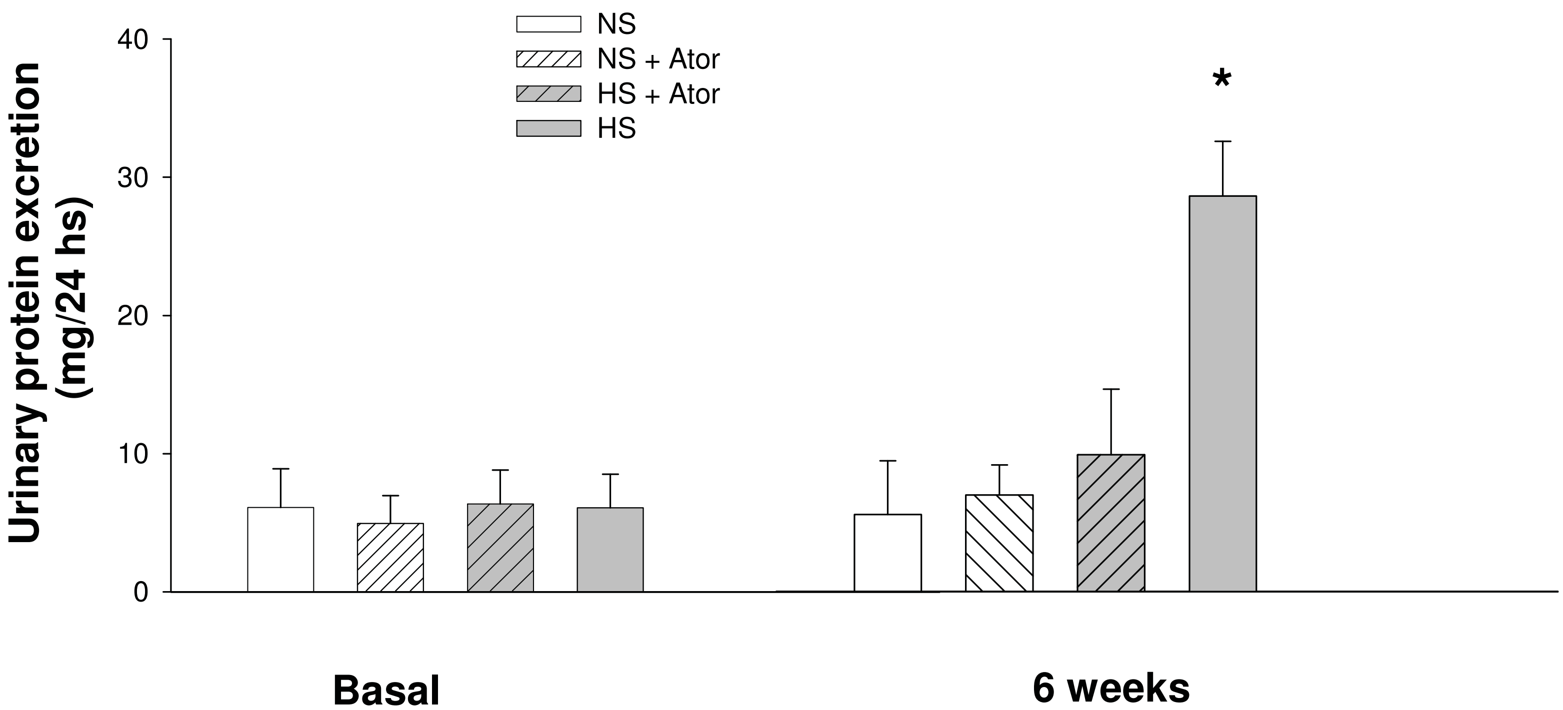
Figure 7. Effect of atorvastatin on renal cortex TGF-β1. NS = normal sodium diet, NS+Ator = normal sodium diet plus atorvastatin, HS = high sodium diet, HS+Ator = high sodium diet plus atorvastatin (* = $p < 0.0038$ vs HS+Ator).

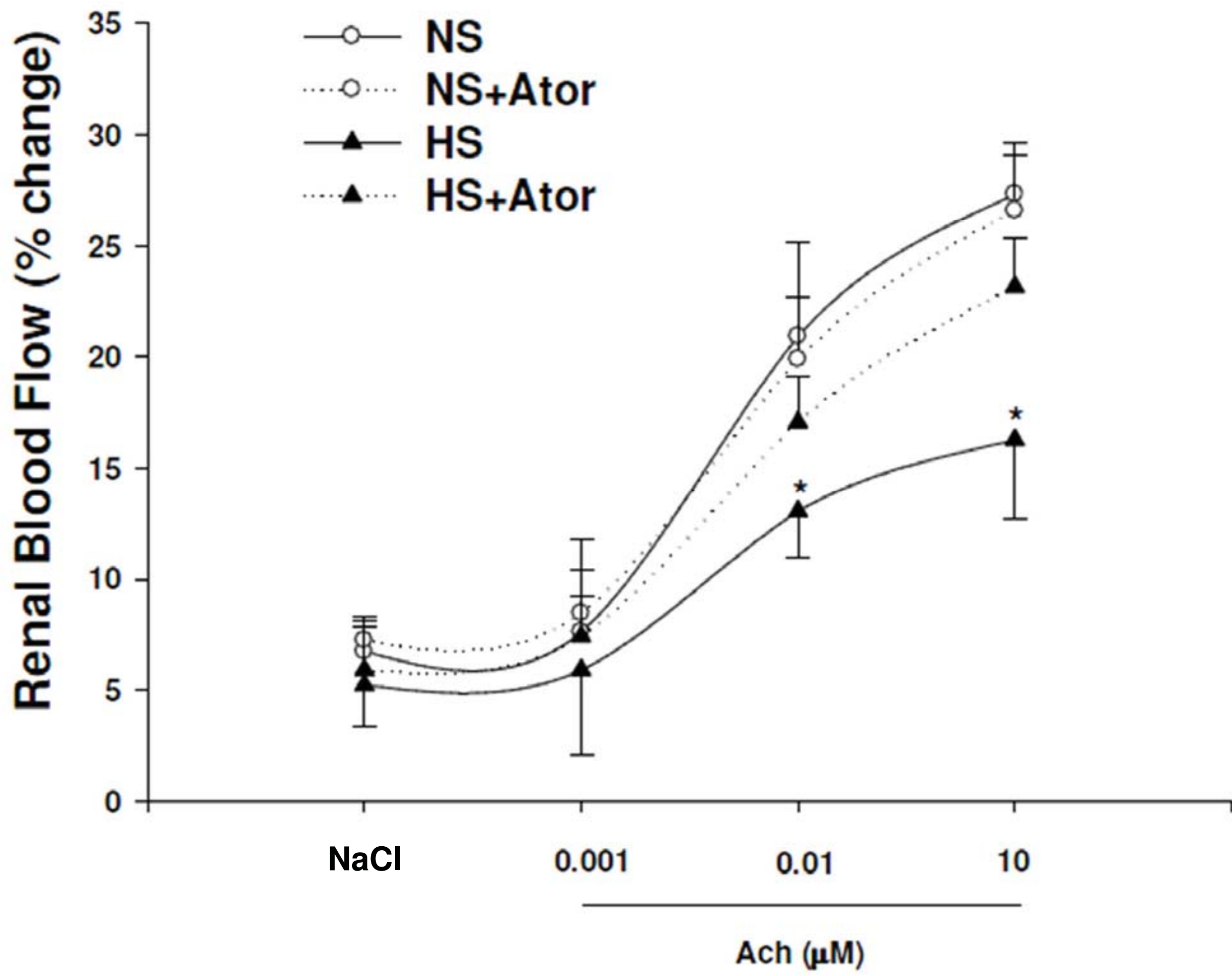
602 Table 1. Physiological parameters during high salt intake.

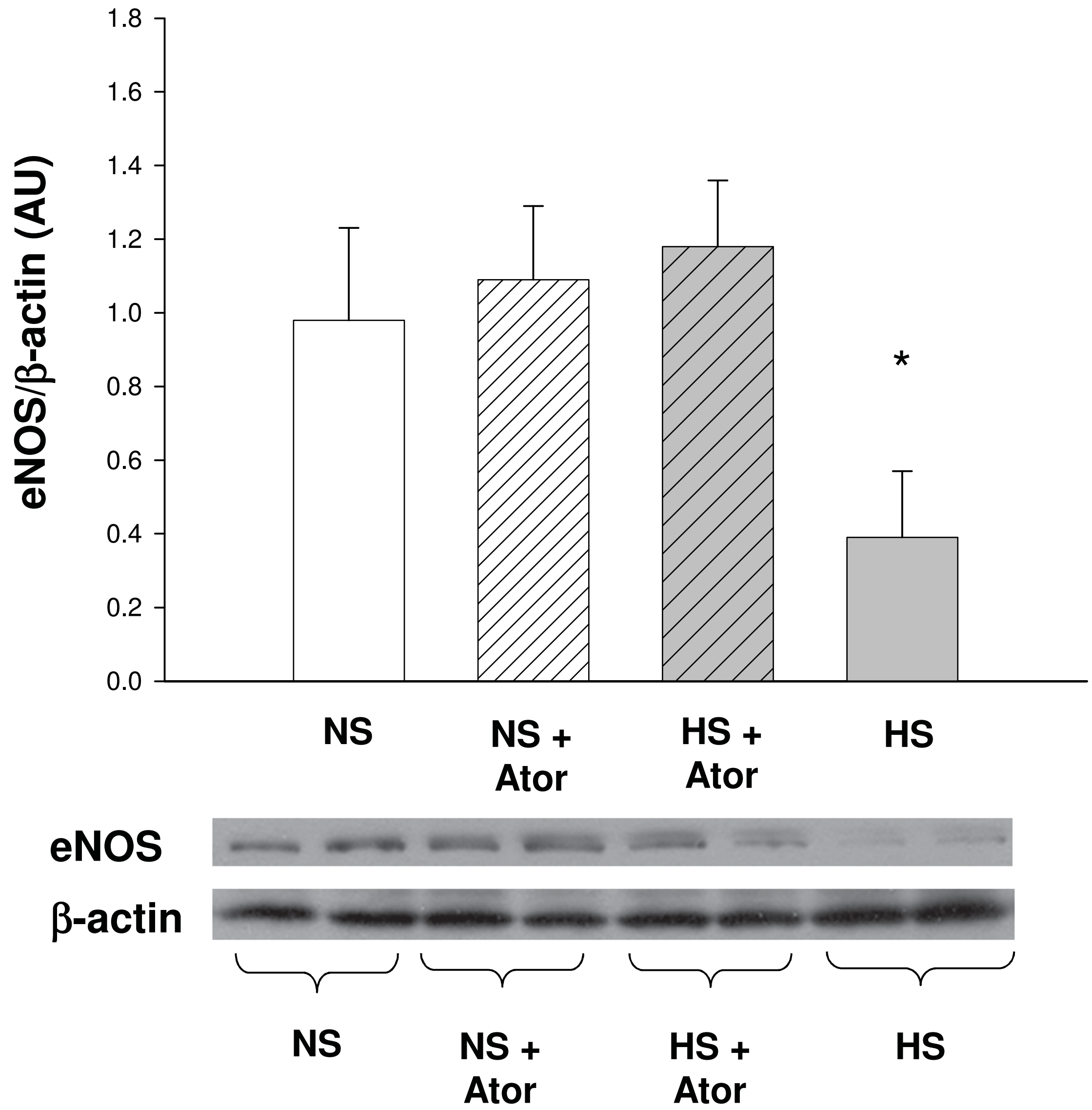
603

604 Table 2. Glomerular volume and renal histology

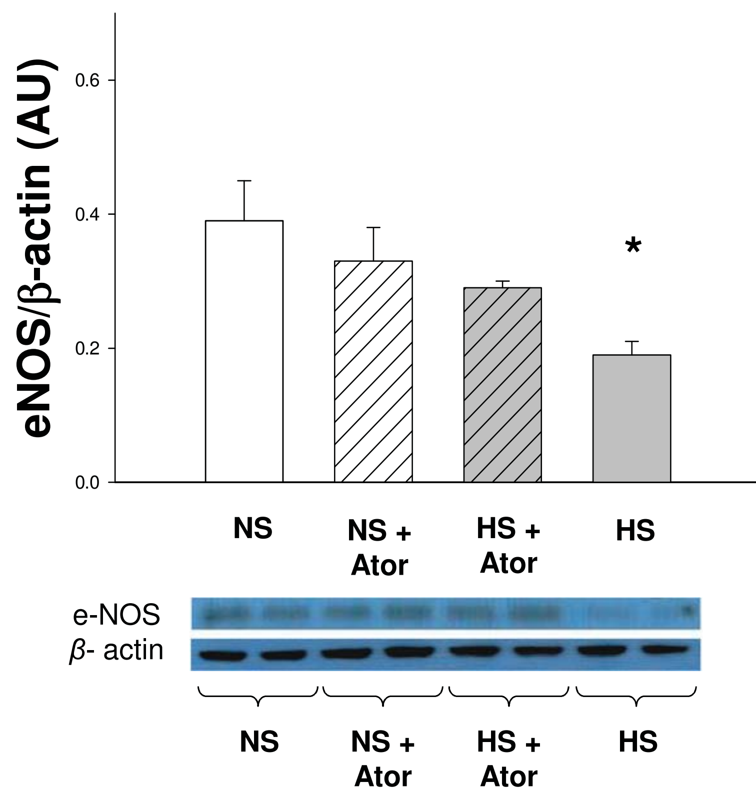
605


Urinary protein excretion (mg/24 hs)
40
30
20
10
0
NS
NS + Ator
HS + Ator
HS
*
Basal
6 weeks

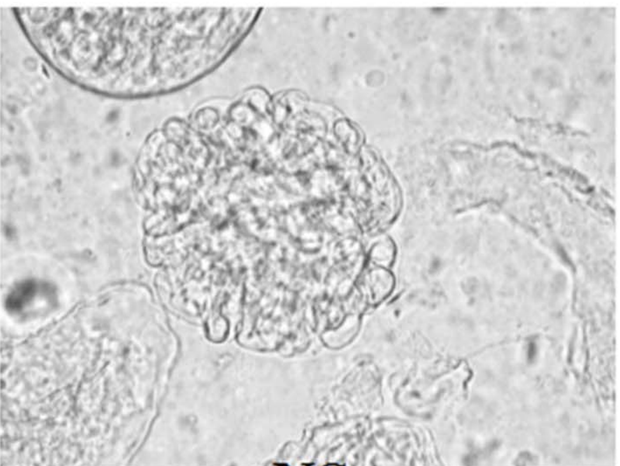

NS
NS+Ator
HS
HS+Ator
Renal Blood Flow (% change)
35
30
25
20
15
10
5
0
NaCl
0.001
0.01
10
Ach (µM)
*
*


eNOS/β-actin (AU)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
NS
NS + Ator
HS + Ator
HS
eNOS
β-actin
NS
NS + Ator
HS + Ator
HS

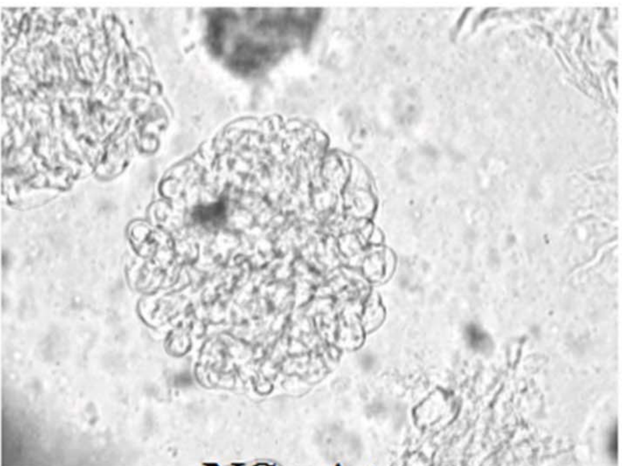
A



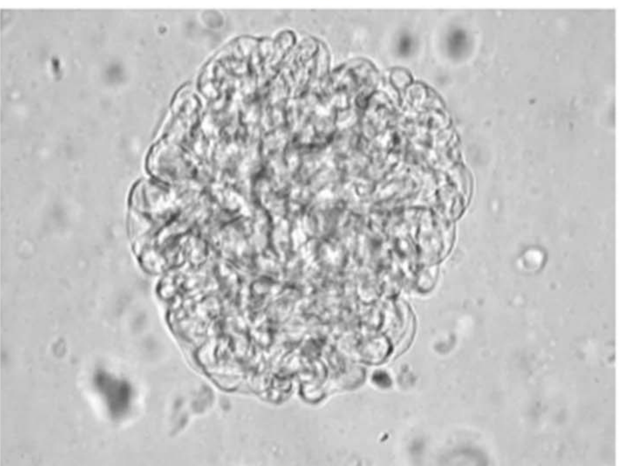
B



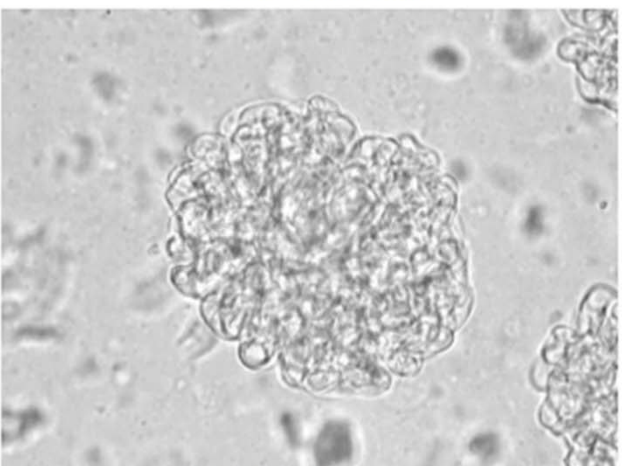
NS



NS+Ator

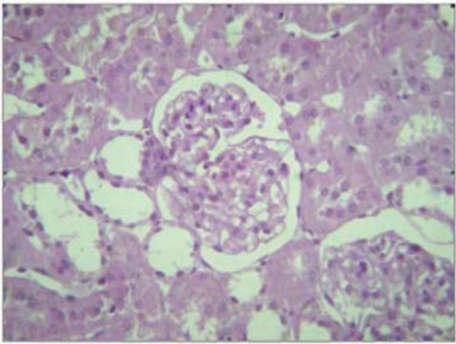


HS



HS+Ator

A



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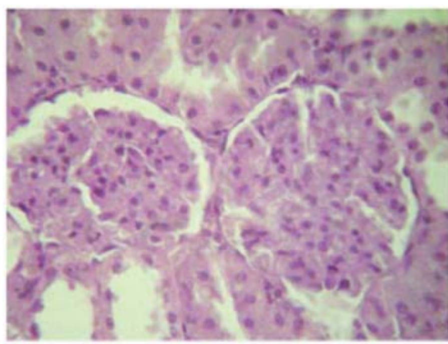

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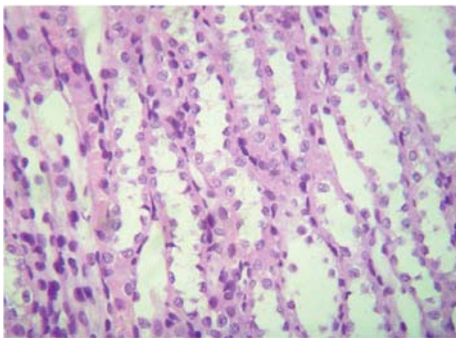
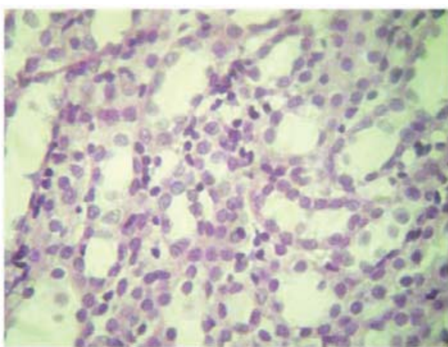
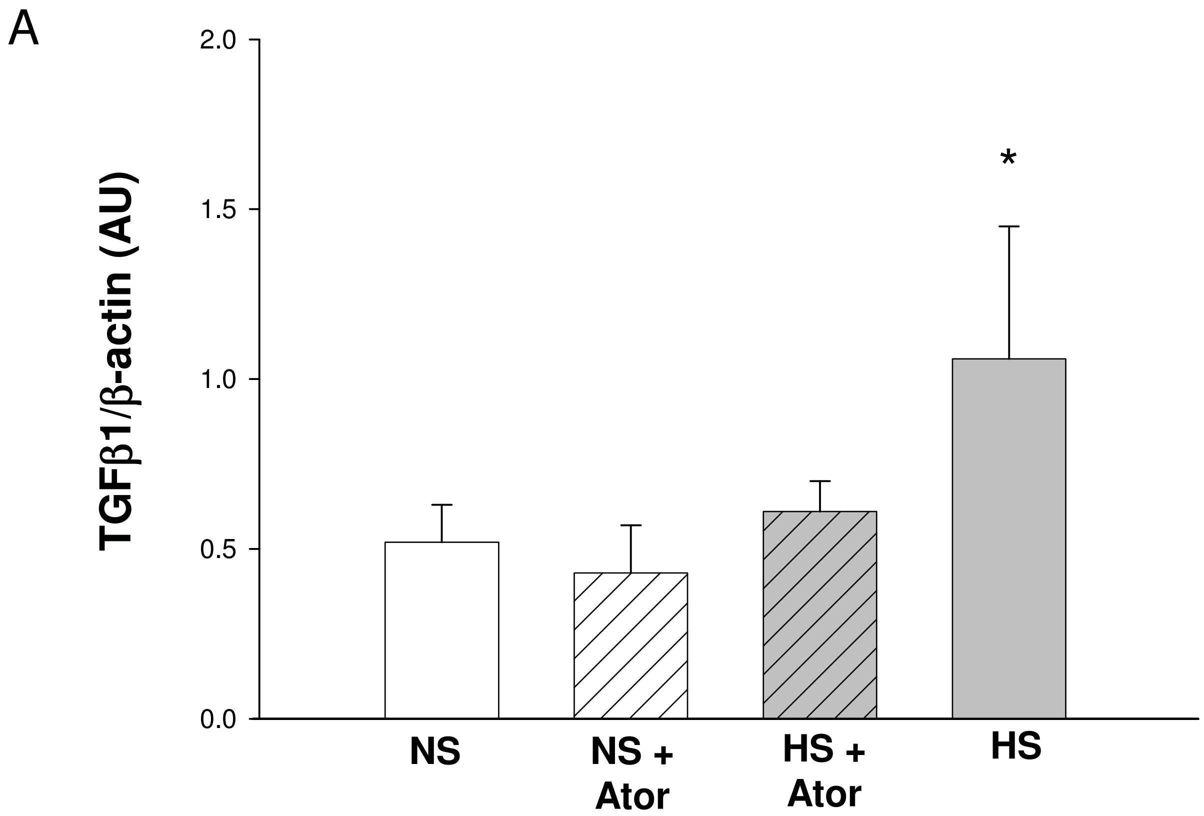


D



A



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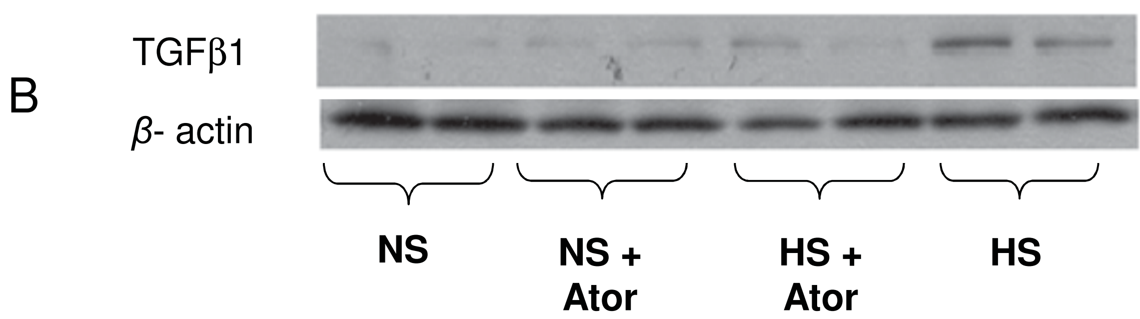

Table 1. Physiological parameters during high salt intake.

Groups	Body Weight (g)		SBP (mmHg)		$U_{Na}V$ (mEq/24 hs)	
	Basal	6 w	Basal	6 w	Basal	6 w
NS (n=10)	187±23	294±26	113±6	116±5	2.2±0.4	2.1±0.7
NS+Ator (n=10)	190±31	293±30	111±4	110±1	1.6±0.5	2.0±0.9
HS (n=9)	190±34	282±22	106±4	116±3	2.7±0.9	11±4.3*
HS+Ator (n=13)	191±27	282±34	110±3	108±3	1.9±0.7	10±4.0*

* $p < 0.05$ vs. Control 6 weeks, # $p < 0.001$ vs. Control Basal

SBP= Systolic blood pressure, $U_{Na}V$, urinary sodium excretion rate

Table 2. Glomerular volume and renal histology.

Groups	Normal Sodium Diet		High Sodium Diet	
	Control	Atorvastatin	Control	Atorvastatin
Glomerular Volume (μ^3)	$3.30 \times 10^6 \pm 0.1$	$3.32 \times 10^6 \pm 0.2$	$4.40 \times 10^6 \pm 0.2\phi$	$3.60 \times 10^6 \pm 0.2\#$
Renal Interstitial infiltration (0 a 4+)	0	0	1.4±0.06*	0
Glomerular sclerosis (0 a 4+)	0	0	1.25±0.5*	0

ϕ p=0.0012, * $p < 0.05$ and # ns vs. Control (ns: no significant).