BASIC RESEARCH

PERSISTENT HYPERTENSION AND PROGRESSIVE RENAL INJURY INDUCED BY SALT OVERLOAD AFTER SHORT TERM NITRIC OXIDE INHIBITION

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Mattar AL, Machado FG, Fujihara CK, Malheiros DMAC, Zatz R. Persistent hypertension and progressive renal injury induced by salt overload after short term nitric oxide inhibition. Clinics.2007;62(6):749-56.

INTRODUCTION: Administration of the NO inhibitor $N^{w\hat{0}}$-nitro-L-arginine methyl ester (NAME) and a high-salt diet (HS) promotes severe albuminuria and renal injury, which regresses upon discontinuation of treatments.
OBJECTIVE: We investigated whether these changes reappear after reinstitution of HS, and whether they are prevented by treatment with the antilymphocyte agent mycophenolate mofetil (MMF) or the AT-1 receptor blocker losartan (L). Adult male Munich-Wistar rats received NAME and HS. A control Group (**C**) received only HS. After 20 days, rats receiving HS and NAME exhibited severe hypertension and albuminuria. After a 30-day recovery period, hypertension was attenuated and albuminuria had virtually disappeared.
MATERIAL AND METHODS: Rats were then distributed among the following groups: HS, receiving HS; NS, receiving a normal salt (NS) diet; HS-MMF, receiving HS and MMF; HS-LOS, receiving HS and L; HS-HDZ, receiving HS and hydralazine (HDZ). Sixty days later, NS rats showed only slight albuminuria and renal damage or inflammation. In contrast, HS rats developed severe hypertension, marked glomerulosclerosis with interstitial expansion and renal infiltration by macrophages and angiotensin II-positive cells. The group treated with losartan had lowered blood pressure and a lack of albuminuria or renal injury. MMF provided similar protection without altering blood pressure, suggesting a nonhemodynamic effect, a hypothesis reinforced by the finding that HDZ lowered blood pressure without preventing renal injury.
RESULTS: These results indicate that treatment with HS and NAME predisposes to the development of hypertension and renal injury upon salt overload, characterizing a new model of chronic nephropathy. **CONCLUSION:** The response to MMF or L, but not HDZ, suggests a key role for inflammatory rather than hemodynamic factors.

KEYWORDS: Chronic kidney disease. Mycophenolate mofetil. Angiotensin II. AT-1 receptor blocker. Salt overload

INTRODUCTION

Chronic nitric oxide inhibition has been amply utilized as a model of hypertension and chronic renal injury.[1-3] Previous observations[4,5,6] suggested that these abnormalities were reversible upon cessation of treatments. However, recent observations indicate that this recovery is incomplete, and that the persistence of several inflammatory elements heralds the development of chronic kidney disease and progressive renal insufficiency in these animals.[5]

Dietary salt overload is known to exacerbate renal injury initiated by a host of other mechanisms.[4,7] The mechanisms responsible for this effect are unclear. Although systemic hypertension is an obvious possibility, increased salt intake may dose-dependently promote renal injury even without a corresponding blood pressure variation,[8] suggesting that nonhemodynamic factors, perhaps directly related to extracellular volume expansion, intervene in this process. Inflammatory mechanisms, such as activation of the NF-k-B system,[9,10] lymphocyte and macrophage infiltration[11] exacerbated production of

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TGFβ[12] and anomalous production of angiotensin II (Ang II)[10] in the renal parenchyma are possible mediators of this effect of salt excess.

Rechallenging animals with a salt-rich diet after termination of NO blockade has been shown to promote the reappearance of hypertension and a worsening of renal injury.[6] Treatment with mycophenolate mofetil (MMF), an antilymphocyte agent, during the early NO inhibition phase largely prevents the development of hypertension and renal injury by salt overload after NO inhibition is removed,[6] suggesting that inflammatory mechanisms play a role in this process. However, the effects of late treatment with MMF or suppressors of the renin-angiotensin system have not been evaluated.

In the present study, we examined the behavior of rats subjected to short-term treatment with NAME and salt overload after only the latter was reintroduced a few weeks later. To assess the role of hypertension and inflammatory phenomena in the development of hypertension and renal injury induced by reinstitution of sodium overload, we treated these animals with MMF, suppressors of the RAS system or hydralazine. This experimental design allowed us to characterize a new model of chronic nephropathy which, unlike the chronic NO inhibition model from which it derives, is characterized by low mortality and nonprogressive hypertension, yet courses with severe albuminuria, culminating with the establishment of well defined glomerulosclerosis and renal interstitial injury.

METHODS

Fifty-six adult male Münich-Wistar rats (initially weighing 200 to 260 g) obtained from a local facility were used in this study. The animals were maintained at 23±1°C on a 12/12 hours light/dark cycle, and were given free access to tap water and either a normal-salt diet (NS (0.5% Na)) or high-salt diet (HS (3.2% Na)). Both diets contained 22% protein and were purchased from Harlan Teklad (Troy, IL). All experimental procedures were approved by the local Research Ethics Committee and conformed strictly to our institutional guidelines and to international standards for the manipulation and care of laboratory animals.

Experimental groups and experimental protocol

The experimental protocol is described in schematic form in Table 1. During the initial part of the protocol (Phase 1), rats received HS chow and the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (NAME) in their drinking water at a concentration of 100 mg/ L, corresponding to a daily intake of 25 mg/kg. At the end of this 20-day period, all treatments ended and rats received NS for the next 30 days (recovery period). Rats were then distributed among five experimental groups according to the treatment received from this time until the end of the study, 60 days later (Phase 2), as is schematically described in Table 1. Group **HS** (n=12) received HS only. Group **NS** (n=10) received NS only. Group **HS-MMF** (n= 8) received HS and the antilymphocyte agent, mycophenolate mofetil (MMF) at 10 mg/kg by gavage once daily. Group **HS-LOS** (n=6) was treated as the preceding group, but received the AT-1 receptor blocker losartan potassium, 50 mg/kg /day in the drinking water, instead of MMF. Group **HS-HDZ** (n=8) was treated as the two preceding groups, but received hydralazine, 6 mg/kg in the drinking water, instead of losartan or MMF. This group served as a blood pressure control for the **HS-LOS** group, in which blood pressure was brought to near normal levels. A control Group (**C**) of 12 rats received HS and no NAME for the first 20 days (Phase 1), and was then switched to NS for the following 30 days, as for all other groups. The control animals were then given HS until the end of the study, 60 days later (Phase 2). This group served as a time control.

Table 1 - Schematic description of experimental groups

GROUP	N	TREATMENT Day 0-20	Day 21-50	Day 51-110
C	12	HS	NS	HS
HS	10	HS+NAME	NS	HS
NS	12	HS+NAME	NS	NS
HS-MMF	8	HS+NAME	NS	HS+MMF
HS-LOS	6	HS+NAME	NS	HS+LOS
HS-HDZ	8	HS+NAME	NS	HS+HDZ

Abbreviations: C, Control; HS, High-salt; NS, normal salt, NAME, N^{ω}-nitro-L-arginine methyl ester; MMF, Mycophenolate mofetil; LOS, Losartan; HDZ, Hydralazine.

Tail-cuff pressure (TCP) and urinary albumin excretion rate ($U_{alb}V$) were measured immediately before treatments with HS and NAME or HS alone were initiated (Day 0), at the end of Phase 1 (Day 20), 30 days after these treatments had been terminated (end of the recovery period) and 60 days later (end of Phase 2).

Measurement of tail-cuff pressure (TCP) and albuminuria ($U_{alb}V$)

TCP was estimated by a tail-cuff method using a blood pressure analysis system (Visitech Systems, USA). Animals were conditioned 2 or 3 times before measurements were taken. Twenty-four-hour urine was collected in individual metabolic cages for assessment of albumin concentration by radial immunodiffusion.[13]

Morphological analysis

At the end of the study, kidneys were perfusion-fixed at the measured arterial pressure with Duboscq-Brasil solution after a brief washout with saline. After fixation, the renal tissue was weighed and 2 midcoronal sections were postfixed in buffered 4% formaldehyde solution. The material was then embedded in paraffin for assessment of glomerular and renal cortical interstitial injury and for immunohistochemical identification of macrophages and of cells staining positively for angiotensin II.

Histomorphometry

2–3 mm thick sections were stained with periodic acid-Schiff or Masson trichrome. All morphometric evaluations were performed in a blinded manner by a single observer. The frequency of glomeruli exhibiting sclerotic lesions (GS) was estimated by consecutive examination of at least 300 glomeruli at 200´. The fraction of the renal cortex occupied by interstitial tissue was quantified in Masson-stained sections by a point counting technique [14] in 25 consecutive microscopic fields, at a final magnification of 100´ under a 176-point grid.

Immunohistochemistry

Immunohistochemical procedures were described in detail previously [15]. Briefly, macrophages and cells positive for AngII were detected by an indirect streptavidin-biotin alkaline phosphatase technique. For macrophage detection, a monoclonal mouse anti-rat ED-1 antibody (Serotec, Oxford, United Kingdom) was used. AngII-positive cells were detected by a monoclonal rabbit anti-human AngII antibody (Peninsula Laboratories, San Carlos, CA). The density of renal interstitial infiltration by macrophages (Mf) and AngII-positive cells was evaluated in a blinded manner at 250x magnification and expressed as cells/mm^2.

Statistics

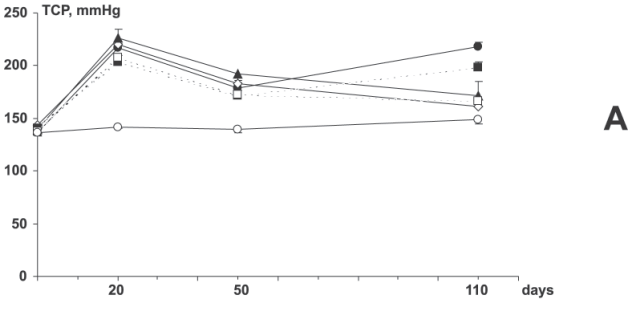
One-way analysis of variance with pairwise comparisons according to the Newman-Keuls method was used in this study to assess the statistical significance of differences between groups. Since the distribution of $U_{alb}V$ was strongly non-Gaussian, these data underwent log transformation prior to statistical analysis. p values of 0.05 or less were considered significant.

RESULTS

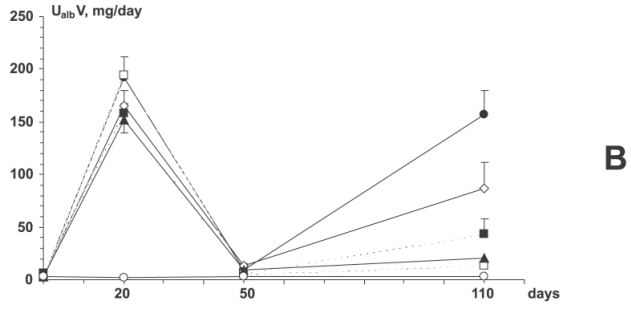
Body growth was similar among groups, although body weight at the end of the study was slightly higher in Group HS compared to the remaining groups (data not shown). As described previously,[4] TCP was markedly elevated after 20 days in all groups receiving HS and NAME treatments (Fig. 1A). This increase was accompanied by deterioration of glomerular barrier function, as indicated by massive albuminuria (Fig. 1B), which reached values 100-fold higher than baseline. No changes in TCP or $U_{alb}V$ were noted in control rats (Group C).

Thirty days after the combined HS and NAME treatment ended (Group NS), TCP decreased to a similar extent in all groups, although the values for each group remained significantly higher than for Group C (Fig. 1A). Albuminuria was drastically reduced in all treated groups after HS and NAME were withdrawn (Fig.1B), bringing the $U_{alb}V$ levels in each group to insignificant values.

By the end of Phase 2 (i.e., sixty days after HS treatment was reinstituted), Group HS again exhibited very se-



	C	NS	HS	HS-MMF	HS-LOS	HS-HDZ
C		p > 0.05	p < 0.001	p < 0.001	p > 0.05	p > 0.05
NS			p < 0.001	p < 0.001	p > 0.05	p > 0.05
HS				p > 0.05	p < 0.001	p < 0.001
HS-MMF					p < 0.05	p < 0.001
HS-LOS						p > 0.05
HS-HDZ						



	C	NS	HS	HS-MMF	HS-LOS	HS-HDZ
C		p > 0.05	p < 0.001	p < 0.001	p < 0.01	p < 0.001
NS			p < 0.001	p < 0.05	p > 0.05	p < 0.001
HS				p < 0.01	p < 0.001	p > 0.05
HS-MMF					p > 0.05	p > 0.05
HS-LOS						p < 0.05
HS-HDZ						

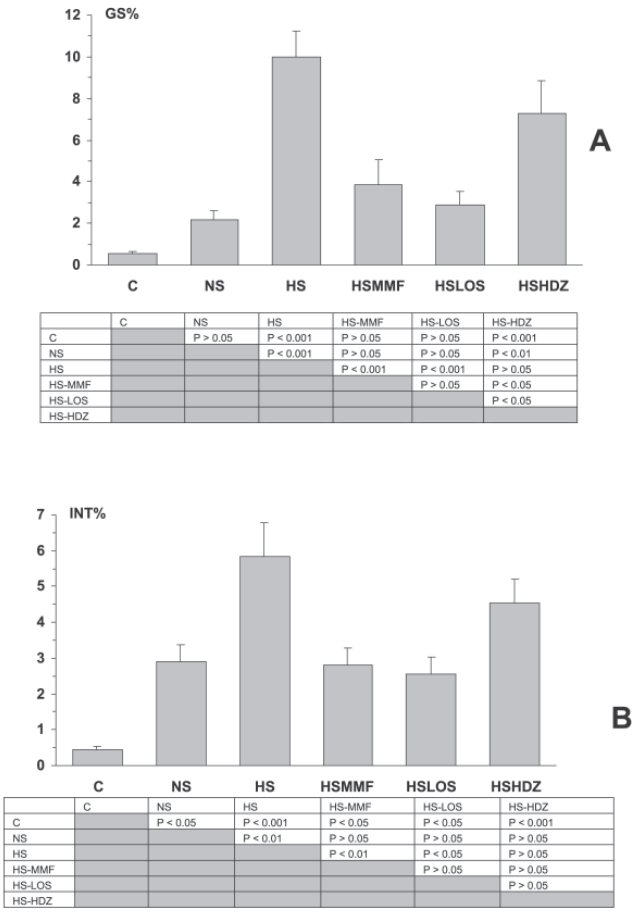
Figure 1 - Time course of tail-cuff pressure, TCP (***A***), and urine albumin excretion rate, $U_{alb}V$ (***B***), in Group C (open circles), NS (open squares), HS (closed circles), HS-MMF (closed squares) , HS-LOS (closed triangles) and HS-HDZ (open diamonds). Results of statistical analyses of differences among groups at the end of the study (Day 110) are shown below the respective panels.

vere hypertension, reaching TCP levels comparable to those observed on Day 20 of Phase 1 (Fig. 1A). Likewise, albuminuria (Fig. 1B) rose to levels similar to the peak values observed during Phase 1, indicating the development of substantial glomerular injury in these rats. In contrast, blood pressure had decreased slightly in Group NS at the end of the study. Accordingly, no elevation in $U_{alb}V$ was observed in this group at the end of phase 2 (Fig. 1B).

Treatment with MMF attenuated hypertension in Group HS-MMF, although TCP increased from the values observed at the beginning of MMF treatment, reaching levels significantly higher than baseline (Fig. 1A). In contrast, both losartan (Group HS-LOS) and hydralazine (Group HS-LOS) treatments of rats receiving HS brought TCP to levels indistinguishable from those observed in Group C. However, the effects of the treatments on $U_{alb}V$ were clearly different from the effects on TCP, as the rise in albuminuria was completely prevented by losartan, strongly attenuated by MMF and only partially prevented by hydralazine (Fig. 1B).

Morphological analysis performed at the end of the study showed that rats rechallenged with HS treatment developed severe renal injury, with 10% of glomeruli examined showing sclerotic lesions (Fig. 2A), while the fraction of the renal cortical area occupied by interstitial tissue increased to nearly 6%, as opposed to 0.6% in group C (Fig. 2B). In rats receiving NS during Phase 2 (Group NS), renal injury was not different from baseline. Treatment with either MMF or losartan prevented the development of glomerular and interstitial injury, both of which remained comparable to baseline (Figs 2A and 2B). Despite its effect on blood pressure, hydralazine treatment during Phase 2 only attenuated the frequency of sclerotic glomeruli and the percent interstitial area, which remained elevated compared to baseline.

Immunohistochemical analysis of the renal tissue at the end of the study showed intense renal macrophage infiltration, especially in interstitial areas (Fig. 3A), in rats preconditioned with HS and NAME and treated with late HS (Group HS). In contrast, renal macrophages were rare and sparse in rats treated with NS during Phase 2 (Group NS) . Renal macrophage infiltration was equally prevented at this phase by treatment with either MMF or losartan, but not by hydralazine. A similar pattern was observed for AngII, which was densely expressed at the interstitial area in Group HS, but not in Group NS (Fig. 3B). Once again, treatment with either MMF or losartan, but not with hydralazine, limited the renal interstitial expression of AngII in rats receiving HS during Phase 2.



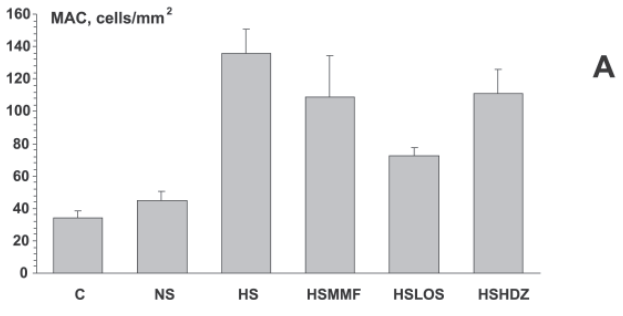
	C	NS	HS	HS-MMF	HS-LOS	HS-HDZ
C		P > 0.05	P < 0.001	P > 0.05	P > 0.05	P < 0.001
NS			P < 0.001	P > 0.05	P > 0.05	P < 0.01
HS				P < 0.001	P < 0.001	P > 0.05
HS-MMF					P > 0.05	P < 0.05
HS-LOS						P < 0.05
HS-HDZ						

	C	NS	HS	HS-MMF	HS-LOS	HS-HDZ
C		P < 0.05	P < 0.001	P < 0.05	P < 0.05	P < 0.001
NS			P < 0.01	P > 0.05	P > 0.05	P > 0.05
HS				P < 0.01	P < 0.05	P > 0.05
HS-MMF					P > 0.05	P > 0.05
HS-LOS						P > 0.05
HS-HDZ						

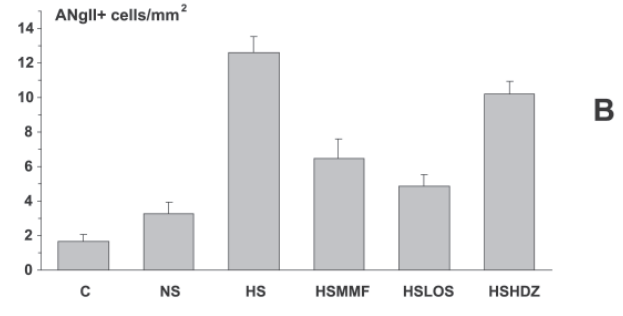
Figure 2 - Frequency of glomeruli with segmental sclerotic lesions (GS, panel A) and percent of cortical area occupied by interstitial tissue (%INT, panel B) at the end of the study (Day 110). Results of statistical analyses are shown below the respective panels.

DISCUSSION

As shown previously,[4,5] short-term chronic NO inhibition, with simultaneous sodium overload, promoted severe hypertension and massive albuminuria. There was a quick but incomplete regression of hypertension upon withdrawal of the treatments, as TCP remained above baseline. The regression of albuminuria was more pronounced, with $U_{alb}V$ falling to levels indistinguishable from baseline. This suggests that the kidney is able to recover from the initial insult, as occurs in most cases of postinfectious glomerulonephritis, for example. However, the regression of renal injury in this model is incomplete. Previous observations of this model [5] showed that the density of cells staining positively for AngII never returned to baseline and that these rats exhibited progressive albuminuria and hypertension, as well as severe glomerular and interstitial injury, when followed for an additional 6 months. These observations suggest that even a short-term treatment with an NO inhibiter in association with salt overload can lead to subtle changes that culminate in the insidious development of chronic kidney disease. The mechanisms underlying why low-grade



	C	NS	HS	HS-MMF	HS-LOS	HS-HDZ
C		P > 0.05	P < 0.001	P < 0.01	P > 0.05	P < 0.01
NS			P < 0.001	P < 0.01	P > 0.05	P < 0.05
HS				P > 0.05	P < 0.05	P > 0.05
HS-MMF					P > 0.05	P > 0.05
HS-LOS						P > 0.05
HS-HDZ						



	C	NS	HS	HS-MMF	HS-LOS	HS-HDZ
C		P > 0.05	P < 0.001	P < 0.001	P < 0.05	P < 0.001
NS			P < 0.001	P < 0.05	P > 0.05	P < 0.001
HS				P < 0.001	P < 0.001	P > 0.05
HS-MMF					P > 0.05	P < 0.01
HS-LOS						P < 0.01
HS-HDZ						

Figure 3 - A – Density (in cells/mm^2) of renal interstitial infiltration by macrophages (MAC, panel A) and cells staining positively for AngII (AngII+, panel B) at the end of the study (Day 110). Results of statistical analyses are shown below the respective panels.

inflammation and unchecked production of AngII persist and progress toward serious damage to the renal parenchyma long after NO inhibition is ended are unclear. One attractive possibility is that these processes are triggered and/or maintained by residual damage to the preglomerular microvessels, which undergo severe constriction and wall thickening during NO inhibition,[1,2] although solid evidence in favor of this possibility is lacking.

Reinstitution of HS, but not NAME, treatment one month after both treatments had been interrupted led to a reappearance of hypertension that eventually reached levels similar to the peak values observed during initial HS/NAME treatment. Likewise, albuminuria, which had decreased to levels not far from baseline one month after HS and NAME treatment was stopped, rose to levels similar to those attained by the end of the initial HS/NAME treatment, while only a slight increase in albuminuria and blood pressure was seen in rats receiving a regular salt diet. These observations indicate that previous treatment with HS and NAME not only leads to insidious progressive chronic disease, but also predisposes to the rapid development of hypertension and renal injury upon salt overload.

Although the administration of a salt-rich diet to otherwise normal animals is not clearly associated with overt hypertension or nephropathy, salt overload is known to exacerbate renal injury initiated by other mechanisms, such as diabetes,[12] 5/6 renal ablation[16] and chronic NO inhibition.[4] The intimate mechanisms responsible for this effect are unclear. Salt overload may cause renal damage as a result of direct hemodynamic changes, such as hypertension or extracellular volume expansion. However, observations made in rats with genetic hypertension indicated that the adverse effects of excess sodium in these rats can occur with minimal blood pressure elevation.[8] Accordingly, acute intravenous sodium overload was shown to promote renal activation of the NF-k-B system, as well as increased production of TGF-β and RANTES, without changes in MAP or GFR.[10] These observations suggest that inflammatory mechanisms may mediate salt-induced renal injury in addition to, or in lieu of, hemodynamic stress.

Angiotensin II is likely to mediate at least part of the deleterious effects of excess sodium.[5,6,10,17] This appears to be a paradox since salt overload would be expected to suppress circulating renin and angiotensin II. However, local production of angiotensin II also occurs at the diseased renal tissue, as shown in several models of chronic kidney disease, even when salt intake is increased.[6,15,17] In rats receiving HS/NAME treatment, AngII was shown to abound in areas of renal interstitial inflammation.[6,17] Accordingly, AngII appeared in inflamed renal interstitial areas in Group HS at the end of the present study, after these rats had received HS for several weeks. Collectively, these findings are consistent with the view that locally produced AngII is independent of the usual physiologic control, as determined by the needs of sodium processing by the organism. Rather, local AngII appears to correlate with the presence of macrophages, and may participate in the inflammatory process associated with chronic kidney disease. Accordingly, abundant evidence now indicates that AngII can influence several events known to be involved in chronic inflammation, such as lymphocyte activation,[18] expression of adhesion molecules[19] and inflammatory mediators,[20] proliferation of a host of resident and inflammatory cells and production of stimulators of fibrogenesis.[21]

Suppression of the RAS with chronic losartan during Phase 2 effectively prevented both hypertension and renal injury, as shown previously in multiple studies of clinical and experimental CKD. The beneficial effect of losartan could be due solely to its hemodynamic action, since renoprotection was associated with near normalization of

blood pressure. However, the favorable effect of losartan was not duplicated by hydralazine, which promoted only partial renoprotection, although blood pressure was maintained at baseline levels with either treatment, suggesting that systemic hypertension was not a decisive factor in the pathogenesis of renal injury. Since glomerular pressure was not measured in this study, the possibility that glomerular hypertension persisted in hydralazine-treated rats despite a normalization of blood pressure cannot be excluded. However, treatment with MMF, an antilymphocyte drug possessing several anti-inflammatory effects, prevented renal injury as effectively as losartan therapy, even though blood pressure remained elevated in MMF-treated rats. These observations are consistent with previous findings that MMF limits renal inflammation and chronic injury in models such as 5/6 renal ablation,[22,23] streptozotocin-induced diabetic nephropathy[24] and chronic NO inhibition.[25] Taken together, these data indicate that both blockade of the AT-1 receptor and inhibition of inflammatory events constitute effective strategies to prevent the progression of CKD in rats rechallenged with HS after short-term HS and NAME treatment. Whether the association of losartan and MMF would afford more complete renoprotection was not addressed in the present study. However, it should be noted that the association of MMF with an AT-1 receptor blocker or an ACE inhibitor prevented CKD much more effectively than either monotherapy in the renal ablation model.[26-28]

In summary, the present observations corroborate previous findings that short-term treatment with HS and NAME leads to reversible hypertension and albuminuria. In addition, we show that reintroduction of HS after a recovery period causes progressive renal injury and inflammation, which can be prevented by treatment with losartan or MMF. Late HS administration following short-term treatment with HS and NAME constitutes a suitable new model of CKD that is considerably less aggressive than 5/6 renal ablation or sustained inhibition of NO synthesis, yet possesses a progressive nature that allows for detailed examination of pathogenic factors, as well as evaluation of the renoprotective effects of pharmacological strategies.

RESUMO

Mattar AL, Machado FG, Fujihara CK, Malheiros DMAC, Zatz R. Hipertensão persistente e lesão renal progressiva induzidas por sobrecarga de sal após inibição temporária do óxido nítrico. Clinics.2007;62(6):749-56.

INTRODUÇÃO: A administração de N^{ω}-nitro-L-arginina metiléster (NAME), um inibidor da produção de NO, com dieta rica em sal (HS) promove albuminúria e dano renal graves, reversíveis ao interromperem-se os tratamentos.
OBJETIVO: Investigamos se tais alterações recrudescem ao reinstituir-se a HS e se são prevenidas pelo micofenolato mofetil (MMF), um agente antilinfócito, ou losartan, um bloqueador do receptor AT-1.
MATERIAL E MÉTODOS: Ratos Münich-Wistar machos adultos receberam NAME e HS. Um grupo controle (**C**) recebeu apenas HS. Após 20 dias, os ratos que receberam HS e NAME exibiam hipertensão e albuminúria graves. Após recuperação de 30 dias, a hipertensão atenuou-se e a albuminúria praticamente desapareceu. Formaram-se então os grupos: HS, recebendo HS; NS, recebendo dieta

normal em sal (NS); HS-MMF, recebendo HS e MMF; HS-LOS, recebendo HS e losartan; HS-HDZ, recebendo HS e hidralazina. Após sessenta dias os ratos NS tinham albuminúria e dano/inflamação renal apenas discretos. Já os ratos HS desenvolveram hipertensão e glomerulosclerose acentuadas, expansão intersticial e infiltração renal por macrófagos e células positivas para angiotensina II. Losartan baixou a pressão arterial e preveniu albuminúria e lesão renal. MMF proporcionou proteção semelhante sem alteração pressórica, sugerindo a ação de mecanismos não hemodinâmicos, hipótese reforçada pelo achado de que a HDZ baixou a pressão arterial sem prevenir a nefropatia.
RESULTADOS: Esses resultados indicam que o tratamento com HS e NAME predispõe ao desenvolvimento de hipertensão e lesão renal induzidos por excesso de sal, caracterizando um novo modelo de nefropatia crônica.
CONCLUSÃO: A resposta ao MMF ou losartan, mas não à hidralazina, sugere o predomínio de fatores inflamatórios.

UNTERMOS: Doença crônica do rim. Micofenolato mofetil. Angiotensina. AT-1 receptor bloqueador. Excesso de sal.

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