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Antiinflammatory effects of AT_1 receptor blockade provide end-organ protection in stroke-prone rats independently from blood pressure fall

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List of non standard abbreviations: α_1-antitrypsin (α_1AT), α_1-major acute phase protein or thiostatin (α_1MAP), kallikrein-binding protein (KBP), serine protease inhibitor-3 (SPI3), major urinary protein (MUP), albumin (SA), transferrin (Tf), magnetic resonance imaging (MRI)

Abstract

Stroke-prone rats (SHRSP) develop hypertension and systemic inflammation, with subsequent brain and renal disorders and early death. We tested the hypothesis that valsartan, an angiotensin II type1 (AT1) receptor antagonist, exerts protective effects in SHRSP through its anti-inflammatory properties, even in the absence of a blood pressure-lowering effect. SHRSP fed a high salt-diet were treated with vehicle or valsartan (1-10 mg/kg/day). The vehicle-treated rats developed hypertension, proteinuria, progressive kidney disease, and, 40±5 days from the beginning of the treatment, brain damage as visualized by magnetic resonance imaging. Rats treated with 1 mg/kg/day-valsartan developed brain damage after 61±3 days ($p<0.01$ *vs* vehicle-treated rats). 80% of the rats treated with 10 mg/kg/day showed no damage after 100 days. Valsartan treatment preserved renal structure, by preventing the infiltration of inflammatory cells, and lowered renal expression of monocyte chemoattractant protein-1, transforming growth factor-beta1 and interleukin-1 beta, as compared with vehicle-treated SHRSP. Urinary excretion of acute-phase proteins increased in the latter but remained negligible in the drug-treated animals. Furthermore, valsartan exerted protective effects also when given after established proteinuria. In SHRSP blockade of AT1 receptor with valsartan prevents the development of proteinuria, delays the appearance of brain damage, preserves renal structure, and increases survival under stressful conditions. Valsartan exerts its beneficial effects independently of any blood pressure fall and by means of broad anti-inflammatory actions both at local and at systemic levels. These observations indicate that the administration of AT1 receptor antagonists may be useful in pathological situations in which an anti-inflammatory effect is required.

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The renin–angiotensin system (RAS) plays a pivotal role not only in the regulation of blood pressure but also in the pathophysiology of a number of vascular and renal diseases (Luft, 2002; Brasier et al., 2002). Activation of the RAS has been recorded in animal models of kidney disorders whether hypertension is present or not (Egido, 1996). Angiotensin II (AngII), the main hormone of the RAS, has significant pro-inflammatory actions on the vascular wall, inducing the production of reactive oxygen species, inflammatory cytokines and adhesion molecules (Brasier et al., 2002). Experimental evidence obtained in recent years indicate that AngII is not only a vasoactive hormone but acts directly as a cytokine and a growth factor (Ruiz-Ortega et al., 2002; Sadoshima, 2000). Infusion of AngII into rats causes the infiltration of inflammatory cells into the glomeruli and the interstitium (Ruiz-Ortega et al., 2001). AngII recruits inflammatory cells into the tissue by directly activating mononuclear cells and regulating the synthesis of adhesion molecules and chemokines, including in particular monocyte chemoattractant protein-1 (MCP-1) (Tummala et al., 1999). The recruited inflammatory cells can in turn activate the RAS and increase the generation of AngII locally, thus generating a cycle of tissue injury (Ruiz-Ortega et al., 2001). AngII exerts these inflammatory effects primarily through the Angiotensin II type 1 (AT1) receptor (Nataraj et al., 1999). *In vivo* studies with animal models of malignant hypertension indicate that AngII type 1 receptor blockers (ARBs) prevent nephrosclerosis independently of blood pressure effects and reduce angiotensin-induced inflammation in the kidney (Hilgers et al., 2001). The spontaneously hypertensive stroke-prone rat (SHR-SP) is an animal model of hypertension with very high plasma levels of AngII; the animals spontaneously develop renal damage and cerebral

abnormalities (Gahnem et al., 1994;). We have recently shown that the appearance of cerebral abnormalities, detected by MRI, is preceded by a systemic inflammation characterized by the accumulation, in serum and urine, of acute-phase proteins, particularly thiostatin, the most usual marker of an inflammatory response in the rat (Sironi et al., 2001; Guerrini et al., 2002). Inflammatory processes play a pivotal role in the pathological events occurring in salt-loaded SHRSP (Sironi et al., 2001; Sironi et al., 2003; Marks et al., 2001). This model may therefore be a useful experimental tool to explore the anti-inflammatory properties of drugs, especially those acting on the RAS. The present report focuses on the effects of valsartan, an AT1-subtype angiotensin receptor antagonist, on the pathological phenomena occurring in SHRSP, with particular attention to the modulation of inflammatory events.

Methods

Animals and treatments

Male SHRSP, aged 4-5 weeks, were obtained from the Charles River Co., Italy (Calco, Lecco). Procedures involving animals and their care were conducted at the Department of Pharmacological Sciences, University of Milan, in conformity with the Institution's guidelines, which comply with national and international rules and policies. Baseline measurements were made in all rats at 6 weeks of age and all animals were then switched to a specific permissive diet (day 0), low in potassium and protein and high in sodium (Japanese permissive diet, JPD; Laboratorio Dr. Piccioni, Gessate, Italy: 18.7% protein, 0.63% potassium, 0.37% sodium) and received 1% NaCl in drinking water. Rats were randomized into one of three groups: group 1 (n=20), the controls, received vehicle; group 2 (n=20) and group 3 (n=20) were given 1 or 10 mg/kg/day, respectively,

of valsartan, dissolved in drinking water. In a fourth group (4), to observe the reversal of the lesions, valsartan (10 mg/kg/day, n=10) or vehicle (n=10) was added to the drinking solution when proteinuria exceeded the value of 40 mg/day. Valsartan, kindly provided by Novartis Pharma (Basel, Switzerland), was dissolved in distilled water at 10 mg/mL according to the manufacturer's instructions and then diluted daily at the selected doses with a small amount (5-10 mL) of 1% NaCl-drinking water. After consumption of this small amount, the animals were given free access to 1%NaCl drinking water. In order to allow the amount of drug administered to be adjusted to changes in body weight, drug concentration in the drinking solution was recalculated weekly. Once a week, all the rats were weighed and their arterial blood pressure was measured; they were then housed individually in metabolic cages for 24 h in order to measure their food and liquid intake, and to collect urine. Systolic arterial blood pressure was measured in conscious rats by means of tail-cuff plethysmography (PB Recorder 8006, Ugo Basile). During each recording session, the blood pressure was measured in each animal 3 times by at least two different operators, expert in these procedures and completely blinded with respect to the experimental group to which each animal did belong. 24-h urine protein was measured according to Bradford, with bovine albumin as a standard. Proteinuria (protein levels $\geq$40 mg/day) predicts the appearance of brain abnormalities in SHRSP (Blezer et al., 1998; Guerrini et al., 2002), and was used to schedule the frequency of MRI investigations. The SHRSP underwent MRI every week until 24-h proteinuria exceeded 40 mg/day, then every other day until brain damage was observed and every day, for 3 days afterwards. Animal treatment lasted up to 100 days.

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Proteomic studies

Urine proteins were concentrated by trichloroacetic acid–acetone precipitation. One-dimensional electrophoresis was run on urine proteins in the presence of sodium dodecyl sulfate (SDS), without sample reduction, in a discontinuous buffer system (Laemmli 680-685) on polyacrylamide gradients 4% to 20% T. The sample load was 3.75 μg per lane. Two-dimensional electrophoresis was according to the manufacturer's protocol (Protean IEF cell, BIORAD). IPG ready strips, 11 cm, pH 3–10 nonlinear gradient (Biorad), were actively rehydrated at 50 V for 24 h. 100 μg of proteins, dissolved in a buffer containing 8 M urea, 2M thiourea, 4% CHAPS, 1% DTT, 20 mM Tris and 2% carrier ampholytes, was loaded on the cathode using the cup loading tray for Protean IEF cell (Biorad) and focused for a total of 20 KVh. After focusing, the strips were first equilibrated for 15 min with a solution containing 50 mM Tris-Hcl, 6 M urea, 30% v/v glycerol, 2% SDS and 2% DTT, and then with the same buffer containing 4.5% iodoacetamide instead of DTT. The focused proteins were then fractionated according to size by SDS-PAGE on 7%–17% polyacrylamide gradients and stained by a silver staining method. The protein patterns were digitalized with a scanner and compared with previously reported maps.

MRI evaluation of brain damage

The rats were anesthetized with 2% isofluorane in 70% N_2–30% O_2, and placed inside a Bruker AMX3 with a micro-imaging accessory. After a sagittal scout image, sixteen contiguous 1-mm thick slices were analyzed caudally to the olfactory bulb using a field of view (FOV) of 4 x 4 cm^2. A turbo spin echo sequence was used with 16 echoes per excitation, 10 ms inter-echo time, and 85 ms equivalent echo time, and 4 s repetition time. The images were 128 x 128 points (zero filled to 256x256); eight images were

averaged in 8'30". The occurrence of lesions was identified as the presence of areas of high signal intensity on T2-weighted MRI.

Histopathology

For the histological examination of brain and kidney, rats (n=6) from each experimental group were sacrificed 3 and 6 weeks after starting the treatment and when in the control group brain abnormalities were first detected. To monitor the initial situation, tissues were prepared from a group of five rats aged 6 weeks. The removed kidneys and brains were fixed in Carnoy reagent (Merck, Darmstadt, D) and embedded in Paraplast (Sigma, St Louis, CO), then 5 µm coronal sections were stained with hematoxylin/eosin and examined by light microscopy. Vascular changes in the kidney slices were evaluated assigning a score as follows:0, absent; 1, initial deposition of hyaline material; 2, mild to moderate thickening of the vascular wall; 3, severe lesion with occlusion of vascular lumens. Tubular cast and tubular atrophy were assigned a score separately and the score values were combined (cast 0: absent, 1: isolated tubular cast in the cortex <5%, 2: large cast in less than 50% of the fields, 3: prominent cast formation; athophy 0: absent, 1: mild, 2: moderate, 3: severe). The evaluations were performed on at least 5 sections of kidney from each animal by a pathologist who was unaware of the nature of the experimental groups.

Immunohistochemistry of kidney

For immunohistochemical studies, paraffin-embedded slides from kidney were dewaxed in xylene and dehydrated. Endogenous peroxidase was blocked by adding 1% H_2O_2 in 50% methanol. Nonspecific binding sites were saturated with goat serum. The sections were incubated overnight at 4°C with the primary antibody *anti*-ED1 (1:100; Serotec, Oxford, UK), then with biotinylated secondary antibodies and streptavidin peroxidase

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(LSAB2 kit, DAKO, Glostrup, DK). Horseradish peroxidase was detected with H_2O_2 and diaminobenzidine (Sigma, St Louis, CO). Intraglomerular ED1-positive cells were counted in all glomeruli of a given kidney section (100 to 300 glomeruli, no selection) and were expressed as cells per glomerular section. Interstitial ED1-positive cells were assessed by a semiquantitative evaluation assigning a score as follows: 0: no staining, 1: few isolated positive cells, 2: moderate staining, 3: strong staining.

Analysis of mRNA for MCP-1, TGF-β1 and IL-1β

Total RNA was prepared by guanidium thiocyanate denaturation from frozen kidney collected from vehicle- and valsartan (10 mg/kg/day)-treated rats sacrificed after 6 weeks of dietary treatment. The expression of monocyte chemoattractant protein-1 (MCP-1), transforming growth factor-β1 (TGF-β1) and interleukin-1β (IL-1β was measured by semi-quantitative RT-PCR (Balduini et al., 2003). GAPDH was amplified as a standard. The RT-PCR products were separated on 1.5% agarose gel and visualized by means of ethidium bromide. The intensity of each band was quantified using the NIH Image software and expressed in arbitrary units. The densities of the MCP-1, TGF-β1 and IL-1β bands were normalized using the corresponding GAPDH signal.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Data were analyzed using a non-parametric Kruskal-Wallis test. Differences between groups, concerning physiological parameters, were computed by ANOVA for repeated measurements, followed by Bonferroni's post-hoc test. $p<0.05$ was taken as statistically significant.

Results

Effects of valsartan on physiological variables of SHRSP

Growth was similar in all groups. In the control rats, however, body mass and weight decreased immediately before the appearance of brain abnormalities ($p<0.05$ valsartan 10 mg/kg/day *vs* vehicle treated-rats after 7 and 8 weeks of treatment), while in all rats treated with valsartan body weight increased up to the end of the experiment (Fig. 1A). SHRSP exposed to salt loading developed a severe hypertension not significantly affected by the treatment with either tested dose of valsartan (1 or 10 mg/kg/day; Fig. 1B). Interestingly, blood pressure remained unaffected also when control animals received valsartan starting on week 6 (Figure 6).

Valsartan delayed the appearance of brain abnormalities

The SHRSP in the salt-loaded control group developed cerebral lesions, as visualized by MRI, 40 ± 5 days from the beginning of the treatment (Figure 2B). Treatment with valsartan (1 or 10 mg/kg/day) significantly delayed the appearance of brain damage (Figure 2A) to 61 ± 3 days ($p<0.01$ *vs* vehicle-treated rats) and 70 ($p<0.01$ *vs* group 1) days. 80% of the animals on the higher dose showed no brain damage during the 100 days test period until they were sacrificed. Affected rats died 7–10 days after brain abnormalities appeared, whatever the treatment. In animals sacrificed at first MRI-detection of brain abnormalities, no difference was found between vehicle- and drug-treated rats in the magnitude of the brain damage revealed by MRI or of the tissue lesions detected by histology (Figure 2B).

Effects of valsartan on protein excretion

The animals given vehicle progressively increased their daily protein loss. After 5-6 weeks of salt loading, 24-h proteinuria increased rapidly and linearly to average 282 ± 22 mg/day. This is the time when brain abnormalities also appeared (Figure 2B). In rats treated with valsartan at 1 mg/kg/day, the proteinuria remained lower than in control

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rats but again quickly increased just before the appearance of brain abnormalities (Figure 1C: $p<0.01$ vehicle treated-rats *vs* valsartan 10 mg/kg/day at week 7 and 8; $p<0.01$ valsartan 1 mg/kg/day *vs* valsartan 10 mg/kg/day treated-rats at week 10 and 11). Development of proteinuria was completely prevented by valsartan at 10 mg/kg/day and protein excretion averaged 79 ± 16 mg/day after 12 weeks of this treatment (Figure1C). In the urine of salt-loaded SHRSP that received vehicle only, the qualitative protein composition also changed over time: there was an accumulation of acute-phase proteins, in particular thiostatin (or α_1-major acute-phase protein; α_1-MAP), as assessed by two-dimensional electrophoresis (2-DE). Figure 3, panel A, shows a representative 2-DE of urine from a control rat after 6 weeks of dietary treatment, compared with that from a rat treated with valsartan at 10 mg/kg/day. High concentrations specifically of thiostatin were observed in the urine of untreated rats whereas it was hardly detectable in the urine of drug-treated SHRSP. The mixture of proteins excreted by untreated SHRSP and by those given valsartan was compared on one-dimensional gels (Fig. 3, panel B). Treatment with either dose of valsartan delayed the appearance of high molecular weight proteins, mainly albumin (SA) and transferrin (Tf), at the expense of MUP (major urinary protein) (Fig. 3).

Effects of valsartan on the progression of renal disorder

Kidneys from control animals sacrificed at different times during salt loading exhibited progressive fibrocellular proliferative lesions, particularly in the glomeruli and arteries. After six weeks of salt loading, the immunohistochemical analysis showed a massive inflammatory cell infiltration around the arteries and the renal tubules. Most of these infiltrating inflammatory cells were positive for ED-1, a marker of macrophages derived from circulating monocytes. Valsartan treatment strongly reduced (lower dose) or fully

prevented (higher dose) the renal lesion and the infiltration of inflammatory cells (Fig. 4). Table 1 reports the morphologic evaluations and a quantification of ED-1 accumulation in the kidney of rats treated for six weeks with vehicle or valsartan.

Expression of MCP-1, TGF-β1 and IL-1β mRNA in the kidneys

Figure 5 shows the expression of the genes of chemokines in kidneys of rats treated with vehicle or 10 mg/kg/day valsartan, and sacrificed after 6 weeks of dietary treatment. Valsartan treatment dramatically prevented mRNA accumulation for the three genes investigated. Drug treatment reduced, IL-1β , MCP-1 and TGF-β1 mRNA to 71% ($p<0.001$, n=5), 64% ($p<0.001$, n=5) and 51% ($p<0.05$, n=5) of untreated SHRSP, respectively (Figure 5).

Effects of valsartan treatment at proteinuria >40 mg/day

When proteinuria reached 40 mg/day in 20 rats on the salt-loading diet, half the animals were given valsartan (10 mg/kg/day) while the remainder received vehicle. During drug treatment body weight progressively increased, whereas the control rats lost weight upon approaching death. Blood pressure increased in the two groups to the same extent. Valsartan treatment stabilized proteinuria for at least three weeks; after this period, proteinuria increased between week 10-13 but remained lower than in controls on week 7. All the rats treated with valsartan after the rise in proteinuria survived 100 days on the salt-loading diet (Fig. 6).

Discussion

We show here that blockade of Ang II with valsartan, a selective AT1 receptor antagonist, prevents the development of proteinuria in spontaneously hypertensive stroke-prone rats, delays the appearance of brain damage, preserves the renal structure and increases the survival under stressful circumstances. Ang II appears therefore a key factor in inflammatory processes. These beneficial effects occur without a significant fall in blood pressure, which was considered necessary for renal protection by other drugs in the same animal model (Abrahamsen et al., 2002; Barone et al., 1996). Similarly, clinical studies indicated that AngII type 1 receptor antagonists (ARBs) improve vascular function independently of the blood pressure-lowering effect (Klingbeil et al., 2002; Viberti and Wheeldon, 2002; Lewis, 2002). Recently, it was reported that diabetes-associated atherosclerosis was improved by AT1 receptor blockade but not by calcium channel antagonism, despite a similar blood pressure reduction (Candido et al., 2004). These experimental and clinical results suggest that ARBs may beneficially affect vascular function beyond controlling blood pressure. In recent years many experimental data have suggest that the RAS participates in immune and inflammatory responses (Tummala et al., 1999; Dzau, 2001). In particular, angiotensin II, acting mainly through the AngII type1 receptor (AT1), regulates several NF-kB-related genes, including growth factors, cytokines and adhesion molecules, which are involved in the pathogenesis of inflammatory lesions, vascular and kidney damage, and hypertension (Ruiz-Ortega et al., 1998; Muller et al., 2000; Dandona et al., 2003). AngII also participates in the recruitment of inflammatory cells into the site of injury, via direct activation of the inflammatory cells or via regulation of adhesion molecules, cytokines, and chemokines such as monocyte chemoattractant protein-1

(MCP-1) (Tummala et al., 1999). Increased expression of TGF-β accompanies the progression of renal disease, and the inhibition of its activity by a specific neutralizing antibody has been reported to prevent nephropathy (Sharma et al., 1996). In the present investigation, valsartan suppresses the up-regulation of MCP-1 and of TGF-β and reduceș macrophage infiltration and the progression of renal disease, independently of any effect on hypertension. Our data indicate that valsartan treatment inhibits the expression of IL-1β mRNA in the kidney. IL-1β is a immunoregulatory and pro-inflammatory cytokine released by various cells, including macrophages and activated mesangial cells (Dinarello, 1996). IL-1β influences by different mechanisms various cellular functions including cell proliferation, growth factors and prostaglandins release and extracellular matrix proteins production. IL-1β clearly plays an important role in the process of glomerular injury (Yu et al., 1999). Our results suggest that the beneficial effects of AT1 blockade in renal disease are due, at least in part, to a local reduction of the activation of pro-inflammatory and pro-fibrotic gene transcription. SHRSP, beside exhibiting severe hypertension, renal and brain damage, develop a systemic inflammation and widespread alteration of vascular permeability characterized by the accumulation, first in serum and then in urine, of several proteins synthesized by the liver such as albumin, transferrin, transthyretin, serine protease inhibitor-3 and thiostatin, the most typical marker of an inflammatory response in the rat (Sironi et al., 2001; Guerrini et al., 2002). Systemic inflammation is the body's overall response to a local inflammation or infection. Epidemiological and experimental studies indicate that the synthesis of a number of acute-phase proteins is rapidly upregulated in hepatocytes under the control of inflammatory mediators, in particular cytokines, originating at the site of persistent inflammation (Pepys and Hirschfield, 2003; Glurich et al., 2002). In

our animal model the accumulation of acute-phase proteins, particularly thiostatin, represents a response to an inflammatory condition which valsartan dramatically delays. Although no unequivocal evidence has been collected on the tissues or organ where inflammation first develops, our results, together with earlier reported data, show that in SHRSP kidney abnormalities, including proliferative and necrotic lesions in arterioles and glomeruli, are detectable shortly after the start of salt loading and may represent the initial insult triggering synthesis of APP (Blezer et al., 1999; Rocha et al., 1999). In patients with end-stage renal disease it has been suggested that there is a complex interaction between the acute-phase inflammatory process and uremia, heart failure, malnutrition, and anemia (Bergstrom et al., 2000). Another recent report associates C-reactive protein (CRP) with renal function abnormalities in a non-diabetic population (Stuveling et al., 2003). CRP, a highly sensitive marker of systemic inflammation, is the major acute-phase protein in humans, as thiostatin is in rats. When tissue is injured or traumatized, CRP is quickly upregulated, mainly in response to circulating mediators, in particular cytokines. Valsartan treatment has been shown to reduce CRP concentration in human plasma whereas ACE inhibitors fail to modulate this inflammatory marker (de Maat et al., 2003; Dandona et al., 2003). In conclusion, our data indicate that in SHRSP the administration of non-hypotensive doses of valsartan retards renal damage and systemic inflammation. Furthermore, the beneficial effects obtained in SHRSP by the *delayed* administration of valsartan (see Figure 6) indicate that this drug is able to reverse the ongoing pathological events in salt-loaded SHRSP. Valsartan exerts its beneficial effects by means of a broad anti-inflammatory action both at local level, reducing macrophage infiltration and expression of MCP-1, TGFβ1 and IL-1β mRNA in the kidney, and at systemic level as indicated by the reduced excretion of acute-phase

proteins in the urine of salt-loaded animals. These observations indicate that the administration of an angiotensin II type 1 receptor antagonist may be useful in pathological situations in which an anti-inflammatory effect is required.

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Legends for figures

Figure 1. Effects of valsartan on physiological variables of SHRSP

Body weight (A), blood pressure (B) and daily proteinuria (C), as a function of the duration of dietary treatment (Japanese Permissive Diet, JPD), for SHRSP receiving vehicle (●), treated with 1 mg/kg/day valsartan (□), or treated with 10 mg/kg/day valsartan ▲ (*$p<0.05$, **$p<0.01$ *vs* 10 mg/kg/day treated rats).

Figure 2. Effects of valsartan on the appearance of brain damage, as evaluated by MRI

Panel A. Event-free survival, as a function of the duration of salt loading, for SHRSP receiving vehicle (●) or valsartan at 1 (□) or 10 mg/kg/day (▲). Panel B. a) Two contiguous coronal brain sections of a vehicle-treated rat sampled at the first detection (after 36 days of dietary treatment) of brain abnormalities (arrows), and 48 hours later. b) Two contiguous coronal brain sections of a valsartan-treated rat sampled after 60 days of dietary-treatment. Histology of the indicated areas show that MRI-hyperintensity corresponds to damaged tissue.

Figure 3. Effects of valsartan on SHRSP urinary proteins

Panel A: representative two-dimensional electrophoresis of urine collected after 6 weeks of salt loading from a vehicle-treated SHRSP (left) and a SHRSP treated with valsartan at 10 mg/kg/day (right). Panel B; representative one-dimensional electrophoresis of urinary proteins, collected weekly, from an SHRSP receiving vehicle (left) or 10 mg/kg/day valsartan (right). Samples of 3.75 µg from a 24-h collection in metabolic cages were loaded per lane. Gels shown are representative of results obtained in four rats.

Figure 4. Effects of valsartan on renal damages progression

Examples of kidney sections stained with hematoxylin and eosin from a SHRSP treated with vehicle (A, magnification 20x in A; B 40x in B) and with valsartan at 10 mg/kg/day (20x in D; 40x in E) after 6 weeks of salt loading. The insert of A shows a fibrocellular proliferative lesion in the renal artery (a so-called onion-skin lesion) and the arrow points to an artery with marked hyperplasia/proliferation. In B the asterisks indicate a collapsed glomerulus. C and F show immunostaining for the macrophage/monocyte marker ED-1 (magnification 40x) of kidney sections from control and valsartan-treated SHRSP after 6 weeks of salt loading. In C, macrophage/monocyte infiltration (ED-1 staining, brown) surrounds the lesions. Each panel is representative of results from at least five rats.

Figure 5. Effect of valsartan on the renal expression of MCP-1, TGF-β, and IL-1β mRNA

RT-PCR analysis for expression of MCP-1, TGF-β and IL-1β mRNA in kidney from rats treated with vehicle or valsartan (10 mg/kg/day) after 6 weeks of salt loading. These gels are representative of five separate experiments. The bar graph reports the densitometric analysis of PCR-bands, normalized to the corresponding GAPDH signals. *$p<0.05$; ** $p<0.01$ valsartan (white column) *versus* vehicle (black column) treated rats.

Figure 6. Effects of valsartan, administered after increase of proteinuria, on physiological variables

Body weight, blood pressure and proteinuria in SHRSP in which vehicle (●) or valsartan (10 mg/kg/day; ▲) treatment was initiated (arrow) when proteinuria reached the value of 40 mg/day (* $p< 0.05$; ** $p< 0.001$).

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Table 1. Effects of 6-weeks treatment with valsartan (10 mg/kg/d) on vascular and tubular changes and ED-1 cells accumulation. The scoring system is detailed in Methods (*Histophatology* section). *p<0.05; **p<0.01 *vs* vehicle

Groups	**Vascular changes** (score)	**Tubular changes** (score)	**ED1 cells**	
			glomerulus (cells/glom)	interstitium (score)
Vehicle	2.8 ± 0.4	2.6 ± 0.4	1.1 ± 0.5	2.6 ± 0.5
Valsartan	1.2 ± 0.4*	1.2 ± 0.4 *	0.6 ± 0.3*	0.8 ± 0.8**

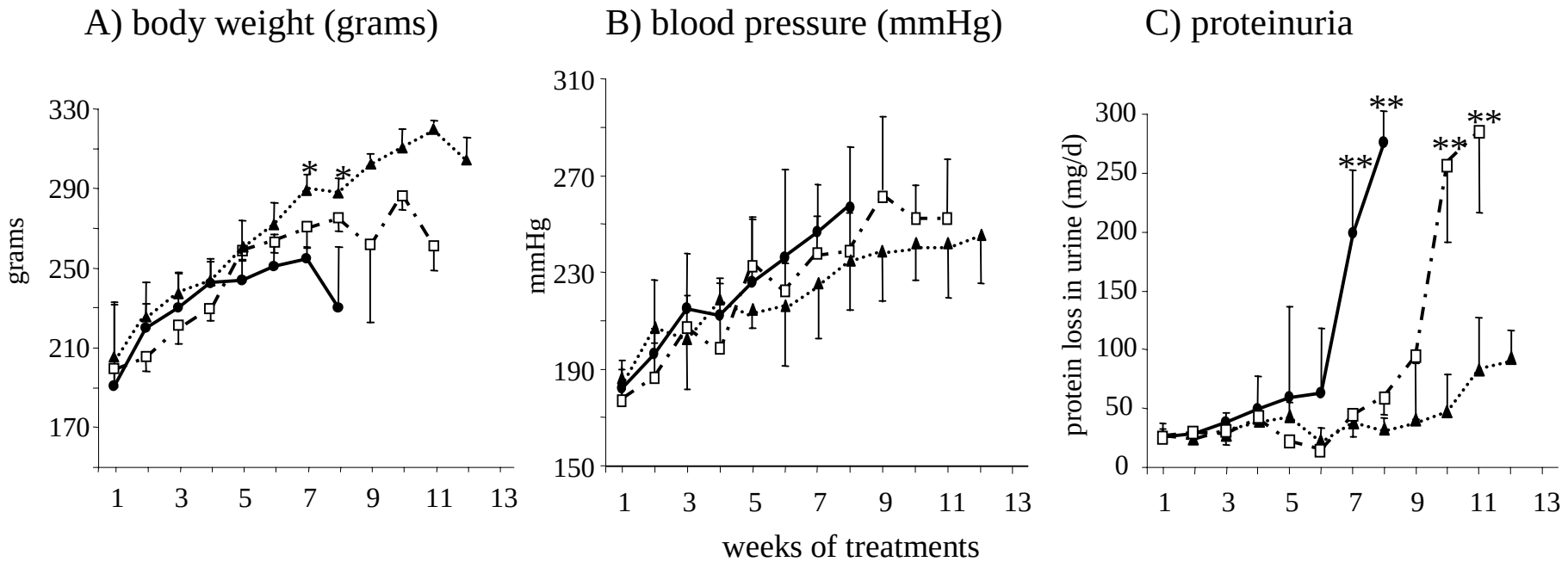

Figure 1

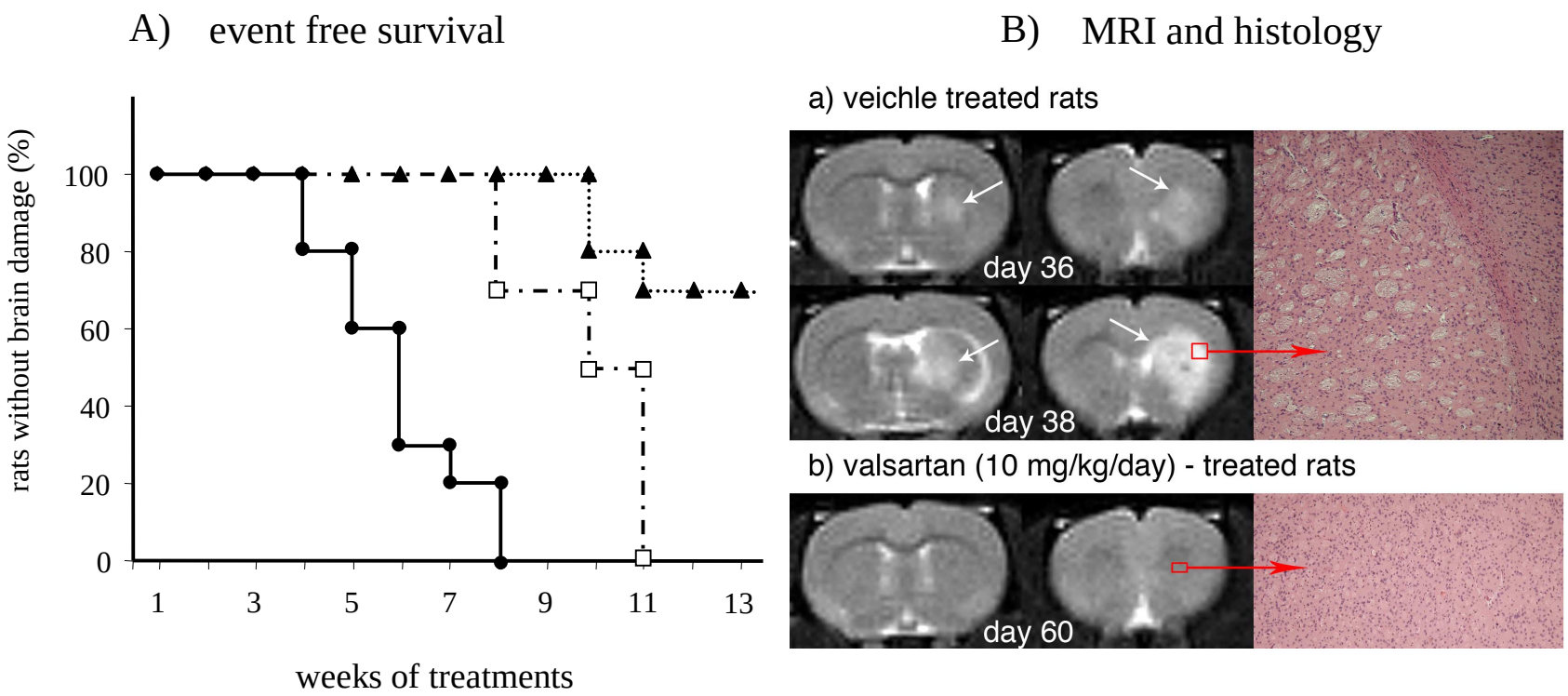

Figure 2

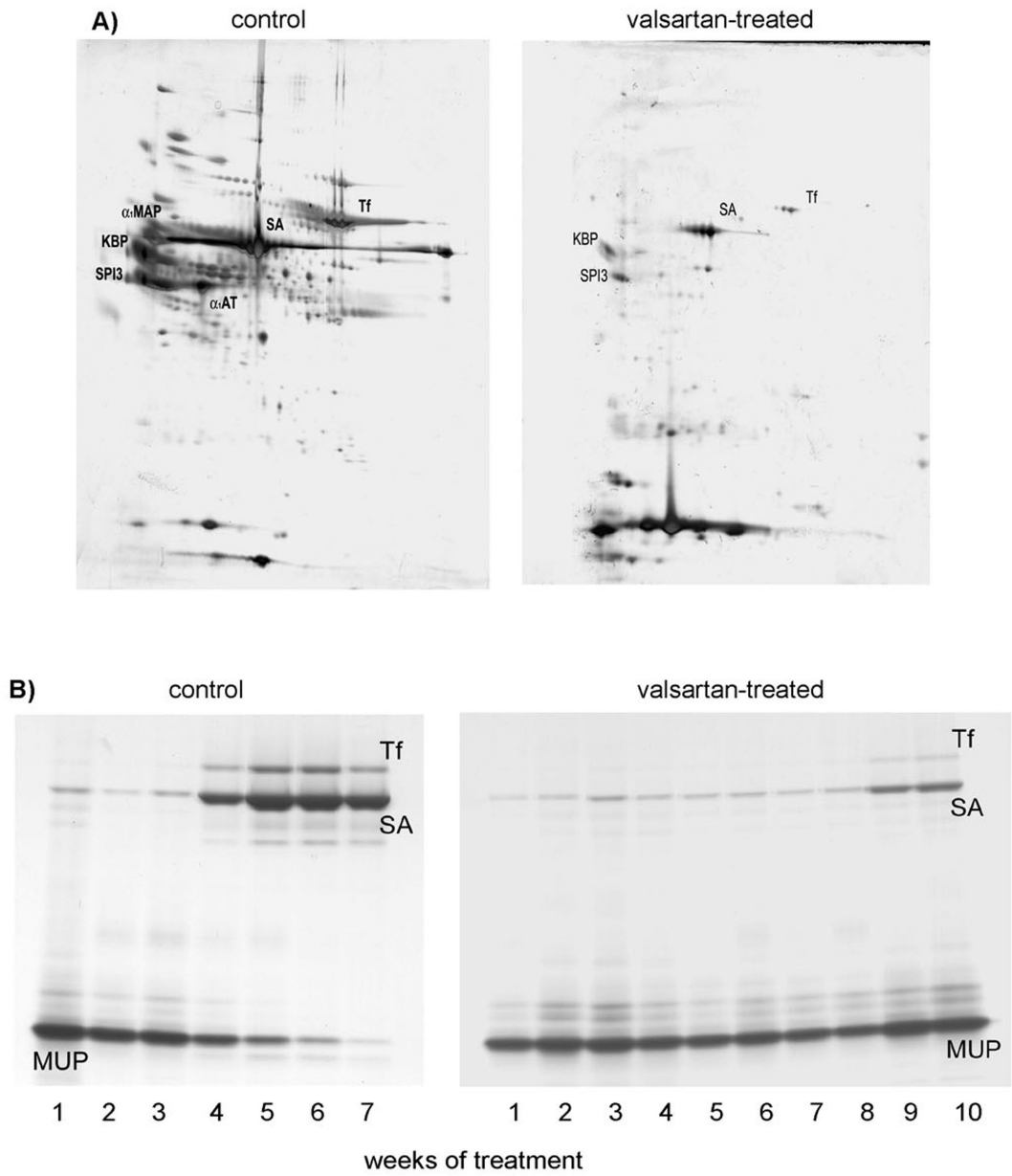

Figure 3

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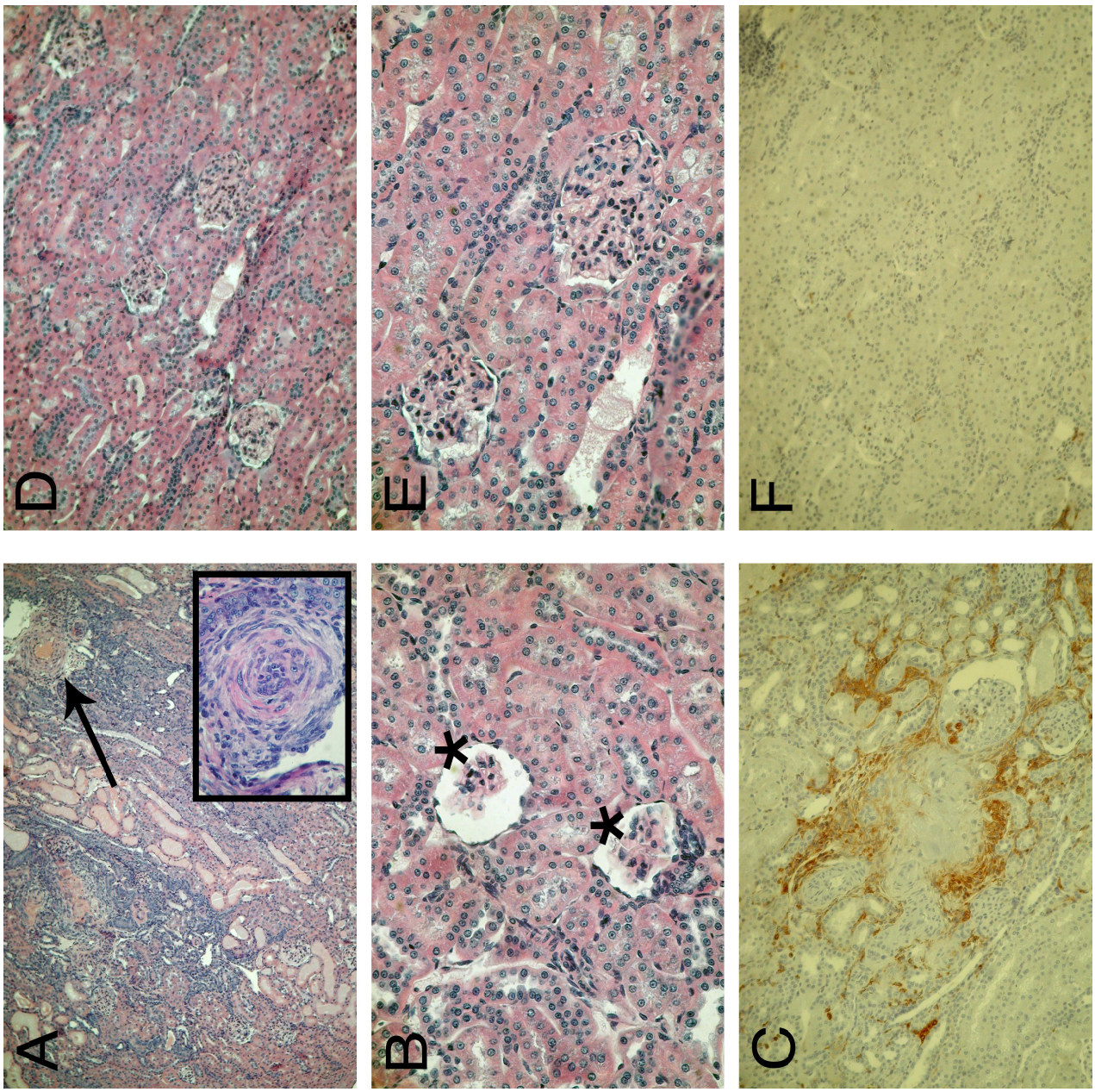

Figure 4

Figure 5

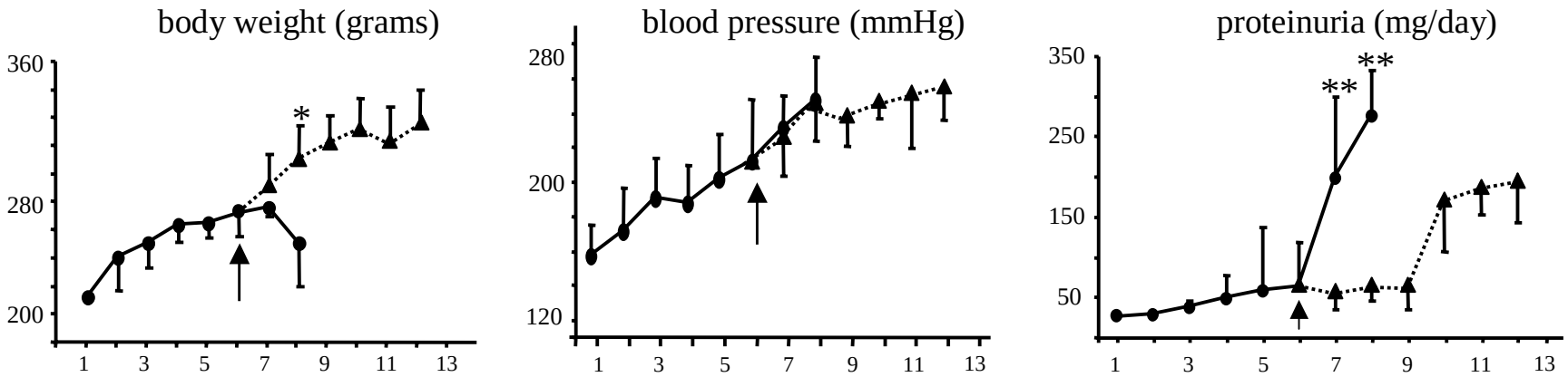

Figure 6