Anti-Inflammatory Effects of AT1 Receptor Blockade Provide End-Organ Protection in Stroke-Prone Rats Independently from Blood Pressure Fall

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ABSTRACT

Spontaneously hypertensive 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 type 1 (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 versus vehicle-treated rats). No damage showed after 100 days in 80% of the rats treated with 10 mg/kg/day. Valsartan treatment preserved renal structure, by preventing the infiltration of inflammatory cells, and lowered renal expression of monocyte chemoattractant protein-1, transforming growth factor-β1, and interleukin-1β, 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.

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 (Brasier et al., 2002; Luft, 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 proinflammatory 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 (Sadoshima, 2000; Ruiz-Ortega et al., 2002). 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 (SHRSP) 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, which is 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 (Marks et al., 2001; Sironi et al., 2001, 2003). 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 paid to the modulation of inflammatory events.

Materials and Methods

Animals and Treatments. Male SHRSP, aged 4 to 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 national permissive diet (day 0), low in potassium and protein and high in sodium (Japanese permissive diet: 18.7% protein, 0.63% potassium, 0.37% sodium; Laboratorio Dr. Piccioni, Gessate, Italy) 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 valsartan, respectively, dissolved in drinking water. In a fourth group (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. 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 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 (PBRecorder 8006, Ugo Basile, Comerio, Italy). During each recording session, the blood pressure was measured in each animal three times by at least two different operators expert in these procedures and completely blinded with respect to the experimental group to which each animal belonged. With bovine albumin as a standard, 24-h urine protein was measured according to Bradford. Proteinuria (protein levels ≥ 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.

Proteomic Studies. Urine proteins were concentrated by trichloroacetic-acetone precipitation. One-dimensional electrophoresis was run on urine proteins in the presence of 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 run according to the manufacturer's protocol (Protein IEF cell; Bio-Rad, Hercules, CA). IPG ready strips, 11 cm, pH 3 to 10 nonlinear gradient (Bio-Rad), were actively rehydrated at 50 V for 24 h. Proteins (100 μg), dissolved in a buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 20 mM Tris, and 2% carrier ampholytes, were loaded on the cathode using the cup loading tray for Protein IEF cell (Bio-Rad) and focused for a total of 20 kV hours. 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 with the same buffer containing 4.5% iodoacetamide instead of DTT. The focused proteins were then fractionated according to size by SDS-polyacrylamide gel electrophoresis on 7 to 17% polyacrylamide gradients and

![A) body weight (grams) B) blood pressure (mmHg) C) proteinuria](https://image.pollard.museum/jpet/fig1.png)  

**Fig. 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), for SHRSP receiving vehicle (○), treated with 1 mg/kg/day valsartan (●), or treated with 10 mg/kg/day valsartan (▲) (*, p < 0.05 and **, p < 0.01 versus 10 mg/kg/day treated rats).
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% isoflurane in 70% N₂-30% O₂ 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 of 4 × 4 cm². A turbo spin echo sequence was used with 16 echoes per excitation, 10 ms of interecho time, 85 ms of equivalent echo time, and 4 s of repetition time. The images were 128 × 128 points (zero filled to 256 × 256); eight images were averaged in 8 h 30 s. The occurrence of lesions was identified as the presence of areas of high signal intensity on T2-weighted MRI.

**Fig. 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, 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 h later (a); two contiguous coronal brain sections of a valsartan-treated rat sampled after 60 days of dietary treatment (b). Histology of the indicated areas show that MRI hyperintensity corresponds to damaged tissue.

**Fig. 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.
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 brain abnormalities were first detected in the control group. 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, Germany) and embedded in Paraplast (Sigma-Aldrich, St. Louis, MO), 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; atrophy 0, absent; 1, mild; 2, moderate; 3, severe). The evaluations were performed on at least five 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 xylen and dehydrated. Endogenous peroxidase was blocked by adding 1% H2O2 in 50% methanol. Nonspecific binding sites were saturated with biotinylated secondary antibodies and streptavidin peroxidase. The primary antibody anti-ED1 (1:100; Serotec, Oxford, UK), then with goat serum. The sections were incubated overnight at 4°C with 1% H2O2 in 50% methanol. Nonspecific binding sites were saturated with biotinylated secondary antibodies and streptavidin peroxidase. The primary antibody anti-ED1 (1:100; Serotec, Oxford, UK), then with goat serum. The sections were incubated overnight at 4°C with 1% H2O2 in 50% methanol. Nonspecific binding sites were saturated with biotinylated secondary antibodies and streptavidin peroxidase. The primary antibody anti-ED1 (1:100; Serotec, Oxford, UK), then with goat serum. 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Effects of 6 weeks of treatment with valsartan (10 mg/kg/day) on vascular and tubular changes and ED-1 cells accumulation

The scoring system is detailed under Materials and Methods (Histophatology section).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Vascular Changes (Score)</th>
<th>Tubular Changes (Score)</th>
<th>ED-1 Cells</th>
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<td>2.6 ± 0.4</td>
<td>1.1 ± 0.5</td>
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<td>Valsartan</td>
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<td>1.2 ± 0.4*</td>
<td>0.6 ± 0.3*</td>
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* p < 0.05; ** p < 0.01 vs. vehicle.

**Effects of valsartan at weeks 7 and 8; p < 0.01 1 mg/kg/day valsartan versus 10 mg/kg/day valsartan-treated rats at weeks 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 (Fig. 1C). 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 α1-major acute-phase protein; α1-MAP), as assessed by two-dimensional electrophoresis. Figure 3, panel A, shows a representative two-dimensional electrophoresis 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 and transferrin, at the expense of 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 arterioles. After 6 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. Valsoartan 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 6 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 (Fig. 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) whereas 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 3 weeks; after this period, proteinuria increased between weeks 10 and 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 to be 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 (Barone et al., 1996; Abrahamsen et al., 2002). Similarly, clinical studies indicated that ARBs improve vascular function independently of the blood pressure-lowering effect (Klingbeil et al., 2002; Lewis, 2002; Viberti et al., 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 suggested that the RAS participates in immune and inflammatory responses (Tummala et al., 1999; Dzau, 2001). In particular, angiotensin II, acting mainly through the AT1 receptor, 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; Dandonà 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 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 reduces 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 an immunoregulatory and proinflammatory 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 prostaglandin release and extracellular matrix protein 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 proinflammatory and profibrotic gene transcription. SHRSP, besides 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 up-regulated in hepatocytes under the control of inflammatory mediators, in particular cytokines, originating at the site of persistent inflammation (Glurich et al., 2002; Pepys and Hirschfield, 2003). In our animal model, the accumulation of acute-phase proteins, particularly thiostatin, represents a response to an inflammatory condition that 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 acute-phase proteins (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 nondiabetic population (Stuveling et al., 2003). CRP, a highly sensitive marker of systemic inflammation, is the major acute-phase protein in humans, like thiostatin is in rats. When tissue is injured or traumatized, CRP is quickly up-regulated, mainly in response to circulating mediators, in particular cytokines. Valsartan treatment has been shown to reduce CRP concentration in human plasma whereas angiotensin-converting enzyme inhibitors fail to modulate this inflammatory marker (Dandonà et al., 2003; de Maat et al., 2003). In conclusion, our data indicate that in SHRSP the administration of nonhypotensive doses of valsartan retards renal damage and systemic inflammation. Furthermore, the beneficial effects obtained in SHRSP by the delayed administration of valsartan (see Fig. 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 at the local level, reducing macrophage infiltration and expression of MCP-1, TGF-β1, and IL-1β mRNA in the kidney, and at the systemic level as indicated by the reduced excretion of acute-phase proteins in

Fig. 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).
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.

References


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