Effect of Bosentan on NF-κB, Inflammation, and Tissue Factor in Angiotensin II–Induced End-Organ Damage


Abstract—Reports on the effectiveness of endothelin receptor blockers in angiotensin (Ang) II–induced end-organ damage are conflicting, and the mechanisms involved are uncertain. We tested the hypothesis that endothelin (ET)A/B receptor blockade with bosentan (100 mg/kg by gavage after age 4 weeks) ameliorates cardiac and renal damage by decreasing inflammation in rats harboring both human renin and angiotensinogen genes (dTGR). Furthermore, we elucidated the effect of bosentan on tissue factor (TF), which is a key regulator of the extrinsic coagulation cascade. We compared bosentan with hydralazine (80 mg/L in the drinking water for 3 weeks) as a blood pressure control. Untreated dTGR featured hypertension, focal necrosis in heart and kidney, and a 45% mortality rate (9 of 20) at age 7 weeks. Compared with Sprague-Dawley controls, both systolic blood pressure and 24-hour albuminuria were increased in untreated dTGR (203±8 versus 111±2 mm Hg and 67.1±8.6 versus 0.3±0.06 mg/dl at week 7, respectively). Bosentan and hydralazine both reduced blood pressure and cardiac hypertrophy. Mortality rate was markedly reduced by bosentan (1/15) and partially by hydralazine (4/15). However, only bosentan decreased albuminuria and renal injury. Untreated and hydralazine-treated dTGR showed increased nuclear factor (NF)-κB and AP-1 expression in the kidney and heart; the p65 NF-κB subunit was increased in the endothelium, vascular smooth muscles cells, infiltrating cells, glomeruli, and tubules. In the heart and kidney, ET_A/B receptor blockade inhibited NF-κB and AP-1 activation compared with hydralazine treatment. Macrophage infiltration, ICAM-1 expression, and the integrin expression on infiltrating cells were markedly reduced. Renal vasculopathy was accompanied by increased tissue factor expression on macrophages and vessels of untreated and hydralazine-treated dTGR, which was markedly reduced by bosentan. Thus, ET_A/B receptor blockade inhibits NF-κB and AP-1 activation and the NF-κB– and/or AP-1–regulated genes ICAM-1, VCAM-1, and TF, independent of blood pressure–related effects. We conclude that Ang II–induced NF-κB and AP-1 activation and subsequent inflammation and coagulation involve at least in part the ET_A/B receptors. (Hypertension. 2000;36:282-290.)

Key Words: angiotensin I ■ endothelin ■ genes ■ albuminuria ■ renin-angiotensin system

The development and progression of malignant, hypertension-induced, end-organ damage is incompletely understood; however, vascular inflammatory responses, extracellular matrix protein accumulation, and thrombosis in small vessels are all involved.1–3 Angiotensin (Ang) II, the key effector of the local and circulating renin-angiotensin system, plays a central role.3,4 Recently, Ang II was shown to be a powerful stimulator of endothelin (ET-1) synthesis and release in vascular smooth muscle and endothelial cells.5–8 Furthermore, Ang II–induced tissue ET-1 fosters vascular hypertrophy.9,10 The importance of ET-1 in cardiovascular disease has recently been reviewed.11 Ang II can activate the nuclear transcription factor NF-κB. NF-κB is primarily responsible for the transcription of MCP-1 and adhesion molecules,12,13 leading to inflammation. NF-κB also regulates the transcription of tissue factor (TF). TF initiates the extrinsic coagulation through an enzymatic factor VII/ factor VIIa complex formation. Constitutive TF expression by mesenchymal cells in the adventitial blood vessel lining normally precludes TF interaction with factor VII in plasma but allows activation of coagulation when the endothelium is damaged.14 TF plays an important role in cardiovascular diseases but also has biological functions independent of the clotting cascade.15–19 Rats harboring both human renin and angiotensinogen genes (dTGR) develop hypertension and severe renal and cardiac damage.20,21 NF-κB activation, MCP-1 production, adhesion molecule expression, and inflammation are prominent features in the damaged kidneys of dTGR.21,22 We provide evidence that bosentan inhibits Ang II–induced NF-κB and AP-1 activation, prevents inflammation, and ameliorates end-organ damage. We show that ET_A/B receptor blockade decreases TF expression in Ang II–induced
vasculopathy, which may ameliorate the microthrombosis featured in this model.

Methods

Four-week-old male age-matched and body weight–matched dTGR and Sprague Dawley (SD) rats were used as described elsewhere.20 All procedures were done according to guidelines from the American Physiological Society. We compared untreated dTGR (n=20), bosentan-treated (100 mg/kg for 3 weeks by gavage once a day; n=15), hydralazine-treated dTGR (80 mg/L in the drinking water for 3 weeks; n=15), and SD control rats (n=10) receiving vehicle (1% sodium carboxymethylcellulose). This bosentan dosage produces a maximal pharmacological effect in rats.23 Systolic blood pressure was measured at weeks 6 and 7 by the tail-cuff method under light ether anesthesia 20 hours after the last drug dose. Urine was collected over a 24-hour period. Rats were killed at age 7 weeks. The kidneys and hearts were washed with ice cold saline, blotted dry, and weighed. For Western blot and NF-κB analysis, the tissues were snap-frozen in liquid nitrogen, for immunohistochemistry in isopentane (−35°C), and stored at −80°C.

Tissue preparation and immunohistological techniques were performed as described before in detail.24 The sections were incubated with primary monoclonal antibodies against rat monocytes/macrophages (ED-1, Serotec), NF-κB subunit p65 (Roche Boehringer), ICAM-1 (1A29, R&D Systems), LFA-1 (WT.1, Pharmingen), VLA-4 (TA-4, Pharmingen), and with polyclonal antibodies against TF (gift of Dr Th. Luther, TU Dresden, Germany), PAI-1 (American Diagnostica), and fibronectin (Paesel-Lorei). Scoring of ED-1–, LFA-1–, and VLA-4–positive cells was performed with the use of the program KS 300 3.0 (Zeiss). Fifteen different areas of each heart and kidney (n=4 to 5 in all groups) were analyzed without knowledge of rat identity. Urinary ET-1 concentration was determined by radioimmunoassay as described previously.25 Quantitative determination of albumin concentration in the urine was performed with the use of a commercially available rat albumin ELISA kit (Celltrend). Urinary ET-1 concentrations were normalized to urinary creatinine concentrations.

Tissue preparation for electrophoretic mobility shift assay (EMSA) was performed as described before in detail.24 Total tissue homogenates were incubated in a binding reaction medium with 0.5 ng of 32P-dATP end-labeled oligonucleotide, containing the NF-κB binding site from the MHC enhancer (H2K, 5′-gatc-CAGGGCTGGGATTCCCCATCTCCACAGG). For AP-1, double-stranded oligonucleotides containing the consensus sequence for AP-1 (Santa Cruz, 5′-GAT CGA ACT GAC CGC CCG CCC GT-3′) were radiolabeled with γ-32P with the use of T4 polynucleotide kinase by standard methods and purified over a column. The DNA-protein complexes were analyzed on a 5% polyacrylamide gel, 0.5% Tris buffer, dried, and autoradiographed. In competition assays, 50 ng unlabeled H2K or AP-1 oligonucleotide was added to the reaction, and the reaction was continued for an additional 20 minutes.

Figure 1. Systolic blood pressure (A) at 6 and 7 weeks in untreated, bosentan (BOS)-treated, and hydralazine (HYD)-treated dTGR compared with SD controls. Bosentan and hydralazine both decreased blood pressure. Cardiac hypertrophy (B) occurred in all dTGR groups compared with SD rats. Bosentan and hydralazine ameliorated cardiac hypertrophy. Untreated and hydralazine-treated dTGR showed significantly increased 24-hour urinary albumin excretion (C) compared with bosentan and SD control values. Results are mean±SEM (⁎P<0.05).

Figure 2. Representative EMSA for NF-κB shows higher activity in untreated and hydralazine (HYD)-treated dTGR renal extracts compared with bosentan (BOS)-treated dTGR or SD control rats (A). Specificity (B) was demonstrated by competition of excess unlabeled oligonucleotides containing κB site from MHC enhancer (H2K). Semiquantification is given for NF-κB DNA binding activity in kidney (C). Bosentan significantly reduced NF-κB DNA binding activity compared with dTGR and hydralazine-treated dTGR. Western blot analysis (D) shows reduced IκBα levels in renal extracts from untreated and hydralazine-treated dTGR compared with bosentan-treated dTGR and SD controls. E, Representative EMSA for AP-1 in heart. Specificity was demonstrated by competition assay (F). Semiquantification is given for AP-1 DNA binding activity in heart (G). Bosentan significantly reduced AP-1 DNA binding activity compared with dTGR and hydralazine-treated dTGR.
tides were used. Homogenates (50 ng) were used for Western blot and stained with polyclonal I-κBα antibody (FL).

Data are presented as mean ± SEM. Statistically significant differences in mean values were tested by ANOVA, blood pressure by repeated ANOVA, and the Scheffé test. A value of P < 0.05 was considered statistically significant. The data were analyzed with the use of Statview statistical software.

Results

The renal tubules were swollen and filled with proteinaceous material in untreated dTGR. Renal and cardiac vessels showed increased intimal and medial thickness as well as microthrombi. Bosentan prevented vasculopathy and extracellular matrix formation. In contrast, hydralazine-treated sections appeared similar to untreated dTGR (data not shown). Nine (45%) of 20 untreated dTGR died at 7 weeks, whereas only 1 (7%) of 20 of the bosentan-treated and 4 (26%) of 15 hydralazine-treated rats died before the end of the study. Urinary ET-1 levels were significantly increased by 50% in untreated dTGR compared with SD rats, respectively (989 ± 52 versus 646 ± 50 pg ET-1/mg creatinine by week 7). Untreated dTGR, bosentan-treated dTGR, and hydralazine-treated dTGR were hypertensive at weeks 6 and 7 compared with SD (Figure 1A). Bosentan and hydralazine decreased blood pressure (Figure 1A) and cardiac weights (expressed as mg/g body wt) (Figure 1B). Body weight was not significantly different between the groups. Albuminuria (Figure 1C) was significantly reduced with bosentan but not with hydralazine.

We next investigated transcription factor activation influencing VCAM-1, ICAM-1, and TF gene expression. EMSA for the detection of NF-κB showed a greater DNA binding activity in the kidneys of untreated dTGR compared with bosentan-treated or SD controls (Figure 2A). On the other hand, hydralazine-treated dTGR were not distinguishable from untreated dTGR with respect to NF-κB activity. Thus, bosentan treatment reduced levels of NF-κB in kidney and heart (data not shown). Binding specificity was demonstrated by competition of excess unlabeled oligonucleotides containing the κB site from the MHC enhancer (H2K) (Figure 2B). Semiquantification is given in Figure 2C. Western blot analysis showed reduced levels of I-κBα in untreated and hydralazine-treated dTGR kidney (Figure 2D), whereas bosentan ameliorated I-κBα reduction. Bosentan but not hydralazine also decreased AP-1 DNA binding activity in heart (Figure 2E) and kidney (kidney data not shown). Binding specificity (Figure 2F) and semiquantification (Figure 2G) corroborated the results.

Immunohistochemical analysis (phase-contrast resolution) shows the localization of the subunit p65 of NF-κB in the kidney (Figure 3). The expression of p65 was increased in the endothelium (Figure 3A), smooth muscle cells of small vessels (Figure 3A), glomeruli (Figure 3B), infiltrating cells (Figure 3, A through C), and tubules (Figure 3C) of untreated dTGR. No immunoreaction was observed in nontransgenic SD rats (Figure 3, D through F). The NF-κB activity in the kidney was markedly reduced by bosentan compared with untreated or hydralazine-treated dTGR. No immunoreaction was observed in nontransgenic SD rats (Figure 3, D through F). The NF-κB activity in the kidney was markedly reduced by bosentan compared with untreated or hydralazine-treated dTGR (Figure 4, A through D). We next investigated TF, which also contains κB and AP-1 binding sites in the promoter. Untreated and hydralazine-treated dTGR showed an increased TF expression (Figure 4, E through H), which resembles the localization of p65 in the vessel wall. Bosentan markedly reduced TF expression. VCAM-1 (Figure 5, A through D) and ICAM-1 (Figure 5, E through H) expression in dTGR kidneys and hearts (data not shown) was increased in the intima, whereas ICAM-1 was also increased in adventitia, glomeruli, tubules,
and in the peritubular space. ICAM-1 and VCAM-1 expression was reduced by ET A/B receptor blockade but not by hydralazine. However, bosentan was more effective in the kidney than in the heart.

Immunohistochemical analysis of the integrins VLA-4 and LFA-1 in the kidney of untreated dTGR showed increased expression in the peritubular space and adventitia. The semiquantification of the counterreceptors for VCAM-1 and ICAM-1 (Figure 6) showed that bosentan reduced VLA-4 by 60% (Figure 6A) and LFA-1 by 70% (Figure 6B) in the kidney compared with untreated dTGR, respectively. There was significant perivascular monocyte/macrophage infiltration in the kidney and heart of untreated dTGR. Cell count analysis (Figure 6C) showed a

![Image of immunohistochemical analysis of NF-κB p65 subunit in kidney of untreated dTGR (A) showed increased expression compared with bosentan (BOS)-treated dTGR (B) and SD controls (D). Hydralazine (HYD)-treated dTGR (C) on the other hand, exhibited p65 staining little different from untreated dTGR. E and F show expression of TF in kidney. TF expression was increased in vessel wall of untreated and hydralazine-treated dTGR. Bosentan markedly reduced TF expression.](http://hyper.ahajournals.org/)

**Figure 4.** Immunohistochemical analysis of NF-κB p65 subunit in kidney of untreated dTGR (A) showed increased expression compared with bosentan (BOS)-treated dTGR (B) and SD controls (D). Hydralazine (HYD)-treated dTGR (C) on the other hand, exhibited p65 staining little different from untreated dTGR. E and F show expression of TF in kidney. TF expression was increased in vessel wall of untreated and hydralazine-treated dTGR. Bosentan markedly reduced TF expression.
significant reduction in the mononuclear cell infiltration after bosentan treatment in kidney and heart compared with the hydralazine-treated and untreated dTGR (heart data not shown). The monocyte recruitment in the vascular wall was accompanied by increased PAI-1 expression (data not shown) in the same areas and accumulation of extracellular matrix protein. Fibronectin (Figure 7, A through D) was completely reduced in the kidney and moderately reduced in the heart by bosentan treatment compared with untreated and hydralazine-treated dTGR (Figure 7, E through H).

**Discussion**
We tested the hypothesis that bosentan treatment inhibits NF-κB activation, prevents inflammatory responses, and
ameliorates Ang II–induced cardiac and renal damage. Furthermore, we investigated the effect of bosentan on TF, which is the major initiator of the coagulation pathway in vivo. Our animal model features hypertension, vasculopathy, severe renal damage, cardiac hypertrophy, inflammation, focal necrosis in heart and kidney, and a 45% mortality rate at 7 weeks. We found that bosentan but not hydralazine prevented cardiac hypertrophy and nephropathy. How-ever, hydralazine did not decrease AP-1 DNA binding activity and extracellular matrix formation in heart and kidney. We speculate that the reduction in cardiac hypertrophy by hydralazine may be overestimated because 26% of hydralazine-treated rats died before the end of the study. Autopsies of all hydralazine rats, which were not included in the cardiac hypertrophy index, showed enlarged hearts. In addition, Ang II is known to stimulate immediate early genes, cell proliferation, and hypertrophic responses.

Recently, 2 groups showed that Ang II induced c-fos, c-jun, and AP-1 activity in vascular smooth muscle cells.27,28 Bosentan was able to reduce AP-1 DNA binding activity and AP-1 regulated fibronectin formation in the heart. Thus, reduction of cardiac hypertrophy by bosentan may be partially mediated by AP-1. In contrast, bosentan reduced blood pressure only to ∼165 mm Hg. Therefore, mechanical load may have counterregulated the reduction of hypertrophy. Altogether, treatment with bosentan also ameliorated cardiac vasculopathy and fibrosis as well as inflammation.

Ang II is a powerful stimulator of ET-1 in vascular smooth muscle and endothelial cells.5–7 Tissue ET-1 induced by Ang II is known to induce vascular hypertrophy.9,10 However, reports on the effectiveness of ET-1 receptor blockers in Ang II–induced end-organ damage are conflicting.30–32 Herizi et al31 showed that bosentan ameliorated end-organ damage in Ang II–infused rats. We were thus not surprised to find that bosentan ameliorated the severity of vascular damage in dTGR. Recently, Randolph et al30 showed reduced left ventricular hypertrophy as well as β-myosin heavy chain and ANP gene expression in the early phase of renovascular hypertension after ETα receptor blockade, independent of blood pressure. In contrast, Li et al32 were not able to show beneficial effects of bosentan in 2-kidney 1-clip renovascular hypertensive rats. However, Li et al32 used rats that had already developed hypertension and presumably already had end-organ damage. Thus, they studied disease regression. In addition, the late phase of 2-kidney 1-clip hypertension may be less dependent on high Ang II levels.

We sought to elucidate molecular mechanisms involved in the pathogenesis of end-organ damage in dTGR and the possible role of endothelin as a mediator. Several reports have demonstrated the participation of macrophages in the onset and progression of various kidney diseases.33,34 Cell surface adhesion molecules could play a major role in mediating cell recruitment. NF-κB, the main factor in the transcription of VCAM-1 and ICAM-1, plays an important role in various cardiovascular diseases. Several studies have shown that NF-κB plays an important role in cardiac and renal end-organ damage. Zhang et al35 have shown that ACE inhibition decreased NF-κB in kidneys with ureteral obstruction. Ruiz-Ortega et al33 have reported that NF-κB and MCP-1 activation in the renal cortex was reduced by ACE inhibition in experimental immune complex nephritis. They also showed that Ang II stimulated NF-κB and MCP-1 in mesangial cells. Morishita et al36 showed that NF-κB inhibition by a decoy technique reduced the extent of myocardial infarction after reperfusion. End-organ damage in dTGR was accompanied by the activation of NF-κB and AP-1 in both the kidney and heart. However, Ang II–induced inflammatory response,
vasculopathy, and the induction of the various NF-κB– and/or AP-1–regulated genes was more prominent in the kidney compared with the heart. Bosentan ameliorated renal and cardiac damage; however, renal protection was more efficient. Recently, we showed that the unselective NF-κB inhibitor PDTC reduced albuminuria by >90%.24 However, PDTC reduced blood pressure, inflammation, cardiac vasculopathy, and cardiac hypertrophy similar to bosentan. Thus, NF-κB plays a role in both cardiac and renal damage. Remuzzi and Bertani37 have shown that filtered albumin is able to activate NF-κB. They demonstrated that increased glomerular permeability is followed by increased filtration of macromolecules, followed by excessive tubular protein reabsorption.37,38 This process leads to abnormal accumulation of proteins in endolysosomes and endoplasmic reticulum. Altogether, these processes foster the activation of NF-κB–

Figure 7. Representative immunohistochemical photomicrographs of fibronectin in kidney (A through D) and heart (E through H) of untreated dTGR, bosentan (BOS)-treated, hydralazine (HYD)-treated dTGR, and SD rats. Bosentan treatment decreased fibronectin staining compared with untreated and hydralazine-treated dTGR.
dependent and NF-κB–independent cytokines, resulting in
renal inflammation. Inhibition of NF-κB by bosentan treat-
ment may have inhibited both the direct activation of NF-κB
by Ang II as well as the subsequent activation induced by the
increased glomerular permeability in our model, thereby
breaking the self-amplifying loop. Thus, NF-κB inhibition
may be an additional mechanism that might further explain
the anti-inflammatory effects of ET-1 receptor blockers in
progressive kidney disease.

TF plays an important role in vasculopathies, repercus-
sion injury, preeclampsia, and kidney disease.15–18,39 Ang II stim-
ulates TF.15,40 TF expression in dTGR was predominantly
located in the walls of damaged vessels, which also stained
positive for p65. Integrin-matrix signaling is also known to
play an important role in inflammation and coagulation.41 The
Ang II–induced formation of fibronectin in the kidney and
heart was reduced by bosentan treatment, an effect that could
have mechanistic implications. Several reports suggest that
the β1 integrin VLA-4 induces monocyte procoagulant activ-
ity.42,43 McGilvray et al42 showed that the VLA-4 integrin
cross-linked on human monocytes induces TF expression by
a mechanism involving mitogen-activated protein kinase. We
found marked infiltration of VLA-4–positive cells in dTGR
kneys, which was reduced after ET A/B receptor blockade.
Thus, it is tempting to speculate that VLA-4/fibronectin
signaling also stimulates TF expression in our model. Re-
cently, Giesen et al18 and Nemerson and Giesen19 showed that
in addition to the scheme whereby coagulation is initiated
after vessel damage and blood exposure to vessel wall–bound
TF, leukocytes are the main source of blood-borne TF. dTGR
showed both vessel wall damage and leukocyte activation,
which may both have influenced TF activity.

In summary, we showed that bosentan decreased blood
pressure and cardiac hypertrophy and provided considerable
renal protection in Ang II–induced end-organ damage. Vas-
cular injury was largely prevented, and mortality rate was
reduced. The amelioration of renal damage was provided by
ET A/B receptor blockade, since hydralazine treatment was not
effective. We provide evidence that long-term ET A/B receptor
blockade in vivo inhibits renal NF-κB activation and TF
expression in Ang II–induced vasculopathy. Bosentan also
markedly reduces the expression of NF-κB–regulated genes
such as VCAM-1 and ICAM-1 and thereby diminishes mono-
cyte infiltration. These findings suggest that ET A/B receptor
blockade may provide an anti-inflammatory and antithrom-
bolic effect that could extend to other kidney diseases.

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