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Angiotensin II (AT_1) Receptor Blockade Reduces Vascular Tissue Factor in Angiotensin II-Induced Cardiac Vasculopathy

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Tissue factor (TF), a main initiator of clotting, is upregulated in vasculopathy. We tested the hypothesis that chronic *in vivo* angiotensin (ANG) II receptor AT_1 receptor blockade inhibits TF expression in a model of ANG II-induced cardiac vasculopathy. Furthermore, we explored the mechanisms by examining transcription factor activation and analyzing the TF promoter. Untreated transgenic rats overexpressing the human renin and angiotensinogen genes (dTGR) feature hypertension and severe left ventricular hypertrophy with focal areas of necrosis, and die at age 7 weeks. Plasma and cardiac ANG II was three- to fivefold increased compared to Sprague-Dawley rats. Chronic treatment with valsartan normalized blood pressure and coronary resistance completely, and ameliorated cardiac hypertrophy ($P < 0.001$). Valsartan prevented monocyte/macrophage infiltration, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) activation, and c-fos expression in dTGR hearts. NF-κB subunit p65 and TF expression was increased in the endothelium and media of cardiac vessels and markedly reduced by valsartan treatment. To analyze the mechanism of TF transcription, we then transfected human coronary artery smooth muscle cells and Chinese hamster ovary cells overexpressing the AT_1 receptor with plasmids containing the human TF promoter and the luciferase reporter gene. ANG II induced the full-length TF promoter in both transfected cell lines. TF transcription was abolished by AT_1 receptor blockade. Deletion of both AP-1 and NF-κB sites reduced ANG II-induced TF gene transcription completely, whereas the deletion of AP-1 sites reduced transcription. Thus, the present study clearly shows an aberrant TF expression in the endothelium and media in rats with ANG II-induced vasculopathy. The beneficial effects of AT_1 receptor blockade in this model are mediated via the inhibition of NF-κB and AP-1 activation, thereby preventing TF expression, cardiac vasculopathy, and microinfarctions. *(Am J Pathol 2000, 157:111–122)*

Tissue factor (TF), a 47-kd transmembrane protein, initiates the extrinsic pathway of coagulation via formation of an enzymatic complex with factor VII/factor VIIa. TF's constitutive expression by mesenchymal cells residing in the adventitial lining of blood vessels normally precludes its interaction with factor VII in plasma, but allows activation of coagulation when the endothelium is damaged.[1] TF also possesses biological functions independent of the clotting cascade. TF is expressed by myocardial cells and plays an important role in embryogenesis[2–5] and promotes vascularization of tumors,[6] cell adhesion, and cell migration.[7] Inflammatory cell infiltration to extravascular sites is an important component of the host response to a variety of stimuli, such as ischemia, bacterial infection, tumor deposits, and atherosclerotic plaques. The surface TF expression as well as release of macrophage products serve to coordinate the local inflammatory responses. Fibrin deposition induced by macrophage TF[8] expression contributes to inflammation. Monocyte adherence to the endothelium stimulates TF expression. This process most likely contributes to local microvascular thrombosis.[9]

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TF expression in cultured cells occurs in response to a variety of stimuli (eg, lipopolysaccharides, tumor necrosis factor-α, phorbol-12-myristate 13-acetate (PMA), and ANG II),[10–15] but until now no evidence has been presented that TF is generated in ANG II-induced cardiac vasculopathy *in vivo*. Because ANG II may use similar signaling pathways as lipopolysaccharides, tumor necrosis factor-α, and PMA *in vivo*, it is quite likely that TF is also induced by ANG II through the angiotensin II (AT_1) receptor, followed by a subsequent activation of the transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1).[10,16] Orthner et al[14] demonstrated that inhibition of NF-κB after stimulation with various agonists resulted in reduced TF activity in endothelial cells.

We investigated the effect of AT_1 receptor blockade on the binding activity of NF-κB and AP-1, as well as TF expression in a model of ANG II-induced cardiac vasculopathy. Our findings demonstrate that ANG II mediates its effect on vascular TF via the AT_1 receptor, followed by the subsequent activation of NF-κB and AP-1.

Materials and Methods

Animals and Study Design

Four-week-old dTGR were divided into two groups, control (n = 26) and valsartan (n = 16) groups. Valsartan was given for 3 weeks by gavage once a day (10 mg/kg). Control dTGR and normotensive Sprague-Dawley (SD) rats (n = 15) rats received vehicle (1% sodium carboxymethylcellulose). The dTGR line and characteristics are described elsewhere.[17] The rats were purchased from Biological Research Laboratories Ltd (Füllinsdorf, Switzerland) and were allowed free access to standard 0.3% sodium rat chow (SSNIFF Spezialitäten GmbH, Soest, Germany) and drinking water. The procedures were approved by the local Council on Animal Care (permit no. G408/97), whose standards correspond to those of the American Physiological Society. Systolic blood pressure was measured weekly by tail cuff under light ether anesthesia, 20 hours after the last drug dose, starting at age 5 weeks. The rats were killed at age 7 weeks. Blood samples for hormone analysis were drawn by aortic puncture into pre-chilled tubes containing EDTA (6.25 mmol/L) and phenantroline (26 mmol/L) as anticoagulant and inhibitor of ANG II breakdown *in vitro*, respectively. Remikiren (10 μmol/L) was added to plasma samples for ANG II measurement to prevent ANG II formation *in vitro*. The hearts were washed with ice-cold saline, weighed, frozen in liquid nitrogen, and stored at −80°C until assayed.

Isolated Perfused Heart

Transgenic rats (n = 5 or 6 in each group) were heparinized and anesthetized with thiopental (150 mg/kg rat, i.p.). Once the rat was deeply anesthetized, the heart was removed by sternectomy and placed in iced Krebs-Henseleit buffer. The heart was cannulated immediately via the aorta and retrograde perfusion was performed in a Langendorff apparatus under constant flow (10 ml/min) with a modified Krebs-Henseleit solution with the following composition: NaCl, 114.7 mmol/L; KCl, 4.7 mmol/L; $MgSO_4$, 1.2 mmol/L; KH_2PO_4, 1.5 mmol/L; $NaHCO_3$, 25 mmol/L; $CaCl_2$, 2.5 mmol/L; and glucose, 11.1 mmol/L. The solution was gassed with 95% O_2/5% CO_2 and adjusted to pH 7.4. Coronary effluent was measured by an electromagnetic flow meter (Narcomatic RT 500, Narco BioSystems Inc., Houston, TX). A high fidelity microtip catheter was inserted via the aorta into the left ventricles to measure heart rate and left ventricular pressure. Protocols were started after 15 minutes' equilibration perfusion. The coronary effluent was collected for 20 minutes into pre-chilled tubes containing the same inhibitor cocktail as was used for plasma ANG II measurements. The Ang II was extracted from the perfusate by reversible adsorption to octadecylsilyl-silica cartridges (Sep-Pak C18, Waters, Milford, MA) separated by high performance liquid chromatography and quantified by direct radioimmunoassay.

Cardiac Immunohistochemistry

For immunohistochemistry, the hearts were cut, snap-frozen in isopentane (−35°C), and stored at −80°C. Frozen specimens were cryosectioned at 6 μm thickness and air-dried. The sections were fixed with cold acetone, air-dried, and washed with Tris-buffered saline (TBS; 0.05 mol/L Tris buffer, 0.15 mol/L NaCl, pH 7.6). The sections were incubated for 60 minutes in a humid chamber at room temperature with primary monoclonal antibodies against rat monocytes/macrophages (ED1; Serotec, Oxford, UK), NF-κB subunit p65 (Roche Boehringer, Mannheim, Germany), VLA-4 (TA-4, Pharmingen, San Diego, CA), and the polyclonal tissue factor antibody, and fibronectin (Paesel & Lorei, Frankfurt, Germany). The p65 antibody recognizes an epitope overlapping the nuclear location signal of p65 subunit and therefore selectively stains released, activated NF-κB after dissociation of its inhibitor I-$\kappa B\alpha$.[18] After washing with TBS, the sections were incubated with a bridging antibody (rabbit-anti-mouse IgG; Dako, Hamburg, Germany) for 30 minutes at room temperature and washed again with TBS. The alkaline phosphatase-anti-alkaline phosphatase complex (Dako, Hamburg, Germany) was applied, and the sections were incubated for 30 minutes at room temperature. The immunoreactivity was visualized by development in a mixture of naphtol-AS-BI-phosphate (Sigma, Deisenhofen, Germany) with neufuchsin (Merck, Darmstadt, Germany). Endogenous alkaline phosphatase was blocked by addition of 10 mmol/L levamisole (Sigma, Deisenhofen, Germany) to the substrate solution. The sections were slightly counter stained in Mayer's hemalaun (Merck), blued in tap water, and mounted with GelTol (Coulter-Immunotech, Hamburg, Germany). Preparations were examined under a Zeiss Axioplan-2 microscope (Zeiss, Jena, Germany) and photographed using a color reversal film Agfa CTX 100. Semiquantitative scoring of ED-1-positive cells in the heart was performed using computerized cell count program (KS 300 3.0,

Zeiss). Fifteen different areas of each heart samples (n = 5 in both groups) were analyzed. The heart samples were examined without knowledge of the rats' identity.

Electrophoretic Mobility Shift Assay (EMSA)

Tissue extracts and EMSA were performed as described earlier.[19] Briefly, frozen total hearts were pulverized in liquid nitrogen with a pestle and mortar, and resuspended in 3 ml 50 mmol/L Tris, pH 7.4, containing a Complete protease inhibitor tablet (Roche Boehringer) and 1 mmol/L Na-ortho-vanadate (Sigma). The suspension was centrifuged (4000 × g, 5 minutes, 4°C). The pellet was resuspended and lysed for 30 minutes in whole cell lysate buffer (20 mmol/L Hepes pH 7.9, 350 mmol/L NaCl, 20% glycerol, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.1 mmol/L EGTA, and 1% NP-40) and again centrifuged (13,000 × g, 10 minutes, 4°C). The supernatant was aliquoted and frozen in liquid nitrogen and stored at −80°C until use. The protein concentration for EMSA was quantified by the Bradford method.[20] For EMSA, total heart homogenates were incubated in binding reaction medium (2 μg poly-dI-dC, 1 μg bovine serum albumin, 1 mmol/L dithiothreitol, 20 mmol/L Hepes pH 8.4, 60 mmol/L KCl and 8% Ficoll) with 0.5 ng of ^{32}P-dATP end-labeled oligonucleotide, containing the NF-κB binding site from the MHC-enhancer (H2K, 5′-gatcCAGGGCTGGGGATTCCCCATCTCCACAGG) at 30°C for 30 minutes. The DNA-protein complexes were analyzed on a 5% polyacrylamide gel (0.5% Tris buffer), dried, and autoradiographed. NF-κB activity could be blocked by excess unlabeled NF-κB probe, suggesting specificity of the activation.

Isolation of mRNA and Gene Expression

After snap-freezing in liquid nitrogen, organs were kept at −80°C. Tissue was homogenized with mortar and pestle under liquid nitrogen. RNA was isolated following the TRIZOL protocol (Gibco Life Technology) and stored at −80°C. Reverse transcriptase-polymerase chain reaction (RT-PCR) primers and TaqMan-probe for GAPDH and TF were constructed with help of Primer Express (ABI Prism 7700 Sequence Detection System, Perkin Elmer, Foster City, CA): GAPDH forward: AAGCTGGTCATCAATGGGAAAC; GAPDH reverse: ACCCCATTTGATGTTAGCGG; GAPDH probe CATCACCATCTTCCAGGAGCGCGCGAT, FAM (6-carboxytetrafluorescein) and TAMRA (quencher) labeled; TF forward: CCACCTTTCTCGGCTTCCTT; TF reverse: CTTTCCCTGGAGGAGTGCC; TF probe: FAM-TCCTTCAGGTGGCCGTTGGTGC-TAMRA; c-fos forward: CCATGATGTTCTCGGGTTTCA, c-fos reverse: GCGCTACTGCAGCGGG, c-fos probe: FAM-CGCGGACTACGAGGCGTCATCC-TAMRA. oligonucleotides were synthesized by BioTez (Berlin-Buch, Germany). Manganese (Mn) and primer concentrations were optimized with a titration curve. The following concentrations were used: GAPDH Mn 3 mmol/L; TF 4 mmol/L, c-fos 4 mmol/L; GAPDH and TF: primer forward 200 nmol/L, primer reverse 600 nmol/L, probe 100 nmol/L, c-fos primer 200 nmol/L, probe 100 nmol/L. Real-time quantitative RT-PCR was performed using the TaqMan system (ABI's Prism 7700 Sequence Detection System, Perkin Elmer, Foster City, CA) and following the instructions of TaqMan EZ RT-PCR TaqMan-kit protocol. 0.5–1μg total RNA was used for each PCR with the following time course: 50°C, 2 minutes; 60°C, 30 minutes; 95°C, 5 minutes; 40 cycles of 94°C, 20 seconds and 60°C, 1 minute. Each sample was tested twice. For quantification, gene expression of the target sequence was normalized in relation to the expressed housekeeping gene GAPDH.

Clotting Test

Thin sections (10 μm) of frozen tissue samples were homogenized with an Ultra Turrax in ice-cold extraction buffer, pH 7.5, containing 5 mmol/L n-octyl-b-D-glucopyranoside and 20 mmol/L HEPES saline (Sigma Chemie). For extraction, homogenates were gently agitated at 4°C for 6 hours followed by centrifugation (300 × g, 15 minutes). The supernatants were aliquoted and stored frozen at −80°C until use. Protein content was determined by the Micro BCA protein assay reagent kit (Pierce, Germany). The procoagulant activity (PCA) of left ventricle extracts was assayed in a one-stage clotting test. In this assay 25 μl of samples were incubated with 25 μl of citrated plasma from rats (Sigma) for 1 minute at 37°C. After addition of 25 μl of 25 mmol/L $CaCl_2$, the clotting time was manually measured. The time recorded was converted to milliunits (mU) of PCA by reference to a TF standard curve derived from a preparation of rat brain acetone powder (Sigma). A clotting time of 50 seconds corresponded to 1000 mU of PCA. PCA was normalized in relation to the expressed total protein (mU/mg).

Transfection of Luciferase Promoter Construct and Analysis

Human coronary artery vascular smooth muscle cells (VSMC) were grown in SmGM2 (Clonetics, San Diego, CA) and Chinese hamster ovary (CHO) cells (cell lines were a kind gift of Dr. Wallukat, MDC, Berlin, Germany) stably overexpressing the AT 1 receptor (CHO-AT_1) and CHO wild-type cells (CHO-WT) in DMEM/Ham's F-12 containing geneticine (63 mg/L), 10% fetal calf serum, 0.1% penicillin/streptomycin, and glutamine to 75% confluence. The human TF luciferase promoters have been described previously.[21] For the promoter studies, 2 μg of the appropriate luciferase promoter construct per milliliter of medium were transfected with Fugene6 (Roche Boehringer) according to the manufacturer's description. Transfected cells were stimulated for 15 minutes with 1×10^{-6}, or 10^{-7} mol/L ANG II. AT_1 receptor was blocked by a 30-minute preincubation with 10^{-6} mol/L valsartan. Cells were harvested and lysed as described earlier.[22] Luciferase activity assay was performed as described elsewhere.[22] Relative luciferase units were calculated as percentage of basal luciferase activity of the nonstimulated cell line. The measurements were performed in

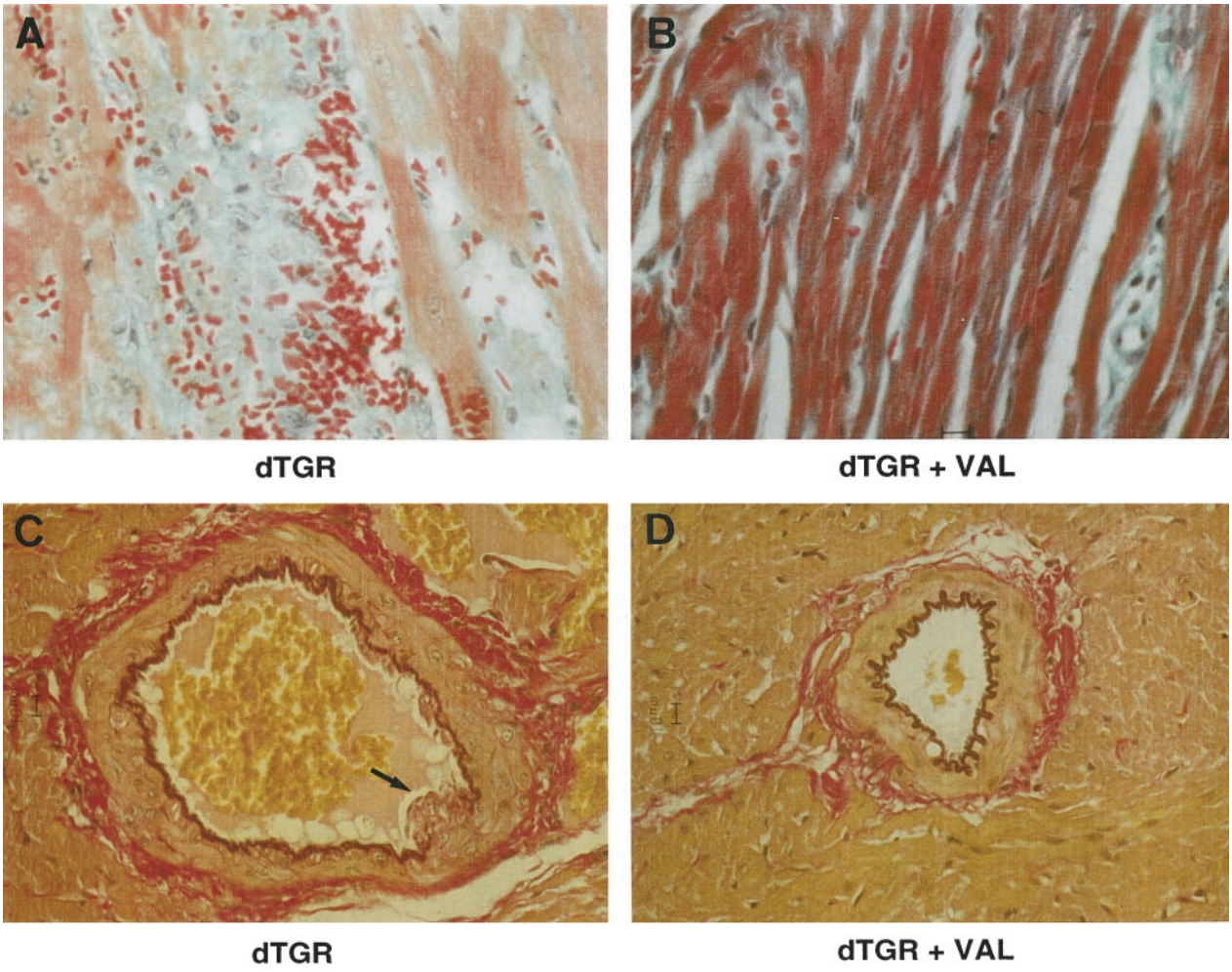

Figure 1. A and **B:** Goldner-Trichrome panel, **C** and **D:** van Gieson-stained section from dTGR- and valsartan-treated hearts. Section of myocardium from dTGR show hemorrhages and patchy areas of necrosis, as well as an interstitial fibroblastic reaction. Valsartan treatment prevented cardiac damage. Untreated dTGR vessel (**C**) shows damaged lamina elastica interna and intimal proliferation (**arrow**). However, valsartan treatment prevented vascular damage.

duplicate. The data were confirmed in 3 to 5 independent transfections.

Statistics

Data are presented as means ± SE. Statistically significant differences in mean values were tested by two-way analysis of variance for repeated measures and the Scheffé test. A value of $P < 0.05$ was considered statistically significant. The data were analyzed using Statview statistical software.

Results

Cardiac Histology and Mortality

dTGR featured hypertension and cardiac hypertrophy. Sections of myocardium from dTGR show hemorrhages and patchy areas of necrosis, as well as an interstitial fibrosis. Twelve of 26 untreated dTGR died before week 7. In contrast, none of the valsartan-treated rats died before the end of the study. Untreated dTGR vessels show signs of vasculopathy indicated by damaged lamina elastica interna and intimal proliferation. Valsartan reduced cardiac hypertrophy, prevented vascular injury, and inhibited extracellular matrix formation (Figure 1, A-D).

Effect of Valsartan on Blood Pressure, Coronary Resistance, and Cardiac Damage

Blood pressure, coronary resistance, cardiac hypertrophy, and left ventricular pressure were markedly increased in untreated dTGR. Valsartan normalized blood pressure, coronary resistance and prevented the development of cardiac hypertrophy ($P < 0.0001$, respectively, Figure 2, A-C). Plasma ANG II concentrations were fivefold higher in untreated dTGR compared to normotensive SD rats. Valsartan did not affect plasma ANG II levels. ANG II release from isolated perfused hearts was increased threefold in dTGR. Plasma lactic acid dehydrogenase (LDH) was significantly increased compared to valsartan-treated and nontransgenic rats (4651 ± 1268 U *vs.* 1574 ± 523 U *vs.* 640 ± 162 U, $P < 0.05$).

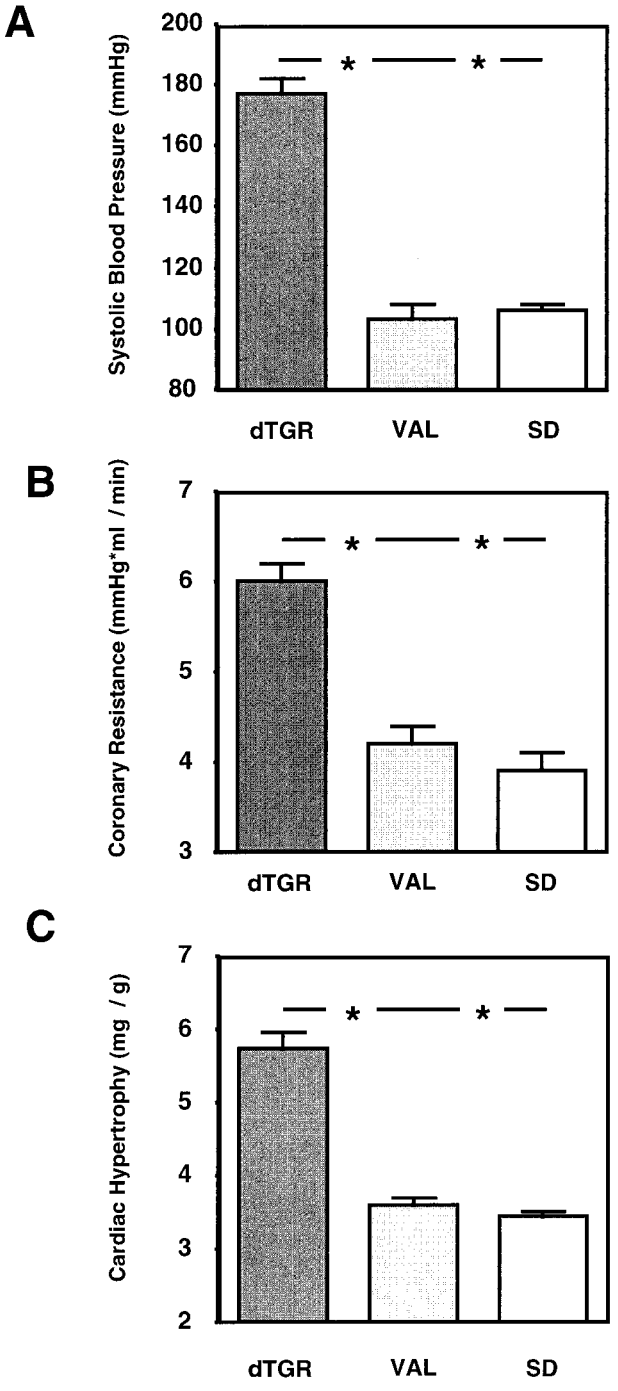

Figure 2. A and **B:** Systolic blood pressure and coronary resistance in dTGR, dTGR treated with valsartan, and SD rats. Valsartan normalized blood pressure (177 ± 5 *vs.* 103 ± 5 *vs.* 106 ± 2 mmHg, $P < 0.0001$, dTGR *vs.* dTGR + valsartan *vs.* SD rats, respectively) and coronary resistance (6.0 ± 0.2 *vs.* 4.2 ± 0.2 *vs.* 3.9 ± 0.1 mmHg* ml/minute) and prevented the development of cardiac hypertrophy (5.7 ± 0.2 *vs.* 3.6 ± 0.1 *vs.* 3.4 ± 0.1 mg/g, **C**). Results are expressed as mean ± SE of 8 to 13 animals per group. *$P < 0.0001$.

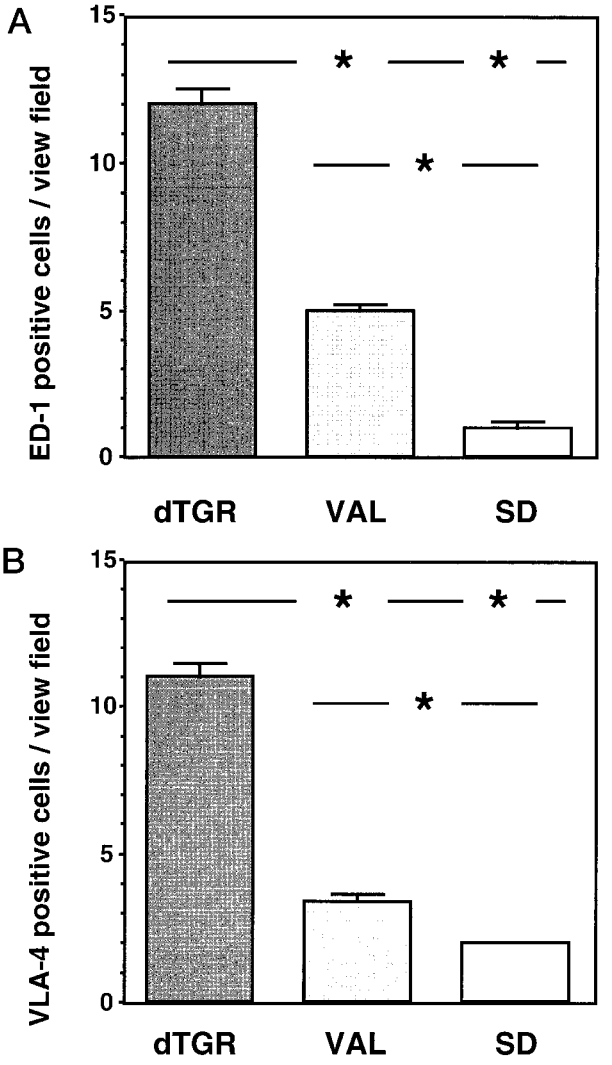

Figure 3. Semiquantitative scoring of ED-1-positive (**A**) and VLA-4-positive (**B**) cells in hearts of dTGR, valsartan-treated dTGR, and SD rats was performed using a computerized cell count program. Ten different areas of each heart were analyzed. Results are expressed as mean ± SE of 5 animals per group. *$P < 0.001$.

Inflammatory Response, VLA-4 Integrin, and Matrix Formation

Monocyte adherence to fibronectin or engagement of VLA-4 has been demonstrated to stimulate TF. Therefore, we analyzed the infiltration monocyte/macrophage- and VLA-4-positive cells in cardiac perivascular space of dTGR. Semiquantitative cell count analysis showed significantly increased infiltration in dTGR with a 61% reduction for ED-1-positive mononuclear cells (Figure 3A), as well as a 69% reduction for VLA-4-positive cells (Figure 3B), after valsartan treatment (both $P < 0.0001$). The localization of VLA-4 in the interstitium was in the proximity of ED-1-positive cells. dTGR hearts showed increased fibronectin, collagen I and IV, and laminin expression, which was prevented by AT_1 receptor blockade (data not shown).

NF-κB and AP-1 DNA Binding Activity and c-fos mRNA in the Left Ventricle

We then investigated the activation of the transcription factors NF-κB and AP-1, which regulate TF gene expression. EMSA for the detection of NF-κB and AP-1 showed

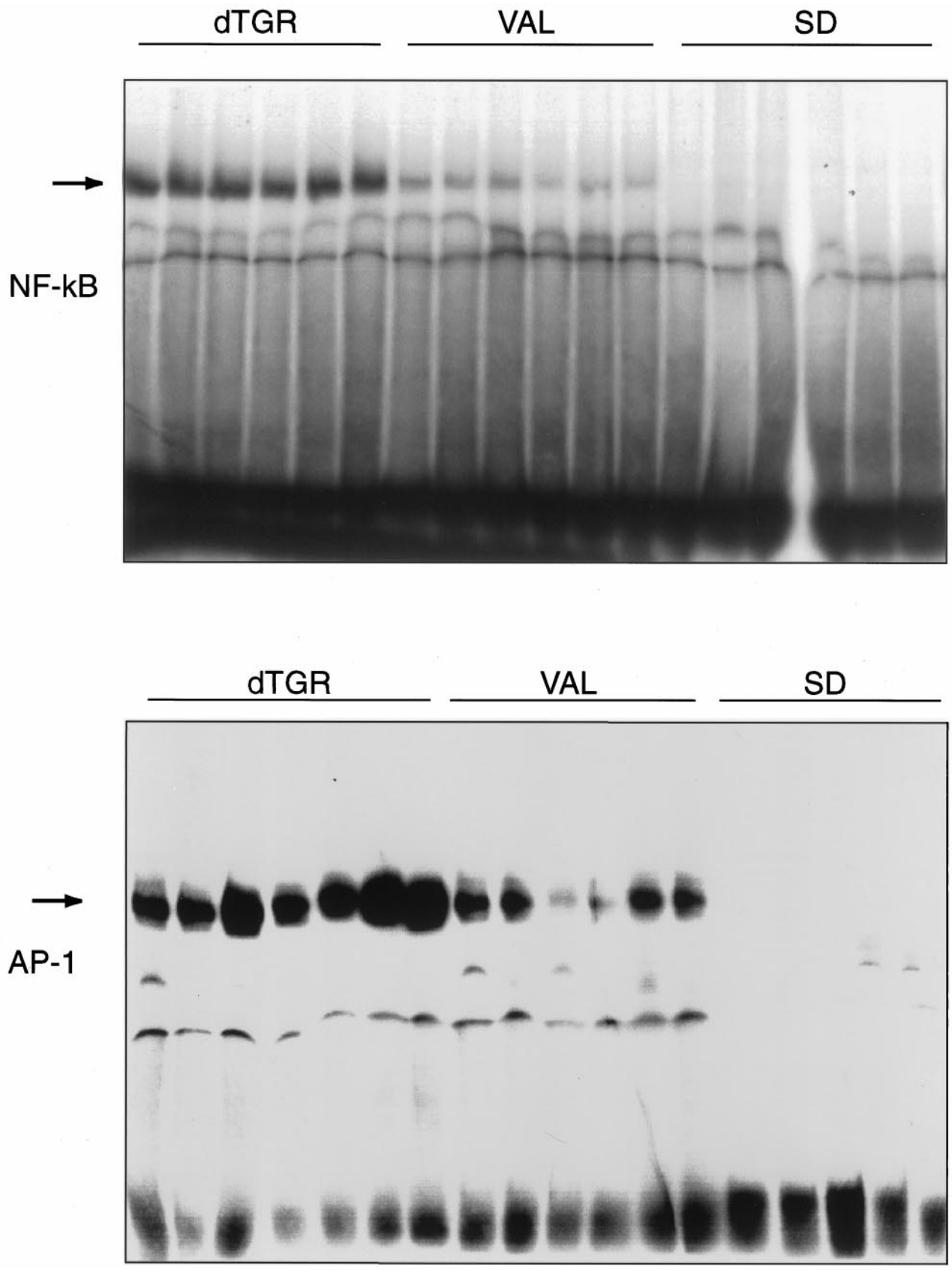

Figure 4. Activation of DNA-binding nuclear factors by ANG II. EMSA for the detection of NF-κB and AP-1 shows a higher binding activity of dTGR heart extracts compared with SD rats. Valsartan treatment reduced levels of NF-κB and AP-1 in the heart. EMSA was performed 3 times independently with similar results.

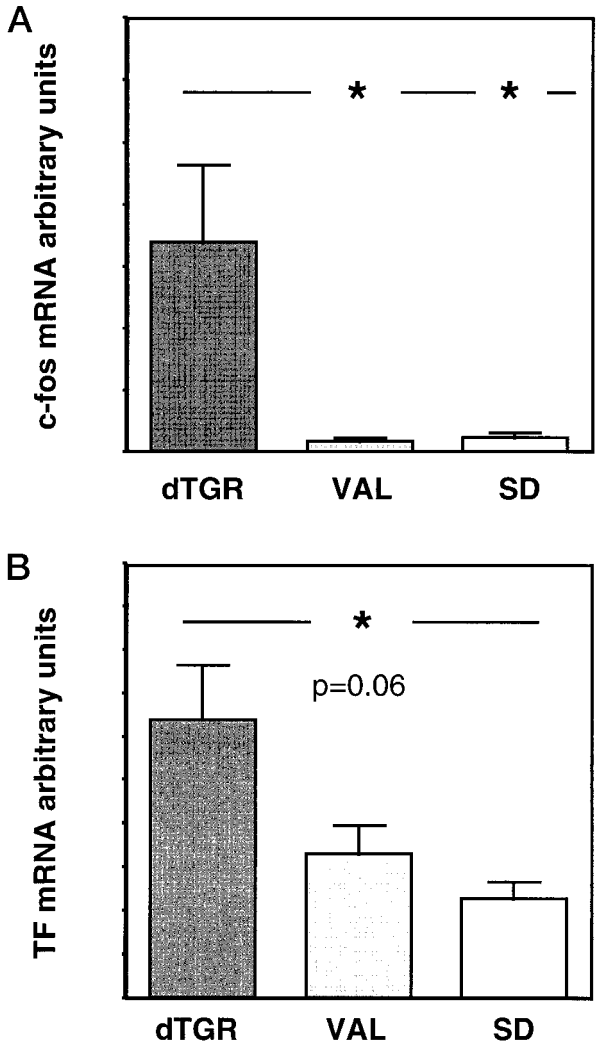

Figure 5. Untreated dTGR show significantly increased c-fos (**A**) and TF (**B**) mRNA levels. Valsartan reduced both c-fos ($^*P < 0.05$) and TF expression ($P = 0.055$). mRNA levels of the target genes were normalized for the housekeeping gene GAPDH. Results are expressed as mean ± SE of 5 to 8 animals per group.

a greater binding activity in heart homogenates of dTGR compared to SD rats (Figure 4). Valsartan treatment reduced binding activity of NF-κB and AP-1 in the heart. The unrelated transcription factor, CAAT enhancer binding protein was used as control and showed no difference between dTGR and SD (data not shown). TaqMan analysis was performed to assess c-fos (Figure 5A) mRNA levels in dTGR hearts. Untreated dTGR show significantly increased c-fos mRNA levels. Valsartan reduced both c-fos expression. mRNA levels of the target gene was normalized for the housekeeping gene GAPDH.

Left Ventricular TF mRNA and Procoagulant Activity (PCA)

TaqMan analysis was performed to assess TF (Figure 5B) mRNA levels in left ventricle of dTGR. Untreated dTGR show significantly increased TF mRNA levels. Valsartan slightly reduced TF expression. mRNA levels of TF gene was normalized for the housekeeping gene GAPDH. However, PCA was not different between the groups when analyzed by ANOVA and Scheffé test. Analyzing median values untreated dTGR showed low levels of TF procoagulant activity per total protein 221 mU/mg (15–4286) compared to relative highest levels in SD rats (549 mU/mg; 25–3192). Valsartan-treated dTGR 280 mU/mg (54–954). These results are in agreement with relative low TF content in human myocardium with pressure-overloaded hearts. In the present study 6 out of 9 dTGR showed a lower PCA compared to the median of the non-transgenic group. Two untreated dTGR with signs of end stage organ damage showed extremely high PCA, indicating that the clotting hemostasis was impaired after microinfarctions. Whereas valsartan treatment inhibited p65 and TF in the vascular wall, valsartan mediated the reduction of hypertrophy, it rather increased PCA per total protein in extracts of the left ventricle. Fluctuation of PCA per total protein within the groups was relatively high, indicating an inhomogeneous distribution of TF in the left ventricle of dTGR.

NF-κB Subunit p65 and TF Expression in Coronary Vessels

We also analyzed p65 and TF expression at the protein level (Figure 6). Immunohistochemical analysis (phase contrast resolution) showed increased expression of the NF-κB p65 subunit in the endothelium, and smooth muscles cells of dTGR vessels, which was reduced by valsartan (Figure 6, A and B). No immunoreaction was observed in the vessel wall of nontransgenic SD rats (Figure 6C). The antibody recognizes an epitope overlapping the nuclear location signal of p65 subunit and therefore selectively stains released, activated NF-κB after dissociation from its inhibitor I-κBα.[18]

Beside TF staining in the myocardium, TF expression was increased in the endothelium and smooth muscle cells in dTGR (Figure 6D). The staining pattern of TF resembles the localization of p65 in the vessel wall. AT_1 receptor blockade reduced ANG II-induced TF expression (Figure 6E). No striking immunoreaction was observed in the vessel wall of nontransgenic SD rats (Figure 6F). Figure 7 shows a representative section of a dTGR heart with increased TF expression in the vessel wall and adventitia as well as infiltrated cells. TF immunostaining was markedly reduced by valsartan.

Analysis of the TF Promoter

To characterizes the effects of ANG II on human TF promoter activity, VSMC and CHO cells overexpressing the AT_1 receptor were transfected with various truncations of plasmids containing the human TF promoter (−244 to 121 bp, relative to the transcription start site) cloned upstream of a firefly luciferase reporter gene. Luciferase activity of cells transfected with the full-length TF promoter was increased 12-fold in VSMC (Figure 8)

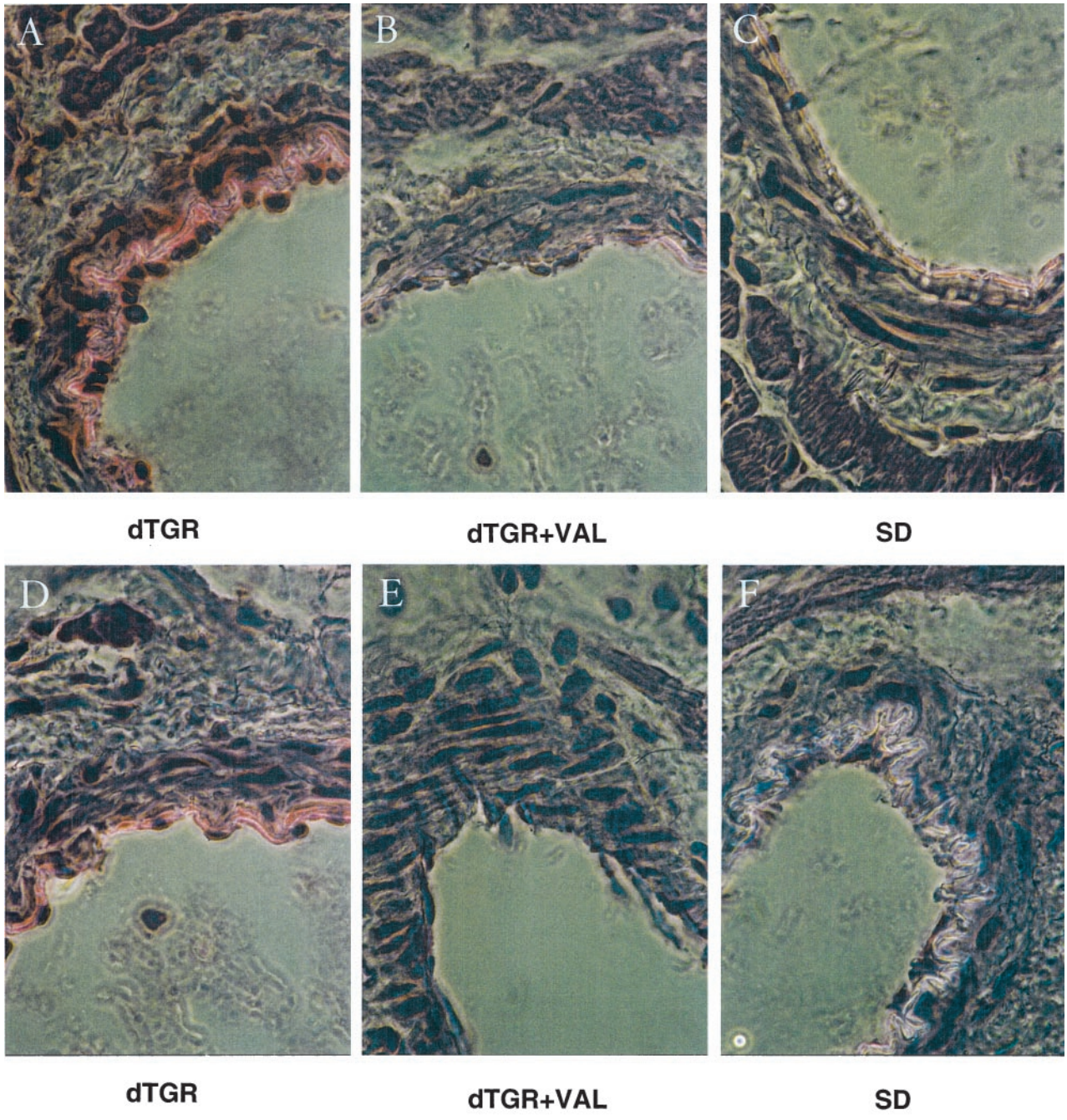

Figure 6. Immunohistochemical analysis (phase contrast resolution) shows the localization of the subunit p65 of NF-κB in a cardiac vessel. NF-κB activity was increased in the endothelium and smooth muscle cells of damaged dTGR vessels (**A–C**). The antibody only recognizes the active form of NF-κB after dissociation of its inhibitor I-κBα. **D–F:** Representative immunohistochemical photomicrographs of TF in dTGR cardiac vessels, valsartan-treated dTGR, and SD rats. TF expression was increased in the endothelium and smooth muscles. The staining pattern of TF resembles the localization of p65 in the vessel wall of dTGR. AT_1 receptor blockade markedly reduced ANG II-induced TF expression. Nontransgenic SD rats were used as negative controls (**C** and **F**).

and 11-fold in CHO-AT_1 (Figure 9), but not in CHO-WT (Figure 9), after 10^{-7} mol/L ANG II. Preincubation with 10^{-6} mol/L valsartan completely abolished luciferase activity. Deletion of both AP-1 and κB sites also abolished ANG II-induced TF gene transcription, whereas the pTF(−194)LUC promoter, which still contains the kB binding site but no AP-1 sites, still showed a fourfold induction of the TF gene in VSMC and CHO-AT_1. However, deletion of both AP-1 and NF-κB sites abolished ANG II-induced TF gene transcription completely in both cell lines. Furthermore, ANG II induced TF promoter in both transfected cell lines in a time- and dose-dependent manner (data not shown). Valsartan alone had no influence on luciferase activity.

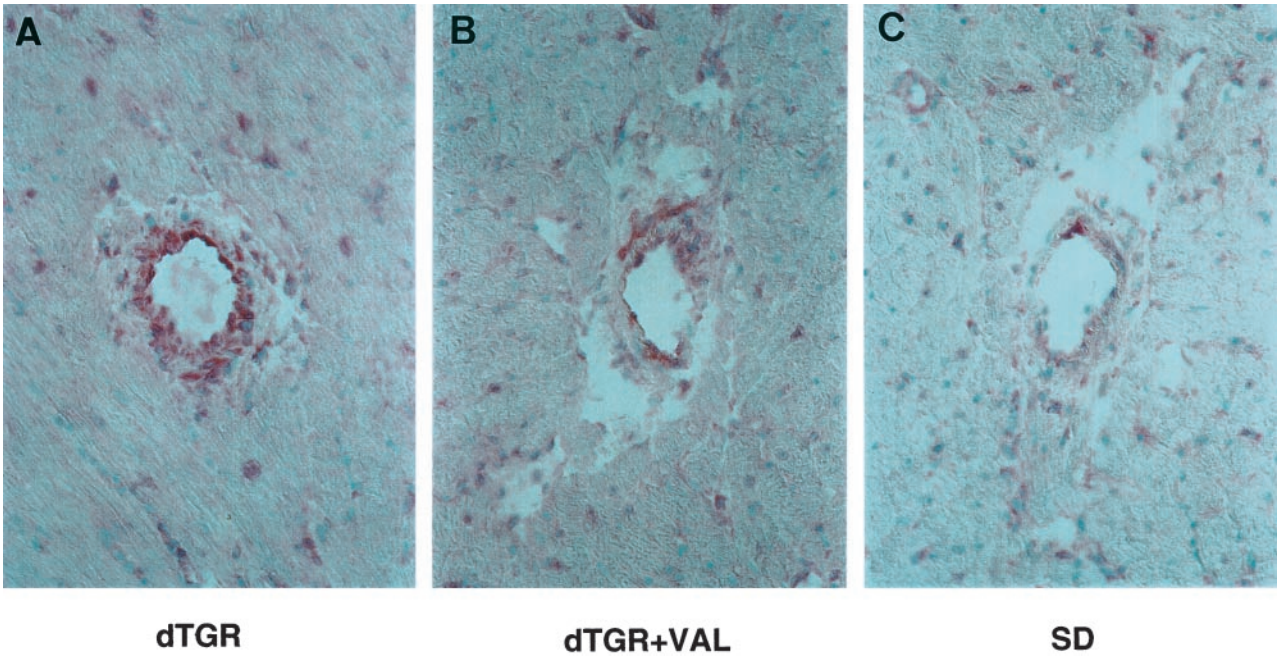

Figure 7. Representative immunohistochemical photomicrographs of TF in the heart of dTGR (**A**), valsartan-treated dTGR (**B**), and SD rats (**C**). TF expression was increased in the endothelium, smooth muscle cells, adventitia, and in infiltrating cells. TF expression was markedly reduced by valsartan.

Discussion

Cardiovascular disease is often associated with chronic inflammation, interstitial fibrosis, and occlusion of capillaries by fibrin. The interaction between the vasoconstrictor ANG II, inflammation, and stimulation of the clotting cascade, is not completely understood. No evidence has been presented that TF is generated in ANG II-induced cardiac vasculopathy *in vivo* and the various functions of TF in the heart are still controversial. We hypothesized that TF might be increased in ANG II-induced vasculopathy. TF is normally absent from circulating blood. Under certain circumstances, however, TF is known to be inducibly expressed by various vascular cells and to promote intravasal coagulation. Indeed, aberrant TF expression within the vascular bed has been associated with the intravascular clotting activation observed in a variety of inflammatory and immunological diseases.[23–25]

This study demonstrating expression of TF within the endothelium and media of coronary dTGR arteries suggests that TF may participate in the pathogenesis of ANG II-induced cardiac vasculopathy. In addition, our data provide both *in vitro* and *in vivo* evidence that inhibition of the transcription factors NF-κB and AP-1 by AT_1 receptor blockade reduces TF expression in vascular cells. In untreated dTGR, we found severe left ventricular hypertrophy with focal areas of necrosis, probably on the basis of fibrinoid necrosis and vascular occlusion. Chronic treatment with valsartan normalized systemic and coronary resistance and prevented cardiac vascular damage. Valsartan prevented inflammation, the activation of NF-κB, AP-1, and c-fos expression in dTGR hearts. TF immunoreactivity in the vessel wall was reduced by valsartan, similar to SD rats. We also investigated a putative mechanism responsible for ANG II induction of human TF gene. TF promoter analysis showed that deletion of both AP-1 and NF-κB sites also abolished ANG II-induced TF gene transcription, whereas the truncated promoter pTF(−194)LUC, which still contains the NF-κB binding site but no AP-1 sites, still showed a partial luciferase activity.

TF is known to possess properties independent of its coagulation capacity. Very recently, Luther et al[26] described TF localization in the myocardium. TF antigen was present in the transversal part of the intercalated disks, where it was colocalized with cytoskeletal proteins such as desmin and vinculin, but not with its coagulation factor VII. The microtopography of TF at cardiomyocyte contact sites and its distribution under physiological and pathophysiological conditions suggest that TF also plays a structural role in the maintenance of the cardiac muscle. In their study, patients with hypertension and left ventricular hypertrophy showed lower TF content compared to healthy patients. dTGR feature hypertension as well as cardiac hypertrophy. Since hypertrophied hearts show less intercalated disks per gram of tissue, it is quite likely that this was the reason for the lower PCA levels in dTGR compared to normal hearts.[26] In the present study, 6 out of 9 dTGR also showed a lower PCA compared to the median of the nontransgenic group. Two untreated dTGR with signs of end stage organ damage showed extremely high PCA, indicating that the clotting homeostasis was impaired after microinfarctions. Although valsartan inhibited p65 and TF in the vascular wall, valsartan increased PCA per total protein in extracts of the left ventricle. Therefore, we speculate that there may be an NF-κB-dependent and independent TF regulation in different compartments of the heart.

ANG II has various effects on function and the pathogenesis of ischemic heart disease. Blockade of the renin-

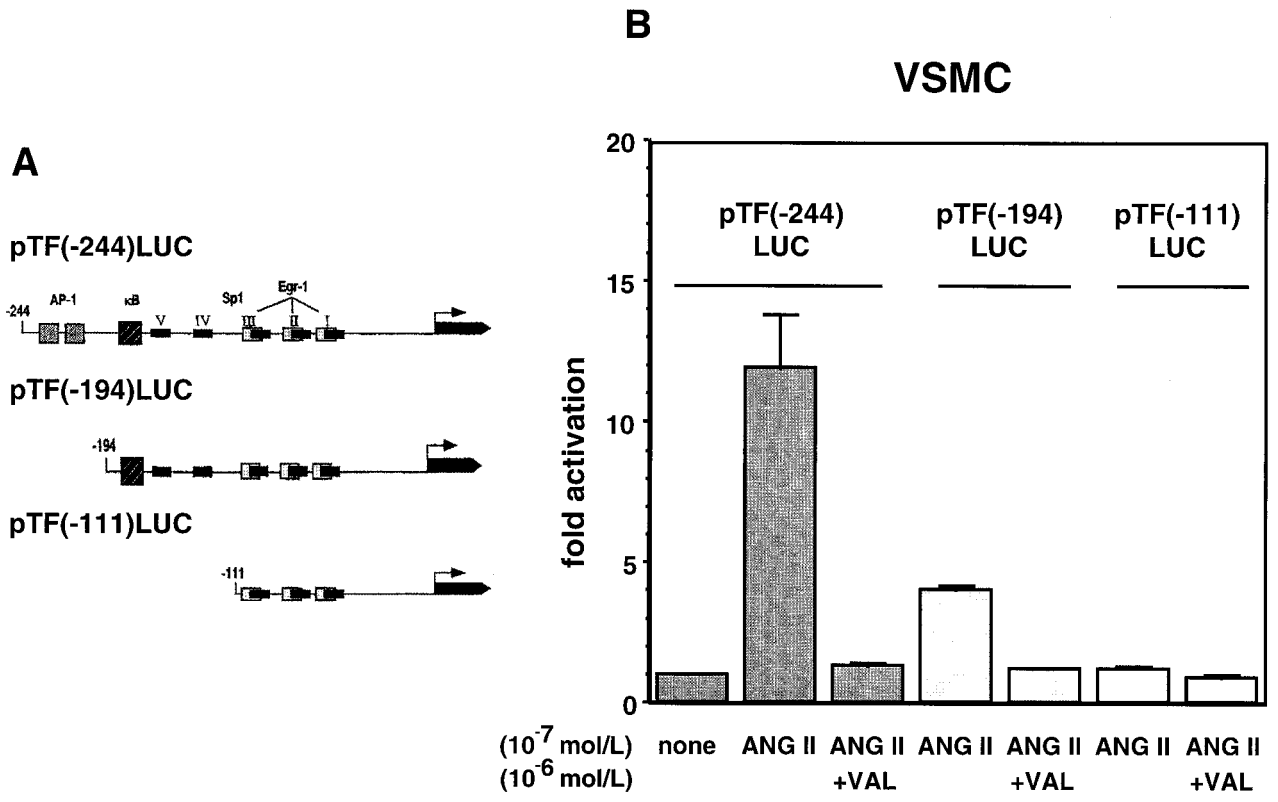

Figure 8. TF promoter activity induced by ANG II in human coronary smooth muscle cells (VSMC). **A** shows various deletion mutants of the human TF promoter. **B** shows the average fold induction of luciferase activity expressed by each plasmid in response to 10^{-7} mol/L ANG II with and without valsartan preincubation. The average fold induction of luciferase activity is expressed as percentage of unstimulated experiment. The measurements were performed in duplicate. Results are expressed as mean ± SE of 3 to 5 independent transfections.

angiotensin system has been shown to reduce the recurrence of cardiovascular events. However, the reduction cannot be completely explained by antihypertensive and antihypertrophic effects of the inhibitors. We suggest that ANG II exerts additional actions such as stimulation of inflammatory processes that may interact with the coagulation system. Nishimura et al[27] showed that ANG II increases plasminogen activator inhibitor-1 (PAI-1) and TF mRNA expression in cultured rat aortic endothelial cells, without changing their counterregulators, tissue type plasminogen activator and tissue factor pathway inhibitor. We have recently shown that ANG II also stimulated PAI-1 in dTGR kidneys, which was suppressed by blockade of the renin-angiotensin system.[28,29]

Little is known about activation and inhibition of NF-κB, AP-1, and TF in the heart. Morishita et al[30] showed that NF-κB inhibition by a decoy technique reduced the extent of myocardial infarction after reperfusion. Moreover, TF expression is increased in cardiac allograft vasculopathy and in cardiac and renal ischemic-reperfusion injury.[31–33] In our model, the surface adhesion molecule expression on the vascular wall of dTGR is reminiscent of histological findings observed in models of reperfusion injury. We also showed increased expression of the NF-κB subunit p65 in the endothelium, smooth muscles cells of dTGR vessels, and infiltrated cells. The staining pattern resembled the localization of the TF expression in the vessel wall. In addition to NF-κB, the transcription factor AP-1 is activated in inflammatory and proliferative processes. EMSA for NF-κB and AP-1 both showed increased DNA binding activity in dTGR hearts compared to SD. Although valsartan reduced cardiac NF-κB binding activity completely, AP-1 activity was only partially inhibited. The latter result was accompanied by suppressed c-fos mRNA and indicated additional jun/jun complex formation, accounting for AP-1 binding activity. Recently, Nemerson and coworkers showed that in addition to the scheme whereby coagulation is initiated after vessel damage and blood exposure to vessel wall-bound TF, even leukocytes are considered as a main source of blood-borne TF.[15,34] dTGR showed both vessel wall damage and leukocyte activation, both of which may have influenced TF activity.

We have recently shown that the inflammatory response in dTGR kidneys is associated with cell surface adhesion molecule expression, as well as their integrins LFA-1 and VLA-4, and can be prevented by blockade of the renin-angiotensin system.[28,29] The surface expression of adhesion molecules, as well as release of a number of macrophage products, serve to coordinate the local inflammatory responses. Within the vascular space, monocyte adherence to the endothelium stimulates monocyte TF expression. Monocyte/macrophage TF induces fibrin deposits, which contributes to the development of inflammation.[8] This process most likely contributes to local microvascular thrombosis,[9] which we also observed in this model.[24] Besides its important role in inflammation, integrin-matrix signaling is also known to

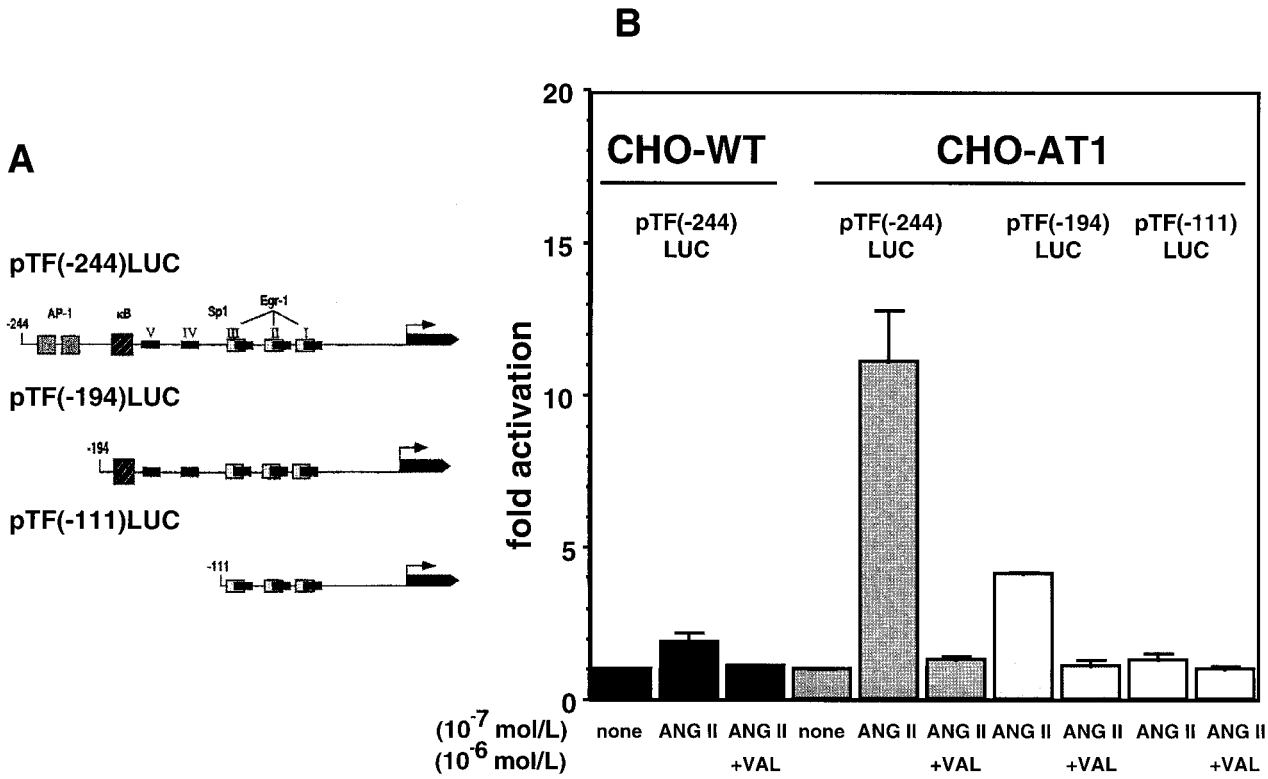

Figure 9. TF promoter activity induced by ANG II in CHO cells overexpressing the AT_1 receptor (CHO-AT_1) and the corresponding wild-type cell line (CHO-WT). **A** shows various deletion mutants of the human TF promoter. **B** shows the average fold induction of luciferase activity expressed by each plasmid in response to 10^{-7} mol/L ANG II with and without valsartan preincubation. The average fold induction of luciferase activity is expressed as percentage of unstimulated experiment. The measurements were performed in duplicate. Results are expressed as mean ± SE of 3 to 5 independent transfections.

influence coagulation.[35] We have previously shown that the AP-1-regulated matrix protein fibronectin is up-regulated in dTGR.[28,29] Several reports suggest that the β1 integrin VLA-4 induces monocyte procoagulant activity.[36,37] McGilvray et al[36] 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 hearts, which was reduced after AT_1 receptor blockade. We suggest that VLA-4/fibronectin signaling also stimulate TF expression via activation of AP-1 in our model. Recent data have demonstrated that TF not only initiates extrinsic coagulation, but also promotes cell adhesion and migration.[7] Thus, TF may participate in cell adhesion and migration, inflammation with the macrophage TF expression, and coagulation.

In summary, our data provide the first evidence that inhibition of the transcription factors NF-κB and AP-1 by AT_1 receptor blockade reduced TF expression in ANG II-induced cardiac vasculopathy. Chronic treatment with the AT_1 receptor blockade normalized systemic and coronary resistance and prevented cardiac damage. Valsartan prevented inflammation, the activation of NF-κB, AP-1, and c-fos expression in dTGR hearts. TF protein expression in the vessel wall were markedly reduced by valsartan, almost to control values. Analysis of the human TF promoter showed increased luciferase activity after ANG II stimulation. TF transcription was completely reduced by AT_1 receptor blockade and the deletion of both AP-1 and NF-κB sites in the TF promoter. In contrast, PCA of left ventricular extracts, representing TF content of myocardium as well as heart vessels, seem not to be reduced uniformly. Therefore, it is obvious that TF regulation in the heart is quite complex. Future studies will clarify the various functions of TF in the different compartments of the heart.

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References

1. Edgington TS, Mackman N, Brand K, Ruf W: The structural biology of expression and function of tissue factor. Thromb Haemost 1991, 66:67–79
2. Bugge TH, Xiao Q, Kombrinck KW, Flick MJ, Holmback K, Danton MJ, Colbert MC, Witte DP, Fujikawa K, Davie EW, Degen JL: Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci USA 1996, 93:6258–6263
3. Luther T, Flossel C, Mackman N, Bierhaus A, Kasper M, Albrecht S, Sage EH, Iruela Arispe L, Grossmann H, Strohlein A, Zhang Y, Nawroth PP, Carmeliet P, Loskutoff DJ, Muller M: Tissue factor expression during human and mouse development. Am J Pathol 1996, 149:101–113
4. Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlaenderen I, Demunck H, Kasper M, Breier G, Evrard P, Muller M,

Risau W, Edgington T, Collen D: Role of tissue factor in embryonic blood vessel development. Nature 1996, 383:73–75
5. Toomey JR, Kratzer KE, Lasky NM, Broze GJ Jr: Effect of tissue factor deficiency on mouse and tumor development. Proc Natl Acad Sci USA 1997, 94:6922–6926
6. Zhang Y, Deng Y, Luther T, Muller M, Ziegler R, Waldherr R, Stern DM, Nawroth PP: Tissue factor controls the balance of angiogenic and antiangiogenic properties of tumor cells in mice. J Clin Invest 1994, 94:1320–1327
7. Randolph GJ, Luther T, Albrecht S, Magdolen V, Muller WA: Role of tissue factor in adhesion of mononuclear phagocytes to and trafficking through endothelium in vitro. Blood 1998, 92:4167–4177
8. Clark EA, Brugge JS: Integrins and signal transduction pathways: the road taken. Science 1995, 268:233–239
9. Lo SK, Cheung A, Zheng Q, Silverstein RL: Induction of tissue factor on monocytes by adhesion to endothelial cells. J Immunol 1995, 154:4768–4777
10. Cui MZ, Parry GC, Edgington TS, Mackman N: Regulation of tissue factor gene expression in epithelial cells. Induction by serum and phorbol 12-myristate 13-acetate. Arterioscler Thromb 1994, 14:807–814
11. Bierhaus A, Zhang Y, Deng Y, Mackman N, Quehenberger P, Haase M, Luther T, Muller M, Bohrer H, Greten J, Martin E, Baeuerle PA, Waldherr R, Kisiel W, Ziegler R, Stern DM, Nawroth PP: Mechanism of the tumor necrosis factor alpha-mediated induction of endothelial tissue factor. J Biol Chem 1995, 270:26419–26432
12. Brisseau GF, Dackiw AP, Cheung PY, Christie N, Rotstein OD: Post-transcriptional regulation of macrophage tissue factor expression by antioxidants. Blood 1995, 85:1025–1035
13. Hall AJ, Vos HL, Bertina RM: Lipopolysaccharide induction of tissue factor in THP-1 cells involves Jun protein phosphorylation and nuclear factor kappaB nuclear translocation. J Biol Chem 1999, 274: 376–383
14. Orthner CL, Rodgers GM, Fitzgerald LA: Pyrrolidine dithiocarbamate abrogates tissue factor (TF) expression by endothelial cells: evidence implicating nuclear factor-kappa B in TF induction by diverse agonists. Blood 1995, 86:436–443
15. Taubmam MB, Marmur JD, Rosenfield CL, Guha A, Nichtberger S, Nemerson Y: Agonist-mediated tissue factor expression in cultured vascular smooth muscle cells: role of Ca2+ mobilization and protein kinase C activation. J Clin Invest 1993, 91:547–552
16. Mackman N, Morrissey JH, Fowler B, Edgington TS: Complete sequence of the human tissue factor gene, a highly regulated cellular receptor that initiates the coagulation protease cascade. Biochemistry 1989, 28:1755–1762
17. Bohlender J, Fukamizu A, Lippoldt A, Nomura T, Dietz R, Menard J, Murakami K, Luft FC, Ganten D: High human renin hypertension in transgenic rats. Hypertension 1997, 29:428–434
18. Zabel U, Henkel T, Silva MS, Baeuerle PA: Nuclear uptake control of NF-kappa B by MAD-3, an I kappa B protein present in the nucleus. EMBO J 1993, 12:201–211
19. Dechend R, Maass M, Gieffers J, Dietz R, Scheidereit C, Leutz A, Gulba DC: Chlamydia pneumoniae infection of vascular smooth muscle and endothelial cells activates NF-kappaB and induces tissue factor and PAI-1 expression: a potential link to accelerated arteriosclerosis. Circulation 1999, 100:1369–1373
20. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976, 72:248–254
21. Mackman N, Fowler BJ, Edgington TS, Morrissey JH: Functional analysis of the human tissue factor promoter and induction by serum. Proc Natl Acad Sci USA 1990, 87:2254–2258
22. Katz S, Kowenz Leutz E, Muller C, Meese K, Ness SA, Leutz A: The NF-M transcription factor is related to C/EBP beta and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells. EMBO J 1993, 12:1321–1332
23. Osterud B, Flaegstad T: Increased tissue thromboplastin activity in monocytes of patients with meningococcal infection related to an unfavourable prognosis. Thromb Haemost 1983, 49:5–7
24. Rao LV: Tissue factor as a tumor procoagulant. Cancer Metast Rev 1992, 11:249–266
25. Taylor FB, Jr., Chang A, Ruf W, Morrissey JH, Hinshaw L, Catlett R, Blick K, Edgington TS: Lethal E. coli septic shock is prevented by blocking tissue factor with monoclonal antibody. Circ Shock 1991, 33:127–134
26. Luther T, Dittert D-D, Kotzsch M, Erlich J, Albrecht S, Mackman N, Müller M: Functional implications of tissue factor localization to cell-cell contacts in myocardium. J Pathol 2000 (in press)
27. Nishimura H, Tsuji H, Masuda H, Nakagawa K, Nakahara Y, Kitamura H, Kasahara T, Sugano T, Yoshizumi M, Sawada S, Nakagawa M: Angiotensin II increases plasminogen activator inhibitor-1 and tissue factor mRNA expression without changing that of tissue type plasminogen activator or tissue factor pathway inhibitor in cultured rat aortic endothelial cells. Thromb Haemost 1997, 77:1189–1195
28. Luft FC, Mervaala E, Muller DN, Gross V, Schmidt F, Park JK, Schmitz C, Lippoldt A, Breu V, Dechend R, Dragun D, Schneider W, Ganten D, Haller H: Hypertension-induced end-organ damage: a new transgenic approach to an old problem. Hypertension 1999, 33:212–218
29. Mervaala EMA, Muller DN, Park J-K, Schmidt F, Breu V, Dragun D, Ganten D, Haller H, Luft FC: Monocyte infiltration and adhesion molecules in a rat model of high human renin hypertension. Hypertension 1999, 33:389–395
30. Morishita R, Sugimoto T, Aoki M, Kida I, Tomita N, Moriguchi A, Maeda K, Sawa Y, Kaneda Y, Higaki J, Ogihara T: In vivo transfection of cis element "decoy" against nuclear factor-kappaB binding site prevents myocardial infarction. Nat Med 1997, 3:894–899
31. Holschermann H, Bohle RM, Zeller H, Schmidt H, Stahl U, Fink L, Grimm H, Tillmanns H, Haberbosch W: In situ detection of tissue factor within the coronary intima in rat cardiac allograft vasculopathy. Am J Pathol 1999, 154:211–220
32. Golino P, Ragni M, Cirillo P, Avvedimento VE, Feliciello A, Esposito N, Scognamiglio A, Trimarco B, Iaccarino G, Condorelli M, Chiariello M, Ambrosio G: Effects of tissue factor induced by oxygen free radicals on coronary flow during reperfusion. Nat Med 1996, 2:35–40
33. Ushigome H, Yoshimura N, Sano H, Nakamura K, Oka T: Expression of tissue factor in renal ischemic-reperfusion injury of the rat. Transplant Proc 1998, 30:3764–3765
34. Nemerson Y, Giesen PL: Some thoughts about localization and expression of tissue factor. Blood Coagul Fibrinolysis 1998, 9:S45–47
35. Cicala C, Cirino G: Linkage between inflammation and coagulation: an update on the molecular basis of the crosstalk. Life Sci 1998, 62:1817–1824
36. McGilvray ID, Lu Z, Bitar R, Dackiw APB, Davreux CJ, Rotstein OD: VLA-4 integrin cross-linking on human monocytic THP-1 cells induces tissue factor expression by a mechanism involving mitogen-activated protein kinase. J Biol Chem 1997, 272:10287–10294
37. Fan ST, Mackman N, Cui MZ, Edgington TS: Integrin regulation of an inflammatory effector gene: direct induction of the tissue factor promoter by engagement of beta 1 or alpha 4 integrin chains. J Immunol 1995, 154:3266–3274