Dihydropyridines calcium antagonists ameliorate end-organ damage. In a nephrectomy model and in diabetic animals the drugs retarded the progression of glomerular disease and inhibited the development of glomerulosclerosis in experimental hypertension. The beneficial effects are a function of blood pressure reduction. However, dihydropyridines may influence the cellular mechanisms directly. In vitro studies provide supportive evidence. For instance, dihydropyridines suppress mesangial cell growth and chemokine expression. They also decreased vascular cell infiltrates and expression of adhesion molecules in endothelial cells. Dihydropyridines inhibit the activation of the protein kinases in vitro. Calcium signaling may be involved; however, we presented evidence indicating that other signal transduction systems such as protein kinase C are the drug targets. Thus, dihydropyridines could influence cellular mechanisms of renal damage via the intracellular signaling pathways and reduce the activity of downstream signaling. To test this hypothesis, we used an angiotensin (Ang) II-dependent transgenic rat model with both human renin and angiotensinogen genes. The rats develop moderate hypertension and severe renal and cardiac damage resulting in 50% mortality at age 7 weeks.

**Methods**

All procedures, as outlined elsewhere, were done according to guidelines from the American Physiological Society and were approved by local authorities. The lacidipine dTGR groups (n = 10 each) received the drug for 3 weeks by gavage once daily at two doses (0.3 or 3 mg/kg by gavage). Control dTGR (n = 10) and SD rats (n = 10) received vehicle (1% sodium carboxymethylcellulose). Systolic blood pressure was measured weekly by tail-cuff method under light ether anesthesia 20 hours after the last drug dose. Urine samples were 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 immunohistochemistry, western blot, and analysis of nuclear factor (NF)-κB and AP-1, the tissues were snap-frozen in liquid nitrogen, for immunohistochemistry in isopentane (−35°C), and stored at −80°C. Our histology, immunohistochemistry, Western blotting analyses, and electrophoretic mobility shift assay techniques have been described in detail elsewhere.

**Key Words:** Angiotensin II • nitric oxide synthase • calcium antagonists • transcription • cell adhesion molecules
Data are presented as means±SEM. Statistically significant differences in mean values were tested by ANOVA and the Tukey multiple range test. A value of $P < 0.05$ was considered statistically significant. The data were analyzed using SYSTAT® statistical software (SYSTAT Inc).

Results

Lacidipine was given at two dosages 0.3 mg/kg body (low lacidipine group) or 3 mg/kg body weight (high lacidipine group). Systolic blood pressure in the untreated transgenic animals at week 7 was 203±11 mm Hg, compared with 108±3 mm Hg in the wild-type SD control animals. Lacidipine in the high treatment group lowered blood pressure by 54% to 149±7 mm Hg ($P < 0.001$) compared with the untreated group) as shown in Figure 1 (top). In contrast, treatment in the low lacidipine group lowered blood pressure by only 9% to 185±10 mm Hg ($P < 0.05$) compared with the untreated group). The difference in blood pressure between the two groups was 36±14 mm Hg ($P < 0.05$). Despite the significant difference in blood pressure between the two treatment groups, the lower dose of lacidipine showed the same effect on albumin excretion as the higher lacidipine (bottom). Albuminuria in the untreated transgenic animals was 11±0.2 mg/d compared with 0.3±0.01 mg/d in the SD controls ($P < 0.01$). Lacidipine decreased albumin excretion to 3.1±0.2 mg/d in the low treatment group and to 3.6±0.03 mg/d in the high lacidipine group.

Vehicle-treated dTGR had severe renal damage with focal necrosis, and a 50% mortality at 7 weeks. Small vessels showed increased intimal and medial thickness as well as hyaline deposits. The renal tubules were frequently swollen and filled with proteinaceous material. Treatment with lacidipine prevented vascular injury in small renal vessels and extracellular matrix formation (data not shown). The beneficial effects of lacidipine were observed with both concentrations of lacidipine.

We quantitated the effects of lacidipine on neutrophils and monocytes as shown in Figure 2. Monocytes were present in the perivascular space and between the tubules. In contrast, granulocytes were mostly seen within the glomeruli and, to a lesser extent, in perivascular areas. Treatment with lacidipine prevented cell infiltration almost completely and only a few neutrophils were observed within glomeruli. Lacidipine had also an inhibitory effect on monocyte infiltration. Semiquantitative cell count analysis confirmed the significant reduction of both neutrophil and mononuclear cell infiltration after lacidipine treatment in kidney ($P < 0.01$).

We next investigated the effect of lacidipine on the expression of adhesion molecules, as shown in Figure 3 (top panels). Intercellular adhesion molecule-1 (ICAM-1) expression in the kidney was increased in the intima, adventitia, and in the perivascular space of the small vessels in untreated dTGR. Glomeruli and tubules showed increased ICAM-1 expression. As in the case of ICAM-1, treatment with lacidipine prevented the upregulation of VCAM-1 at both doses.

We next investigated the effects of lacidipine on iNOS expression as shown in Figure 3 (lower panels). We observed
a strongly increased expression in the glomeruli and in the vessel wall of renal arterioles from dTGR. Treatment with lacidipine greatly reduced the iNOS immunoreactivity both in the blood vessels and the glomeruli at both doses.

Since matrix expression is involved in scarring, we analyzed the effects of lacidipine on the expression of collagen IV and fibronectin. In untreated dTGR, collagen IV was observed in the peritubular space, as shown in Figure 4. Treatment with lacidipine at both doses prevented the increased expression of collagen IV almost completely, and no significant difference between the lacidipine-treated animals and the SD control animals was observed at week 7. We also observed an increased expression of fibronectin in the renal interstitium. Lacidipine reduced the increased expression of fibronectin significantly at both doses.

We studied MAP kinase activation by using specific antibodies that only detect the phosphorylated, active form of the enzyme (p-ERK). In untreated dTGR, phosphorylated MAP kinase was increased in the glomeruli and in the peritubular space (data not shown). A similar increase was also present in the medulla. Treatment with lacidipine at either dose abolished this staining pattern both in the cortex and in the medulla.

We next analyzed the activation of the transcription factors NF-κB and AP-1 since both regulate ICAM-1 and iNOS gene expression, as shown in Figure 5. Both, NF-κB and AP-1 showed greatly increased activity in the kidneys of dTGR compared with SD rats. Lacidipine-treatment reduced the increased levels of NF-κB and AP-1 activity. This effect was evident at either dose.

Discussion

We tested the hypothesis that the lipophilic calcium antagonist lacidipine ameliorates renal failure and inhibits inflammatory changes independent of its blood pressure-lowering activities. We used a transgenic animal model of angiotensin II-mediated organ damage and analyzed the effects of lacidipine on adhesion molecule expression, cell infiltration, iNOS expression, and matrix molecule expression. In addition, we investigated whether specific intracellular signaling
pathways are influenced by lacidipine. We showed that lacidipine reduces NF-κB activation, prevents inflammatory responses, and altogether ameliorates renal damage. Our animal model features hypertension, albuminuria, severe inflammatory changes with renal damage and focal necrosis, and a 50% mortality at 7 weeks.\textsuperscript{20} We have previously demonstrated that Ang II is responsible for these effects.\textsuperscript{21} Our findings strongly suggest that lacidipine influences the angiotensin-induced cellular mechanisms directly in a blood pressure-independent manner.

Chronic treatment with a high dosage of lacidipine led to a decrease in blood pressure compared with animals treated with a lower concentration of the dihydropyridine. The decrease in blood pressure with lacidipine may have contributed to the renoprotective effect of the dihydropyridine. However, we do not believe that the blood pressure lowering effect of lacidipine had a major effect because effective antihypertensive treatment by hydralazine, reserpine, and hydrochlorothiazide in our model only partially prevented tissue damage and did not reduce inflammation.\textsuperscript{22}

Treatment with the calcium antagonist lacidipine inhibited the inflammatory process in our animal model considerably. Lacidipine almost abolished neutrophil infiltration. We have previously shown that the specific inhibition of ICAM-1 prevents leukocyte infiltration in the kidney thus ameliorating tissue injury.\textsuperscript{23,24} Neutrophils are important mediators of injury in many inflammatory diseases.\textsuperscript{25,26} The prevention of neutrophil infiltration by lacidipine may partially explain the anti-inflammatory effects of dihydropyridines in our model. Furthermore, lacidipine inhibited the expression of the endothelial cell adhesion molecule ICAM-1. Because ICAM-1 is responsible for the neutrophil adhesion to the endothelium and subsequent infiltration,\textsuperscript{25} it is most likely that lacidipine inhibited neutrophil infiltration via inhibition of ICAM-1. This assumption is supported by earlier findings from Cominacini and coworkers that lacidipine inhibits the ICAM-1 expression in endothelial cells in vitro.\textsuperscript{13,16} Others have also demonstrated that dihydropyridines affect direct cell infiltration and macrophage activation in vivo.\textsuperscript{27–29} We have recently shown that dihydropyridines also lead to down-regulation of ICAM-1 and VCAM and influence endothelial cell permeability.\textsuperscript{30,31}

We also demonstrated that monocyte infiltration was reduced by lacidipine. Several reports have demonstrated the participation of monocyte/macrophages in the onset and progression of various renal diseases.\textsuperscript{32} Since monocytes play a role in long-term pathological changes and chronic renal failure and have been associated with matrix accumulation and fibrosis,\textsuperscript{33} our findings may implicate that lacidipine has a direct influence on these processes. It has recently been shown that dihydropyridines improve long-term outcome in renal transplantation,\textsuperscript{34} and the positive effect of lacidipine on chronic vascular changes in the kidney may partially be due to its direct effects on monocyte/neutrophil infiltration.

Lacidipine greatly reduced iNOS expression in the dTGR. Nitric oxide (NO) regulates numerous physiological processes, including smooth muscle contractility, platelet reactivity, and the cytotoxic activity of leukocytes. Because of the ubiquitous nature of NO, inappropriate release of this mediator has been linked to the pathogenesis of a number of disease states.\textsuperscript{35} While NO serves beneficial roles as a messenger and host defense molecule, excessive NO production can be cytotoxic. The result of NO’s reaction with reactive oxygen and nitrogen species, leads to peroxynitrite anion formation, protein tyrosine nitration, and hydroxyl radical production. NO may contribute to the evolution of several commonly encountered renal diseases, including immune-mediated glomerulonephritis, postischemic renal failure, radiocontrast nephropathy, obstructive nephropathy, and acute and chronic renal allograft rejection.\textsuperscript{36} NO synthase inhibitors are potentially beneficial in the treatment of conditions associated with an overproduction of NO, including inflammation.\textsuperscript{37,38} It is therefore likely that the inhibition of the increased NO expression by lacidipine plays an important role in the anti-inflammatory effect of this compounds.

Lacidipine reduced the increased activity of the transcription factors NF-κB and AP-1 considerably. NF-κB is the main factor in the transcription of NOS, ICAM-1 and VCAM-1 and lacidipine could influence expression of these molecules through inhibition of NF-κB.\textsuperscript{39} Cominacini and coworkers have previously shown that lacidipine inhibits lipopolysaccharide-induced NF-κB activation in human mesangial cells in vitro.\textsuperscript{16} Activation of NF-κB could occur through several mechanisms. Ruiz-Ortega et al showed that ANG II stimulated NF-κB in mesangial cells and that activation of NF-κB in the renal cortex was reduced by ACE inhibition.\textsuperscript{40} Another possibility is the activation of NF-κB through oxygen free radicals. Reactive oxygen species represent an important signal transduction pathway inside the cell and also participate in the expression of adhesion molecules on the endothelial cells.\textsuperscript{41,42} Our results indicate that dihydropyridines interfere with the MAP kinase activation proximal of NF-κB activation.

Mostly, the effects of dihydropyridines have been explained via their inhibitory effects on the L-type calcium channel. Recently, several other hypotheses have been postulated. We have observed an inhibitory effect of calcium antagonists on protein kinase C activity without any change in intracellular free calcium concentration.\textsuperscript{19} The calcium antagonist-mediated effects on gene expression were observed at pharmacological concentrations that are one to two orders of magnitude lower than those required for inhibition of depolarization-induced opening of voltage sensitive L-type calcium channels. Similar observations have been made by other groups.\textsuperscript{11,19} Recently, Orth et al investigated the effects of calcium antagonists in mesangial cells and could not associate the observed inhibitory effects on cell proliferation with changes in intracellular calcium.\textsuperscript{42}

In summary, we have shown that the dihydropyridine lacidipine ameliorates angiotensin II–induced renal disease independent of its blood pressure lowering effect. In a model of severe organ damage with endothelial cell activation, leukocyte infiltration and sclerosis, we observed an inhibitory effect of lacidipine on adhesion molecule expression, leukocyte infiltration, iNOS activation and matrix molecule expression. We could also demonstrate that intracellular pathways such as MAP kinase and activation of transcription factors were influenced by lacidipine. It remains open whether the
observed effects are mediated by calcium channel inhibition or whether other cellular effector mechanisms play a role in the beneficial therapeutic effect of lacidipine.

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