

## Cellular and molecular mechanisms of tissue protection by lipophilic calcium channel blockers

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**ABSTRACT** Long-acting third-generation dihydropyridine calcium channel blockers (CCBs) improve endothelial dysfunction and prevent cardiovascular events in humans, but their cellular and molecular mechanisms of tissue protection are not elucidated in detail. We assessed organ (renal) protection by the highly lipophilic CCB lercanidipine in a double-transgenic rat (dTGR) model with overexpression of human renin and angiotensinogen genes. We randomly treated dTGR with lercanidipine (2.5 mg/kg/day;  $n=20$ ) or vehicle ( $n=20$ ) for 3 wk. Furthermore, we explored the influence of lercanidipine on protein kinase C (PKC) signaling *in vivo* and *in vitro* using endothelial and vascular smooth muscle cell cultures. Cumulative mortality was 60% in untreated dTGR, whereas none of the lercanidipine-treated animals died ( $P<0.001$ ). We found significantly less albuminuria and improved renal function in lercanidipine-treated dTGR (both  $P<0.05$ ). Lercanidipine treatment also significantly ( $P<0.05$ ) reduced blood levels of the endogenous NOS inhibitor asymmetric dimethylarginine. On histological examination, we observed significantly less tissue inflammation and fibrosis in lercanidipine-treated animals (both  $P<0.05$ ). Lercanidipine significantly inhibited angiotensin (ANG) I-mediated PKC- $\alpha$  and - $\delta$  activation *in vivo* and *in vitro*, partly due to reduced intracellular calcium flux. As a result, lercanidipine improved endothelial cell permeability *in vitro*. Lercanidipine prevents tissue injury and improves survival in a model of progressive organ damage. These effects may result, at least in part, from inhibition of tissue inflammation as well as improved NO bioavailability. Modulation of PKC activity may be an important underlying intracellular mechanism.—Menne, J., Park, J.-K., Agrawal, R., Lindschau, C., Kielstein, J. T., Kirsch, T., Marx, A., Muller, D., Bahlmann, F. H., Meier, M., Bode-Böger, S. M., Haller, H., Fliser, D. Cellular and molecular mechanisms of tissue protection by lipophilic calcium channel blockers. *FASEB J.* 20, 000–000 (2006)

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DIHYDROPYRIDINE CALCIUM CHANNEL blockers (CCBs) are widely used therapeutics in cardiovascular medicine (1). Their predominant vascular action is inhibition of calcium channels in vascular smooth muscle cells (VSMCs), and the resulting decrease in the VSMCs intracellular  $Ca^{2+}$  concentration causes relaxation of the vessel wall (2,3). In experimental models of hypertension and/or renal damage, dihydropyridine CCBs including lercanidipine retard the progression of glomerular injury, i.e., glomerulosclerosis (2,4). This beneficial effect is thought to be, at least in part, a result of blood pressure reduction, but dihydropyridines also have direct cellular effects that are unrelated to their effect on blood pressure. For instance, they suppress mesangial cell growth and chemokine production and decrease the expression of adhesion molecules in glomerular endothelial cells *in vitro* (5). Dihydropyridine CCBs also diminish the intracellular activation of protein kinases, probably as a result of altered calcium signaling. Thus, it is plausible to assume that these compounds modify cellular mechanisms of renal injury via intracellular pathways and diminished down-stream signaling.

We have tested this hypothesis in an angiotensin (ANG) II-dependent model of organ (renal) damage in which both human renin and angiotensinogen genes are overexpressed, i.e., in double-transgenic rats (dTGR). These rats develop severe ANG II-mediated organ damage resulting in 50% mortality as early as 7 wk of age (6–8). Previous studies exploring this model have revealed that ANG II induces inflammation, increases collagen deposition, and stimulates smooth muscle cell proliferation. Molecular mechanisms mediating these changes include activation of various signaling pathways such as protein kinase C (PKC), mitogen-activated protein kinases, and RhoA/Rho kinase. We have randomly treated dTGR with the long-acting

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highly lipophilic third-generation CCB lercanidipine or vehicle and thoroughly analyzed the influence of lercanidipine on ANG II-mediated mechanisms of renal injury. Moreover, we have performed ancillary *in vitro* experiments to elucidate its action on the molecular concentration.

## MATERIALS AND METHODS

### Protocol and laboratory measurements

The Animal Care Committee of Lower Saxony approved the study protocol. We assessed baseline blood pressure and renal function in 4-wk-old dTGR. Thereafter, the animals received in random order 2.5 mg/kg lercanidipine ( $n=20$ ) or vehicle ( $n=20$ ) for 3 wk once daily. Lercanidipine and the vehicle were given to each rat individually on a biscuit to minimize variability of dosage intake. In addition, 10 Sprague-Dawley (SD) rats received vehicle. We measured systolic blood pressure weekly 20 h after administration of the last dose in conscious animals by using the tail-cuff method (TSE-blood pressure system). For this measurement, animals were kept at 37°C.

For measurement of albumin excretion, urine samples were collected over a 24 h period in metabolic cages. Albuminuria was quantified by using a commercially available ELISA kit (Nephro-Rat, Exocell). In addition, blood was collected from the retro orbital region and after centrifugation serum creatinine concentration was measured using the Creatinine Analyzer 2 (Beckman). We also measured asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and L-arginine in serum collected immediately before the death of the animals. ADMA is a potent endogenous NOS inhibitor, whereas SDMA is the stereoisomer of ADMA with no direct inhibitory effect on NOS. It is excreted by the kidney and increases in parallel with decline in glomerular filtration rate. The amino acid L-arginine is the precursor substance for NO. These compounds were analyzed with a recently developed liquid chromatography-mass spectrometry (LC-MS) method, as described in detail elsewhere (9).

### Organ preparation and immunohistochemistry

Seven-week-old rats were euthanized under anesthesia after 3 wk of treatment, and the kidneys were removed. The kidneys were washed in cold saline, blotted dry, and weighted. The left kidney was fixed for 20 h in 3% paraformaldehyde in Soerensen's phosphate buffer and was then paraffin embedded. Immunohistochemistry was performed on 2  $\mu\text{m}$  paraffin sections using primary polyclonal antibodies against rat inducible NOS (iNOS; ABR, Golden, CO) and fibronectin (Paesel+Lorei, Frankfurt, Germany) and a monoclonal mouse antirat ED1 antibody (Ab) for monocyte/macrophage (Serotec, Oslo, Norway).

### Real-time quantitative PCR in renal tissue extracts

We analyzed the mRNA content of dimethylarginine dimethylaminohydrolase (DDAH), i.e., the enzyme metabolizing ADMA, in whole kidney extracts from SD rats and from untreated and lercanidipine-treated dTGR. For real-time qPCR, 2  $\mu\text{g}$  of DNase-treated total RNA were reverse transcribed using a mix of random hexamers and oligo(dT)<sub>12-15</sub> oligonucleotides (Stratagene) and Superscript II Reverse

Transcriptase (Invitrogen). QPCR was performed on a SDS 7700 system (Applied Biosystems) using 10 ng of transcribed RNA, Rox dye as internal control (Invitrogen), FastStart taq Polymerase (Roche Diagnostics), and gene-specific primers in combination with SYBR-green chemistry (Molecular Probes). PCR amplification was carried out at 10 min 95°C, 40 cycles at 10 s 95°C, and 1 min at 60°C. Specificity of the amplification product was verified by melting curve analysis. For each group, three RNA samples were used. For normalization of the samples, distribution of 18S rRNA was measured. Quantification was carried out using qgene software. Primers were designed using Primer Express software (Applied Biosystems) based on Unigene clusters or Genbank accession numbers respectively (given in parenthesis). The primer sequences are as follows: (5'-3'-direction): r18S (NC\_001665), ACATC-CAAGGAAGGCAGCAG (Primer 1) and TTTTCGTCACTACTCCCCG (Primer 2); and DDAH II, CCAATCATCGAG-GAGCTGAGAT (Primer 1) and TCAGTCAGTGCCATTGC (Primer 2).

### Measurement of calcium signaling in cells and immunocytochemistry

Human umbilical vein endothelial cells (HUVECs) were loaded with fluo 3-acetoxymethyl ester (AM; 5  $\mu\text{M}$ ) for 15 min at 37°C. After being washed twice with buffer, the cells were again incubated for 15–20 min at 37°C. Measurements of calcium signaling were done on a confocal microscope. HUVECs loaded with fluo 3-AM were stimulated by addition of ANG II to the cell culture medium (final concentration  $10^{-7}$  M). The same experiment was performed after preincubation of the cells for 60 min with lercanidipine ( $10^{-7}$  M). Measurements were performed in three independent cell preparations. Each was stimulated 15 times.

Because PKC isoforms play a central role in several endothelial cell functions, such as adhesion and permeability, we tested the hypothesis that the calcium antagonist lercanidipine influences PKC activation. ANG II was administered with a concentration of  $10^{-7}$  M in the culture medium. HUVECs were fixed after different time intervals and stained for PKC. For immunocytochemistry, HUVECs were fixed with 4% paraformaldehyde and permeabilized with ice-cold 80% methanol. After incubation with 3% skimmed milk in PBS (SM/PBS) for 60 min, the preparations were incubated for 1 h at room temperature with the mouse or rabbit anti-PKC-antibody (PKC- $\delta$ , Transduction laboratories, cat Nr.P36520; PKC- $\alpha$ , UBI). The preparations were washed three times with PBS and then exposed to the secondary Ab (Dianova, Cy2 labeled) for 60 min. For confocal microscopy, the preparation was mounted with Aqua Poly/Mount. A Bio-Rad MRC 1024 confocal imaging system with an argon/krypton laser at 488 nm excitation was used. At least 25–30 cells from three independent experiments were examined under each experimental condition. Images were acquired in the normal scanning mode with a Kalman filter of 3.

### Transfection of cells with GFP and confocal microscopy of PKC-GFP translocation

Transient transfection was carried out using SuperFect. Rat VSMCs were seeded on fourwell glass chamber slides for 1 day before transfection and washed twice with PBS without  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$ . They were subsequently incubated with SuperFect, 0.5  $\mu\text{g}$  plasmid-DNA/well in culture medium for 3 h at 37°C, and 5%  $\text{CO}_2$  and washed twice with PBS without  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$ . After the transfection, VSMCs were cultured at 31.5°C to obtain optimal fluorescent intensity of GFP. The fluorescence

of GFP-tagged PKC proteins (10) was monitored 24 h after transfection.

PKC-GFP fluorescence was measured using an argon/krypton laser at 488 nm excitation and a Nikon Flour 40× (numerical aperture 1.15) water immersion lens. Transfected cells were washed twice with HEPES buffer (5 mM HEPES, pH 7.3, 135 mM NaCl, 5.4 mM KCl, and 5 mM glucose) containing 1.5 mM CaCl<sub>2</sub>. Afterward, the cells were incubated for at least 2 to 4 min in HEPES buffer with 1.5 mM CaCl<sub>2</sub> for adaptation to the medium. The cells expressing PKC-GFP (10–40% of the total cell population) were analyzed with confocal microscopy. PKC-GFP signals were collected using the Bio-Rad time-course software function with single line excitation (488 nm) in different, stimuli-dependent time intervals (1, 5, 10, 15, 20, up to 90 s) between images (>15 cells in 3 independent transfections were analyzed).

### Western blotting

The excised organs were divided with forceps and scissors. The renal tissue was treated with cold buffer [50 mM Tris-HCl, pH 7.4, 2% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 8.8 mM egtazic acid (EGTA), 25 mM leupeptin, and 5 mM phenylmethylsulfonyl acid (PMSF)] and immediately homogenized by sonification. Afterward, the tissue was spun at 1,600 g for 15 min to remove cell debris. The homogenate was then spun in a TLA 100–2 rotor (Beckman) at 100,000 rpm for 10 min, and the supernatant was used as the cytosolic fraction. The pellet was resuspended in buffer containing 1.0% Triton X-100 and shaken at 4°C for 30 min. The homogenate was centrifuged at 100,000 rpm for another 10 min and then used as the particulate fraction. After determination of protein content, the immunoblotting was carried out as previously described (11). Briefly, 60 µg of each sample were run on a 10% polyacrylamide gel and electroblotted onto a poly screen polyvinylidene difluoride (PVDF) transfer membrane (NEN, Boston, MA). The membrane was then treated according to a commercially available protocol (Serva, Heidelberg, Germany). The membrane was incubated with an Ab directed against the catalytic region of PKC-α (UBI). A final incubation was carried out with peroxidase conjugated antimouse IgG (Pierce Chemicals, Oud-Beijerland, Netherlands). Visualization was achieved by chemiluminescence (Renaissance; DuPont, Boston, MA).

### Permeability measurements *in vitro*

It has been shown that PKC-α activation is an important mediator of endothelial permeability (12). Because PKC-α activation was inhibited by lercanidipine, we tested if lercanidipine also modulates the permeability of a confluent endothelial layer. For these experiments, we used porcine endothelial cells from the aorta and studied the permeability across the endothelial cell monolayer in a two-compartment system separated by a filter membrane as described previously (12). In brief, both compartments contained modified Tyrode's solution. There was no hydrostatic pressure gradient between both compartments. Trypan blue-labeled albumin (50 µM) was added to the luminal compartment. The appearance of trypan blue-labeled albumin in the abluminal compartment was continuously monitored by pumping the liquid through a two-wavelength photometer (Specord S10, Zeiss; Jena, Germany; wavelength for measurement of trypan blue 580 nm, control wavelength 720 nm). The albumin flux (F, expressed as mol·s<sup>-1</sup>·cm<sup>-2</sup>) across the monolayer with the surface area S was determined from the rise of albumin concentration.

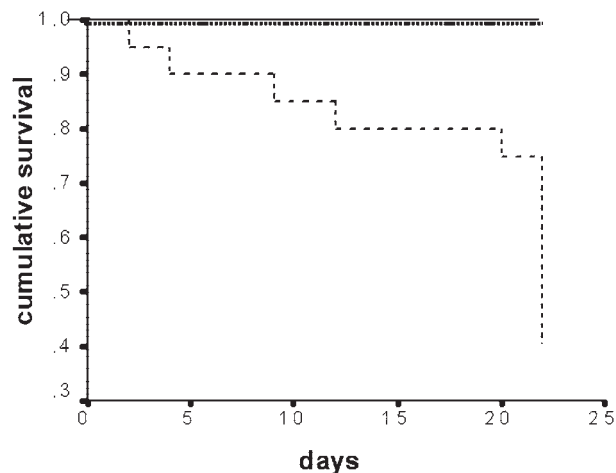
### Statistical analysis

We used the statistical Packages for the Social Sciences (SPSS) package (SPSS 11.51 for Windows). We compared baseline and end point characteristics between the three groups of animals in the *in vivo* experiment with ANOVA and appropriately corrected *t* tests for random data. Differences were considered significant at *P* < 0.05; data are shown as mean ± SE (blood pressure results ± SD). Furthermore, we analyzed survival in untreated and in lercanidipine-treated dTGR with a log-rank analysis and created Kaplan-Meier survival curves. Data from *in vitro* experiments were analyzed with appropriate tests.

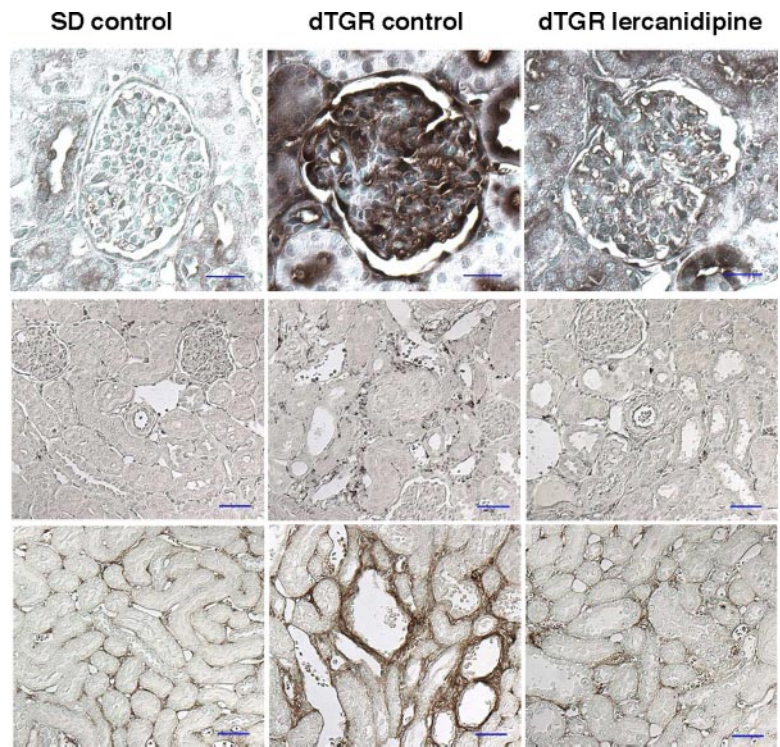
## RESULTS

### Animal survival, renal function, and albumin excretion rate

Cumulative mortality was 60% in untreated dTGR, whereas no lercanidipine-treated animal died (Fig. 1); this difference was highly significant (*P* < 0.001). No SD rat died during the observation period as well. Baseline systolic blood pressure was 163 ± 4 mmHg in lercanidipine-treated dTGR, 167 ± 9 mmHg in control dTGR, and 124 ± 4 mmHg in SD rats. At the end of the 3 wk study period, systolic blood pressure was significantly higher (*P* < 0.05) in surviving untreated dTGR (260 ± 19 mmHg) as compared with lercanidipine-treated dTGR (215 ± 20 mmHg). Systolic blood pressure in SD rats was significantly lower (*P* < 0.01) throughout the observation period than in both groups of dTGR. At the end of the study, serum creatinine was significantly lower in SD rats (28 ± 2 µmol/l) and treated dTGR (36 ± 5 µmol/l) as compared with surviving untreated dTGR (66 ± 5 µmol/l; both *P* < 0.05 vs. untreated dTGR). In addition, albumin excretion rate was 0.24 ± 0.05 mg/24 h in SD rats. It was sevenfold higher in the untreated dTGR (1.70 ± 0.16 mg/24 h), whereas ler-



**Figure 1.** Kaplan-Meier survival curves in treated (grey line) and untreated dTGR (dotted curved line) rats and in control SD rats (dotted straight line). Difference in survival between both groups of dTGR was highly significant (*P* < 0.001).



**Figure 2.** Representative immunohistochemical photomicrographs of iNOS staining (*upper panel*), monocyte infiltration (*middle panel*), and fibronectin expression (*lower panel*) in kidney tissue of control SD rats, and of untreated and lercanidipine-treated dTGR. We observed a marked increase of iNOS expression in the glomeruli and in the vessel wall of renal arterioles from dTGR. Treatment with lercanidipine reduced iNOS immunoreactivity both in blood vessels and in the glomeruli. Similarly, lercanidipine treatment reduced monocyte infiltration and fibronectin expression in renal tissue of dTGR.

canidipine treatment significantly reduced albuminuria in dTGR ( $0.81 \pm 0.1$  mg/24 h;  $P < 0.01$  vs. untreated dTGR).

### Renal histology and immunohistochemistry

On histological examination, untreated dTGR had severe renal damage with focal necrosis and arteriolar hyalinosis. 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 lercanidipine prevented vascular injury in small renal vessels and extracellular matrix formation (data not shown). We observed markedly increased iNOS expression in glomeruli and in vessel walls of renal arterioles from dTGR (**Fig. 2**). Treatment with lercanidipine reduced the iNOS immunoreactivity both in blood vessels and in the glomeruli. Monocytes (ED-1 positive) were present in the perivascular space and between the tubules (**Fig. 2**). In renal tissue of SD rats,  $5 \pm 1$  monocytes were observed per microscopic field as compared with  $26 \pm 1$  in untreated dTGR. Lercanidipine treatment significantly prevented cell infiltration, and we observed only

$12 \pm 2$  monocytes per microscopic field ( $P < 0.05$  vs. untreated dTGR). Since matrix expression is involved in scarring, we analyzed the effects of lercanidipine on the expression of fibronectin in kidney tissue. We observed an increase in fibronectin expression in renal interstitium of untreated dTGR, and administration of lercanidipine abolished the increased expression of fibronectin (**Fig. 2**).

### ADMA, SDMA, and L-arginine blood levels and DDAH mRNA tissue levels

Mean ADMA blood concentration in untreated dTGR was 35% percent higher than in control SD rats (**Table 1**); the difference was statistically significant. Administration of lercanidipine significantly reduced ADMA blood levels in treated dTGR. In contrast, no differences between groups were observed with respect to L-arginine blood levels, whereas SDMA blood levels were significantly higher in untreated dTGR than in SD rats and treated dTGR (**Table 1**). The DDAH mRNA levels in kidney tissue were lower in untreated dTGR as compared with SD rats and lercanidipine-treated

TABLE 1. Mean ADMA blood concentration

|                                  | SD rat           | Untreated dTGR          | Treated dTGR    |
|----------------------------------|------------------|-------------------------|-----------------|
| L-Arginine ( $\mu\text{mol/l}$ ) | $190 \pm 17$     | $209 \pm 52$            | $225 \pm 50$    |
| ADMA ( $\mu\text{mol/l}$ )       | $0.83 \pm 0.032$ | $1.09 \pm 0.04^*$       | $0.93 \pm 0.01$ |
| SDMA ( $\mu\text{mol/l}$ )       | $0.24 \pm 0.06$  | $0.37 \pm 0.14^\dagger$ | $0.23 \pm 0.04$ |
| DDAH (relative units)            | $1.00 \pm 0.31$  | $0.66 \pm 0.2$          | $0.86 \pm 0.28$ |

\* $P < 0.05$  vs. SD rat;  $^\dagger P < 0.01$  vs. SD rat and lercanidipine-treated dTGR.

dTGR, but the difference did not reach statistical significance ( $P=0.055$ ).

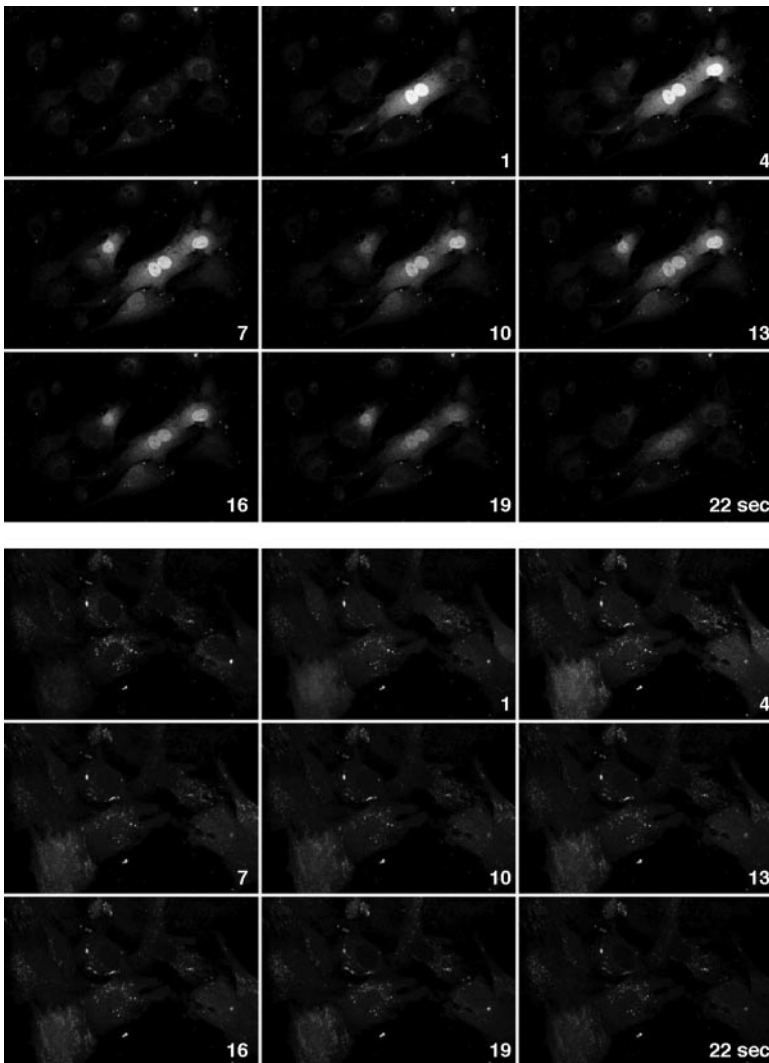
### Effects of lercanidipine on ANG II-mediated intracellular and molecular effects

**Figure 3** shows HUVECs loaded with fluo 3-AM and stimulated by addition of ANG II to the cell culture medium. Within 1 s, a marked calcium uptake into the cytoplasm and the nucleus was visible (*upper panel*). After 22 s, the calcium levels were nearly back to normal. If the same experiment was performed after preincubation of the cells with lercanidipine, the calcium uptake was markedly reduced and the uptake was delayed (*lower panel*). The maximal intracellular calcium concentration was reached after  $\sim 4$  s.

HUVECs stained for PKC- $\alpha$  are presented in **Fig. 4**. ANG II induced a strong activation of nuclear PKC- $\alpha$  after 1 min, which decreased after 5 min. After 20 min, the nuclear activation had disappeared (data not shown). Pretreatment with lercanidipine for 90 min not only reduced the initial activation of PKC- $\alpha$  but also diminished the nuclear response to ANG II,

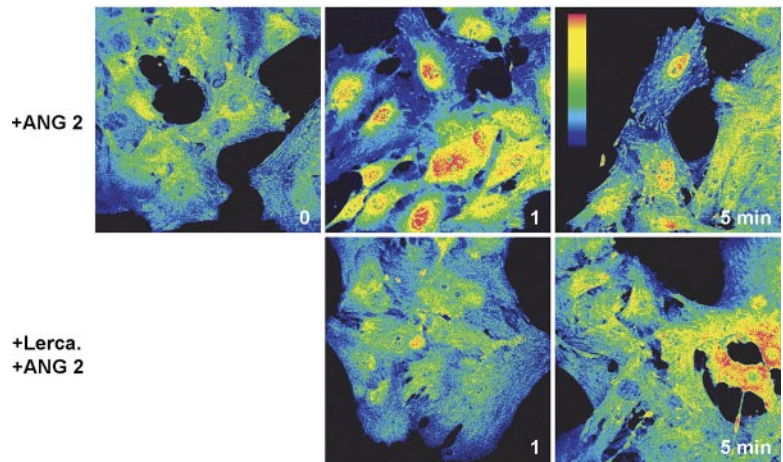
which can be easily seen after 5 min. Lercanidipine not only influenced calcium-dependent PKC isoform but also other PKC isoforms. ANG II induced a strong activation of nuclear PKC- $\delta$  after 1 min, which was back to normal after 5–10 min (**Fig. 5**). Pretreatment with lercanidipine for 90 min reduced the initial response of PKC- $\delta$  but prolonged the nuclear activation of PKC- $\delta$ . To visualize PKC movement after exposure of rat VSMCs with ANG II, we analyzed translocation of PKC-GFP using confocal microscopy. Addition of ANG II at a concentration of  $10^{-7}$  M to the culture medium induced movements of PKC- $\alpha$ -GFP along the cytoplasmic membrane, resulting in a punctuated pattern after 45 s (**Fig. 6**). Preincubation with lercanidipine for 90 min inhibited this movement substantially. For PKC- $\delta$ -GFP, a strong punctuated pattern appeared within a second after cell stimulation with ANG II (**Fig. 7**). These aggregations were also abolished after preincubation of cells with lercanidipine.

To analyze the effects of lercanidipine on PKC translocation *in vivo*, we performed Western blots from the cytoplasm and particulate/membrane fraction of renal



**Figure 3.** Confocal microscopy of ANG II-induced changes in intracellular distribution of calcium at 0–22 s in endothelial cells with (*lower panel*) and without (*upper panel*) addition of lercanidipine.  $[Ca^{2+}]_i$  is not only visible in cytoplasm but is also prominent within nucleus.

#### Effect of Lercanidipine on Angiotension 2-induced PKC- $\alpha$ Activation



**Figure 4.** Confocal microscopy of PKC- $\alpha$  immunoreactivity at baseline (time point 0 min) and 1 and 5 min after exposure of HUVECs to ANG II with (*lower panel*) or without (*upper panel*) lercanidipine preincubation. Signal intensity is indicated by a color scale, where red color is highest intensity, i.e., PKC- $\alpha$  activation.

tissue. We found a significantly ( $P < 0.05$ ) lower membrane association of PKC- $\alpha$  in lercanidipine-treated dTGR in comparison to untreated dTGR (**Fig. 8**).

#### Effects of lercanidipine on endothelial permeability

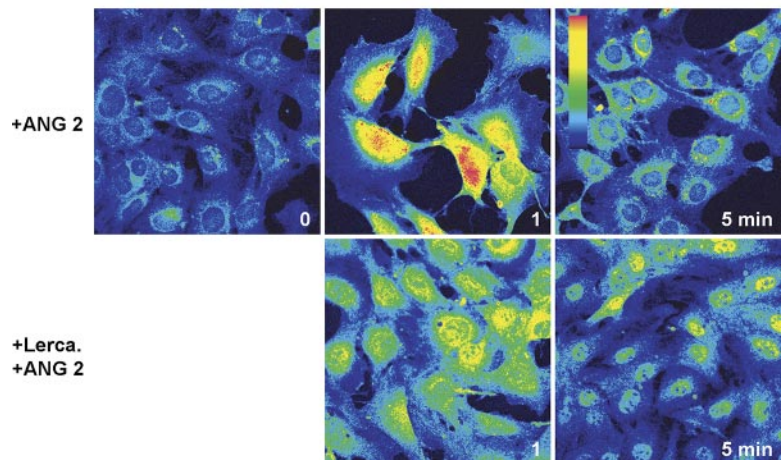
Administration of potassium-cyanate and desoxy-glucose leads to ATP depletion in the endothelium, which resembles an ischemic condition. After their administration, we observed a marked increase of the albumin flux across the endothelial cell layer that peaked after 30 min (**Fig. 9**). This increase was reduced by 50% ( $P < 0.05$ ) when lercanidipine was added to the cell culture medium at a concentration of  $10^{-8}$  M.

#### DISCUSSION

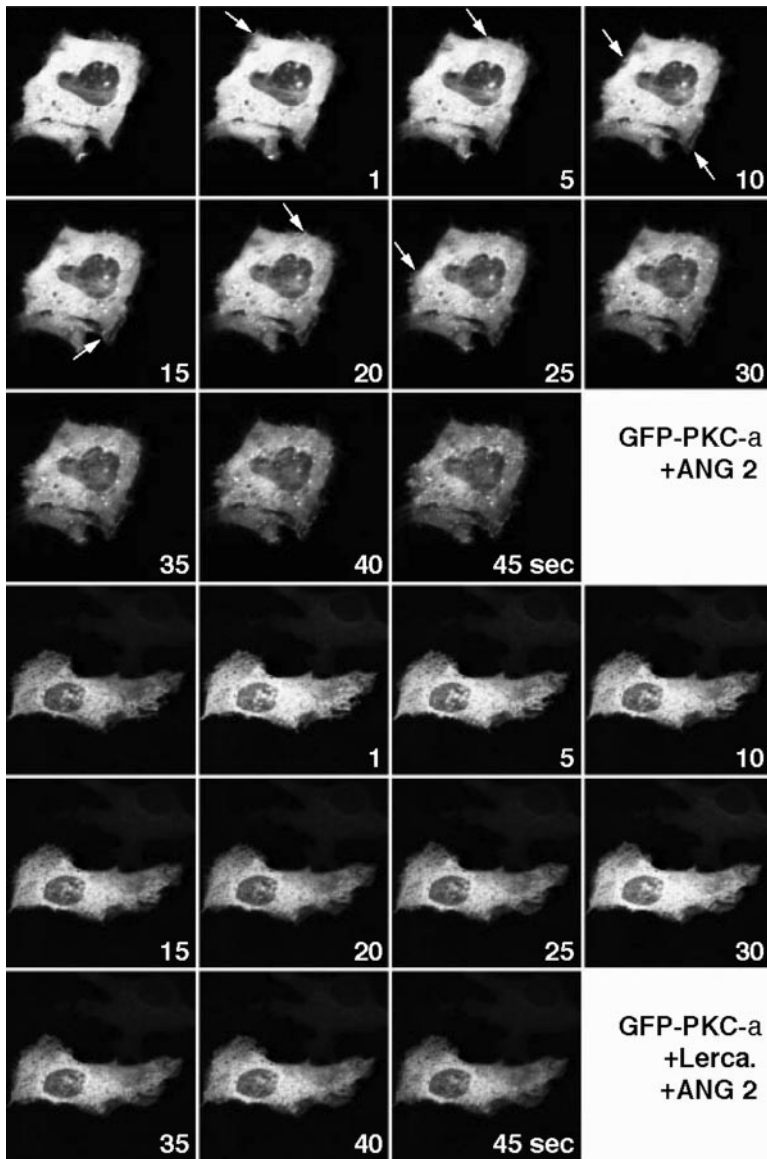
In the present study, we explored cellular and molecular mechanisms by which lipophilic CCBs may affect endothelial function, prevent tissue injury, and hence improve survival in a transgenic animal model of ANG II-mediated organ damage. For this purpose, we assessed the effect of the highly lipophilic third-genera-

tion CCB lercanidipine on renal function and tissue histology, particularly on inflammatory cell infiltration, iNOS expression, and expression of the matrix molecule fibronectin. We could demonstrate that administration of lercanidipine significantly reduced tissue inflammation and tubulo-interstitial fibrosis. These changes in renal histology were accompanied by preserved renal function and reduced albuminuria in lercanidipine-treated dTGR, resulting in improved outcome. Several previous reports have revealed that participation of monocyte/macrophages is crucial for the initiation and perpetuation of progression in various models of renal injury including that mediated by ANG II (13,14). Monocyte infiltration in progressive renal diseases has been associated with matrix accumulation and fibrosis, and our data support the notion that lercanidipine prevents this unfavorable scenario. Furthermore, in an experimental setting lercanidipine reduced expression of intracellular adhesion molecules (ICAM) and E-selectin in HUVECs stimulated with tumor necrosis factor (TNF)- $\alpha$  (15). We could extend this finding by demonstrating that lercanidipine also inhibits P-selectin expression in TNF-alpha stimulated HUVECs (data not shown).

#### Effect of Lercanidipine on Angiotension 2-induced PKC- $\delta$ Activation



**Figure 5.** Confocal microscopy of PKC- $\delta$  immunoreactivity at baseline (time point 0 min) and 1 and 5 min after exposure of HUVECs to ANG II with (*lower panel*) or without (*upper panel*) lercanidipine preincubation. Signal intensity is indicated by a color scale, where red color is highest intensity, i.e., PKC- $\delta$  activation.

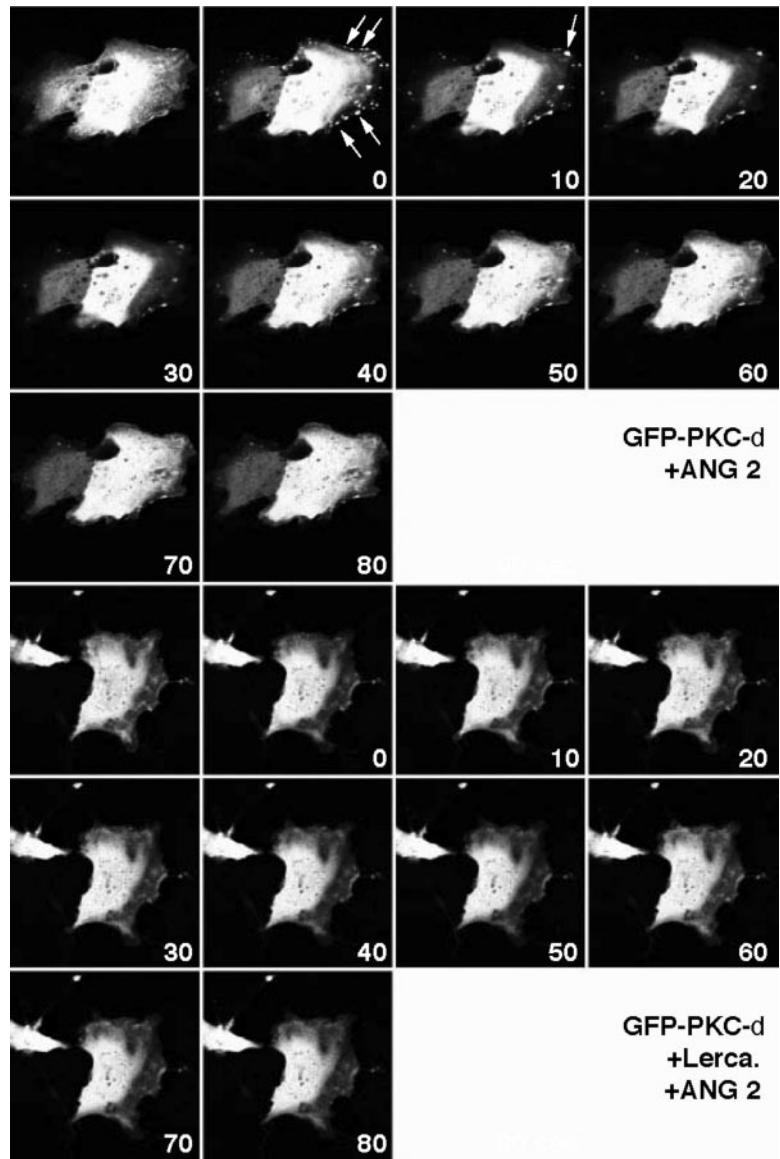


**Figure 6.** Confocal microscopy showing PKC- $\alpha$ -GFP translocation 1–45 s after exposure to ANG II with (*lower panel*) and without (*upper panel*) lercanidipine preincubation in VSMCs. Arrows indicate accumulation of PKC- $\alpha$  along cell membrane.

We provide first *in vitro* evidence that the beneficial effects of long-acting lipophilic CCBs may result, at least in part, from inhibition of PKC. Among the various signaling pathways that mediate intracellular effects of ANG II, PKC isoforms have been shown to be activated in several cell types, e.g., VSMCs, endothelial, and mesangial cells (16–18). We analyzed the effect of lercanidipine on PKC- $\alpha$  and PKC- $\delta$  activation by ANG II in HUVECs and on translocation using GFP-tagged PKC- $\alpha$  and PKC- $\delta$  isoforms in transfected VSMCs under a confocal laser scanning fluorescent microscope. We have monitored the movement of this fusion protein after addition of lercanidipine and could clearly demonstrate inhibition of the ANG II-mediated translocation of PKC- $\alpha$  and PKC- $\delta$ . That inhibition of PKC activation and translocation to cellular membranes by lercanidipine might also be relevant *in vivo* is supported by our observation that less PKC- $\alpha$  is found in the particulate/membrane fraction of renal tissue of lercanidipine-treated dTGR. The effect of lercanidipine on PKC activation and translocation may explain some

of the observed clinical findings in our animal model of ANG II-mediated organ damage. For example, increased PKC- $\alpha$  activity is thought to be responsible for the enhanced leakage of the endothelial cell layer for albumin (12,19). Indeed, we were able to show that administration of lercanidipine reduces the albumin permeability of ischemic endothelial cells. Furthermore, it has been demonstrated that PKC- $\alpha$  activation is involved in the development of albuminuria (20), cardiac hypertrophy and fibrosis (21), and heart failure (22,23). *In vitro* PKC- $\alpha$  interacts with L-selectin (24) and stimulates ICAM mRNA and iNOS expression (25,26). PKC- $\delta$  is involved in the development of pulmonary fibrosis (27). Furthermore, PKC- $\delta$  enhances ICAM and vascular cell adhesion molecule expression *in vitro* (28,29). Finally, some of the beneficial renal effects of ANG-converting enzyme (angiotensin I-converting enzyme) inhibitors seem to result from PKC inhibition *in vivo* (30).

We have also demonstrated that lercanidipine interferes with the calcium influx into endothelial cells, and



**Figure 7.** Confocal microscopy showing PKC- $\delta$ -GFP translocation 0–80 s after exposure to ANG II with (*lower panel*) and without (*upper panel*) lercanidipine preincubation in VSMCs. Arrows indicate focal (“clumpy”) accumulation of PKC- $\delta$  beneath cell membrane.

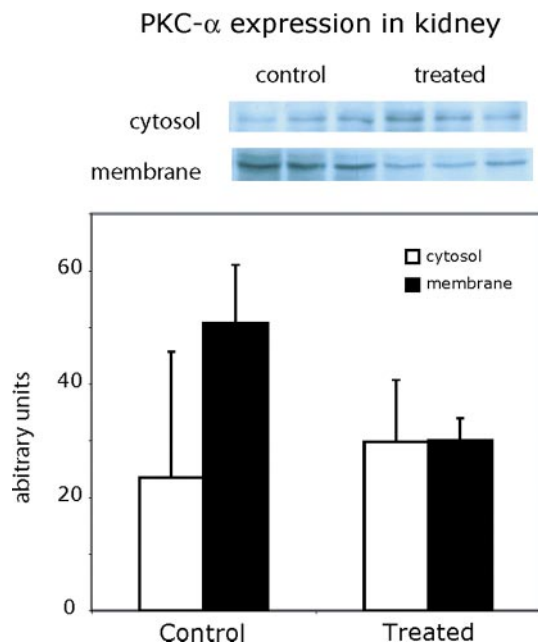
this could be a pivotal mechanism by which CCBs modulate PKC activation in endothelial cells and probably also in VSMCs (31). However, this action would only explain the observed effects on classical (calcium sensitive) PKC isoforms, such as PKC- $\alpha$ . Another putative pathway by which highly lipophilic CCBs may modulate PKC activity is reduced release of phospholipid components such as diacylglycerol from the plasma membrane (32). It previously has been shown that these phospholipids may affect signaling of classical and novel PKC isoforms (33).

In the present experimental setting, we have unfolded a novel mechanism by which lipophilic dihydropyridine CCBs may exert organ protection. Treatment with lercanidipine increased DDAH activity and reduced ADMA blood levels in dTGR. This endogenous NOS inhibitor has attracted much attention in cardiovascular medicine recently, and its role in endothelial dysfunction, atherosclerosis, and cardiovascular mortality has been studied in various clinical conditions such as hypertension, renal disease, insulin resistance, pe-

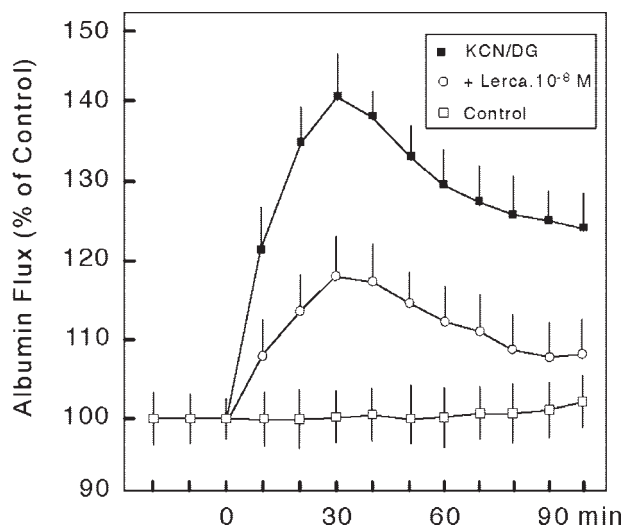
ripheral vascular disease, and acute coronary syndrome (34–40). We have recently demonstrated that ADMA blood concentrations encountered in these conditions inhibit NO production, impair cardiac and renal function, and increase blood pressure in healthy subjects (36). Thus, chronically elevated ADMA blood levels may contribute to endothelial and tissue injury resulting from reduced (local) NO availability. In clinical studies, dihydropyridine CCB improved endothelial dysfunction in hypertensive patients and in patients with manifest atherosclerosis (41). The effect is thought to be, at least in part, the result of increased NO bioavailability, but the mechanism(s) whereby CCB modulate NO metabolism have not been elucidated in detail. Dihydropyridine CCBs seem to increase NOS activity *in vitro* and *in vivo* and thus have antiatherogenic properties (41). In addition, Taddei et al. have shown that lercanidipine restores NO availability also through an antioxidant action (42). Our data support the notion that dihydropyridine CCBs may also influence the NO pathway by modulation of ADMA blood

levels and thus the inhibitory effect of ADMA on NOS. We further provide evidence that lercanidipine treatment modulates DDAH activity. This intracellular enzyme is a key regulator of ADMA metabolism. In transgenic animals harboring an additional human DDAH gene, significantly lower ADMA blood levels and higher tissue NO bioavailability have been documented (43). In this respect, the high lipophilicity of lercanidipine could be advantageous, since it passes more easily through the cell membrane to interact with DDAH. Interestingly, we were able to show that lercanidipine also reduces iNOS expression in tissue of the dTGR. Results from experimental studies have revealed that DDAH activity can be directly regulated by S-nitrosylation of its active site by NO, thereby creating a regulatory feedback loop NO production, DDAH activity, and ADMA blood levels (44). The implication of this finding is that under conditions of increased NO production such as in inflammation, where iNOS generates abundant NO, S-nitrosylation diminishes DDAH activity, and this in turn would lead to accumulation of ADMA and to NOS inhibition. Therefore, ADMA blood levels are postulated to be an excellent indicator of vascular and tissue damage as a result of (micro) inflammation (40). Lercanidipine may interrupt this vicious cycle by inhibiting iNOS via inhibition of PKC- $\alpha$  (26) and thus increasing DDAH activity or by directly inhibiting DDAH, or both.

In summary, we present data that the highly lipophilic third-generation dihydropyridine CCB lercanidipine prevents ANG II-induced renal injury and improves survival by reducing tissue inflammation and fibrosis. These beneficial effects may result from impor-



**Figure 8.** Western blot analysis of PKC- $\alpha$  bound to membranes or contained in cytoplasmic fraction in kidney tissue of in lercanidipine-treated and untreated dTGR. Treatment with lercanidipine significantly ( $P < 0.05$ ) reduced the amount of membrane bound PKC- $\alpha$ .



**Figure 9.** Potassium-cyanate (KCN)/desoxy-glucose (DG-2)-induced ischemia increased albumin flux across the endothelial cell monolayer. Data are %control; 100% corresponds to permeability of  $4.8 \pm 0.5 \times 10^{-6}$  cm/s of barrier formed by endothelial monolayer and filter support after 30 min of incubation. Ischemia increased permeability within minutes; the increase reached its maximum at 30 min. Addition of lercanidipine markedly reduced albumin flux.

tant intracellular actions of lercanidipine such as inhibition of PKC activation and modulation of DDAH activity. FJ

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