The T- and L-Type Calcium Channel Blocker (CCB) Mibefradil Attenuates Leg Edema Induced by the L-Type CCB Nifedipine in the Spontaneously Hypertensive Rat: A Novel Differentiating Assay

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Received November 12, 2007; accepted March 5, 2008

ABSTRACT

Among the L-type calcium channel blockers (CCBs), particularly dihydropyridines like nifedipine [1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester], a common adverse effect is vasodilatory edema. Newer CCBs, such as the T- and L-type CCB, mibefradil [(1S,2S)-2-[2][3-(2-benzimidazolyl)propyl]methylaminoethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxyacetate dihydrochloride hydrate], demonstrate antihypertensive efficacy similar to that of their predecessors but seem to have a reduced propensity to cause edema. Using a magnetic resonance imaging (MRI) T2 mapping technique, we investigated the ability of mibefradil to reduce extracellular water accumulation caused by the L-type CCB, nifedipine, in the hindleg skeletal muscle of the spontaneously hypertensive rat. Mibefradil (10 mg/kg i.v.) and nifedipine (1 mg/kg i.v.) lowered mean arterial blood pressure by 97 ± 5 and 77 ± 4 mm Hg, respectively. MRI edema index (expressed as percentage increase of integral T2 over predrug control) was significantly higher with nifedipine (2606 ± 86%; p < 0.05) than with mibefradil (981 ± 171%) measured 30 to 60 min after the start of drug infusion. The hindleg edema caused by nifedipine was dose dependently decreased by coadministration of mibefradil (0, 0.3, or 3 mg/kg). The hindleg edema formation was not due to albumin leakage into the interstitial space based on immunostaining. However, a 4.2-fold increase in the arterial L-/T-type CC mRNA expression ratio was observed compared with the venous L/T ratio as shown by quantitative reverse transcription polymerase chain reaction. These results demonstrate the novel utility of MRI to measure extravascular water after acute exposure to CCBs and indicate that T-type CCB activity may reduce L-type CCB-induced vasodilatory edema in the skeletal muscle vasculature, possibly by a differential effect on arteriole and venule dilatation.

Calcium channel blockers comprise a class of powerful, well tolerated, and safe antihypertensive agents that are widely used either alone or as a key component of combination therapy for hypertension. It is unfortunate that a common adverse effect is vasodilatory edema. Among the L-type calcium channel blockers (CCBs), particularly dihydropyridines like nifedipine [1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester], a common adverse effect is vasodilatory edema. Newer CCBs, such as the T- and L-type CCB, mibefradil [(1S,2S)-2-[2][3-(2-benzimidazolyl)propyl]methylaminoethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxyacetate dihydrochloride hydrate], demonstrate antihypertensive efficacy similar to that of their predecessors but seem to have a reduced propensity to cause edema. Using a magnetic resonance imaging (MRI) T2 mapping technique, we investigated the ability of mibefradil to reduce extracellular water accumulation caused by the L-type CCB, nifedipine, in the hindleg skeletal muscle of the spontaneously hypertensive rat. Mibefradil (10 mg/kg i.v.) and nifedipine (1 mg/kg i.v.) lowered mean arterial blood pressure by 97 ± 5 and 77 ± 4 mm Hg, respectively. MRI edema index (expressed as percentage increase of integral T2 over predrug control) was significantly higher with nifedipine (2606 ± 86%; p < 0.05) than with mibefradil (981 ± 171%) measured 30 to 60 min after the start of drug infusion. The hindleg edema caused by nifedipine was dose dependently decreased by coadministration of mibefradil (0, 0.3, or 3 mg/kg). The hindleg edema formation was not due to albumin leakage into the interstitial space based on immunostaining. However, a 4.2-fold increase in the arterial L-/T-type CC mRNA expression ratio was observed compared with the venous L/T ratio as shown by quantitative reverse transcription polymerase chain reaction. These results demonstrate the novel utility of MRI to measure extravascular water after acute exposure to CCBs and indicate that T-type CCB activity may reduce L-type CCB-induced vasodilatory edema in the skeletal muscle vasculature, possibly by a differential effect on arteriole and venule dilatation.
renin-angiotensin-aldosterone system inhibitors such as angiotensin-converting enzyme inhibitors or angiotensin-receptor blockers to the treatment regimen (Messerli, 2001; Weir et al., 2001). Diuretics may remediate the edema state somewhat, but at the expense of further reducing plasma volume. Traditional measures such as limiting the amount of time that a patient is upright and/or considering use of graduated compression stockings are useful adjunctive therapies.

Not all CCBs seem to have the same propensity for causing edema. For example, in the COHORT study, two lipophilic CCBs, lercanidipine and lacidipine, in elderly hypertensives caused edema in 9 and 4% of patients, respectively, compared with 19% for amloidipine (Leonetti et al., 2002). The mechanism for the reduced incidence of edema with more lipophilic CCBs like lercanidipine, lacidipine, and mibefradil still remains unclear.

Mibefradil (Brogden and Markham, 1997), which is structurally and pharmacologically different from traditional calcium antagonists, recently has been shown in clinical studies to reduce the incidence of leg edema to 5% compared with 26 and 17% for the dihydropyridines, amlodipine and nifedipine, respectively (Kobrin, 1997). Mibefradil represents a new generation of CCBs that blocks both the T- and L-type Ca$^{2+}$ channels, whereas the dihydropyridines selectively block L-type Ca$^{2+}$ channels. Previous work has shown that the L-type Ca$^{2+}$ channel binding affinities for nifedipine and mibefradil, measured at the α1C subunit, had a $K_i$ value of 8.2 and 156 nM, respectively (Huber et al., 2000). The T-type Ca$^{2+}$ channel (i.e., the α1H subunit) binding affinities determined electrophysiologically for nifedipine and mibefradil had $IC_{50}$ values of >10 μM and 140 nM, respectively, with mibefradil having a 10 to 15-fold preference for the T-type versus the L-type Ca$^{2+}$ channel (Martin et al., 2000). These reported Ca$^{2+}$ channel affinity data illustrate the difference in binding capabilities between nifedipine and mibefradil and establish the proposed mechanism in the edema formation between nifedipine and mibefradil.

To explain the functional differences of T- and L-type Ca$^{2+}$ channels in vascular tissue, previous studies have demonstrated the differential dilatory effects of CCBs on arteries and veins (Harris et al., 1980; Magnon et al., 1995; Ozawa et al., 2001). Feng et al. (2004) have recently shown that CCBs with combined T- and L-type activity such as pimozide and mibefradil equally dilate efferent and arteriolar arterioles in the renal circulation and provide protection against hypertensive glomerular injury, unlike L-type CCBs. These studies are consistent with the notion that CCBs with T-type activity have an attenuated ability to cause peripheral edema due to their equal vasodilatory effects on pre- and postcapillary vessels. The molecular mechanism involved may be dependent on the differential T- and L-type Ca$^{2+}$ channels in arterial and venous tissue.

In this study, we examined whether MRI could be used to quantitatively detect CCB-induced peripheral edema in a well-characterized model of hypertension, the spontaneously hypertensive rat (SHR), compared with the normotensive rat. After establishing that peripheral edema can be measured by this technique, we examined whether mibefradil could attenuate edema caused by nifedipine. The functional MRI results indicate that, at equally antihypertensive doses, mibefradil caused less vasodilatory edema than nifedipine, which is consistent with a mechanism that may equalize hydrostatic pressure across the capillary bed by decreasing venous resistance. This equalized capillary hydrostatic pressure may be due to either directly having equal T-type Ca$^{2+}$ channel expression on the arterial and venous sides of the capillary or indirectly through a simple reduction in L-type Ca$^{2+}$ channel expression on the arterial side.

### Materials and Methods

#### Materials

Mibefradil and nifedipine were obtained from Sigma-Aldrich (St. Louis, MO). Intravenous solutions of both drugs were prepared using a cardiovascular-inert vehicle consisting of 5% N-methylpyrrolidone/45% polyethylene glycol 400/50% 50 mM lactic acid.

#### Animal Preparation

The animal handling and imaging procedures were approved by the Pfizer Institutional Animal Care and Use Committee according to the Institute of Laboratory Animal Resources (1996). Thirty-eight SHRs (360 ± 65 g) and six Sprague-Dawley (SD) normotensive rats (450 ± 50 g) were anesthetized with isoflurane in O$_2$ [3% (v/v)] induction in a chamber followed by 1–1.5% (v/v) maintenance via a nose mask. Catheters (PE50) were surgically placed into the left carotid artery (for blood pressure measurement) and the right jugular vein (for drug delivery). The animal was then placed supine on a heated bed, and the hindpaws were secured to the bed. The rat was allowed to breathe spontaneously. Respiration and mean arterial blood pressure (MAP) were monitored throughout the experiment with a chest pneumatic transducer and a blood pressure transducer connected to an animal monitoring system. Temperature was maintained with the rectal fluoroptic thermometer and maintained at 36 ± 1°C.

#### Magnetic Resonance Imaging Protocol

A T$_2$ mapping MRI technique was adapted for the present studies (Patten et al., 2003). In brief, MRI was performed using a 7-Tesla Bruker MRI system. A 72-mm volume coil (Bruker Biospin, Billerica, MA) was used for radio frequency (RF) excitation and reception. Preliminary experiments were performed on phantoms to optimize the sequence for T$_2$ measurement. A vial with water titrated with gadopentetate dimeglumine (Magnevist; Berlex Pharmaceuticals, Wayne, NJ) to a known T$_2$ (served as a quality control imaging phantom) was placed between the hindpaws of the rat. A multislice multiecho spin echo sequence was used to obtain axial images of the upper hindleg region for the calculation of the T$_2$ maps. The imaging parameters were as follows: matrix size, 128 × 128; field of view, 5.5 × 5.0 cm; 16 slices; slice thickness, 2 mm; echo time, 7 ms; 12 echoes; 2500-ms repetition time; and two averages. The edge of the slice pack was carefully positioned at the center of the knee joint, which served as a fiducial mark for reproducible slice planning. Each T$_2$ map acquisition took approximately 11 min.

#### Experiment Protocol

To test for the edema-generating mechanism of the L-type Ca$^{2+}$ channel, nifedipine, both hypertensive SHRs as well as the normotensive SD rats were studied for MRI-measured hindleg edema, femoral artery/vein mRNA, and extravascular skeletal muscle albumin staining. In another set of experiments, the pharmacological effect of the mixed L- and T-type CCB, mibefradil, was assessed both alone and in combination with nifedipine to assess the influence of the T-type Ca$^{2+}$ channel expression on peripheral edema formation in the SHR. MBP was allowed to stabilize for 30 min. The experiment was aborted if the predrug control mean MBP was greater than 130 mm Hg for the SD rat or less than 130 mm Hg for the SHR. Two T$_2$ map scans, 9 min apart, were acquired to establish the baseline condition. If the baseline was not achieved (i.e., there were statistical differences between average T$_2$ values in these two scans), the animal was excluded from experiment in the postprocessing stage. The rat was then dosed i.v. with nifedipine (1, 0.1, or 0.01 mg/kg; n = 3 rats/dose), mibefradil (10, 1, or 0.1 mg/kg; n = 3 rats/dose), or vehicle (1 ml/kg bolus +1 ml/kg/80 min; n = 3 rats). The compound was delivered as an i.v. loading dose over 2 min.
immediately followed by a maintenance infusion for over 60 min using a syringe infusion pump. Two $T_2$ map scans were acquired, one at 30 and 50 min after the start of the infusion, respectively. These scans from the drug infusion were averaged together for comparison to the predrug control scans. The blood pressure lowering was determined as the difference between baseline and a point with a minimal blood pressure, which invariably occurred right at the end of loading dose delivery.

The protocol for the combination of mibebradil with nifedipine involved the same MRI scanning paradigm as described above. The dose selection criteria for the combination of mibebradil and mibebradil were determined in separate experiments (data not shown) that demonstrated low, medium, and maximal blood pressure lowering.

**MRI Data Analysis.** Data were zero-padded to matrix size of $256 \times 256$ and analyzed using the AFNI software package (National Institutes of Health, Bethesda, MD). $T_2$ maps were calculated by monoexponential fitting of multiecho images using the least square error fitting. Percent increase in $T_2$ during drug treatment was calculated pixel-by-pixel. To limit the number of false positives, all increases in $T_2$ of less than 10% were set to zero. Regions of interest (ROI) were drawn on the muscle tissue on seven contiguous slices on anatomical images 8 mm distal from the knee joint (tibial plateau), which served as a fiducial mark. The stack of these ROIs was treated as a single volume ROI. Regions with bone structures (tibia and fibula) were excluded from ROI. The number of nonzero voxels (N) and the average change in $T_2$ ($\Delta T_2$) over these voxels was calculated in a volume ROI. The integral increase in $T_2$ $(I = N \times \Delta T_2)$ was used as quantitative biomarker for the severity of edema (MRI edema index). This could also be described as the sum of $\Delta T_2$ of all voxels within volume ROI, which changed for more than 10%. The quality control phantom had an average $T_2$ value of 44 ms. The data set was considered good for further analysis if there were no voxels in the phantom showing change in $T_2$ more than 10% during a single experiment.

The AFNI software package was used for volume measurements of the hindleg skeletal muscle in selected data sets. For volume calculations, two-dimensional ROIs were manually drawn on the anatomical images to cover the same regions (i.e., the same number of slices) that were analyzed for $T_2$ maps. The best judgment of human operator was used to discriminate between muscle and nonmuscle tissue. Volume was calculated as the sum of the ROI areas. The percentage increase in muscle volume was calculated from the predrug and postdrug scans.

**Immunohistochemistry.** In a separate set of animals for immunohistochemistry, formalin-fixed, paraffin-embedded sections were used for each SHR proximal hindleg skeletal muscle specimen to assess whether extracellular protein accumulation induced by the L-type CCB, nifedipine, caused hindleg edema. In brief, 1 mg/kg nifedipine or vehicle was delivered as an i.v. loading dose over 2 min, immediately followed by a maintenance infusion for 30 min using a syringe infusion pump to three SHRs. Animal euthanasia was immediately performed after the i.v. infusion of nifedipine or vehicle.

Results

**Effects of Nifedipine and Mibebradil on Edema as Measured by MRI.** To ascertain whether MR imaging is capable and sensitive to measure static, interstitial water as a marker for peripheral edema, the L-type CCB, nifedipine (total dose after 60 min equals 1 mg/kg), which is known to elicit peripheral edema, was assessed by $T_2$ mapping in the rat hindleg skeletal muscle. As shown in Fig. 1, representative $T_2$ maps after 60 min of nifedipine constant i.v. infusion show that nifedipine increases the $T_2$ values measured in a 7-Tesla MRI system compared with the vehicle. The $T_2$ change became measurable after only 30 min into the nifedipine infusion and continued to increase slightly during the following 20 min, whereas MBP dropped immediately after
bolus dose delivery and gradually recovered during infusion of the rest of the compound (Fig. 2).

To confirm that the observed increase in the integral $T_2$ reflects edema, we measured the volume of muscle tissue on the same MRI scans we used for $T_2$ calculations in both the vehicle- and nifedipine-treated groups, which represent two extremes, baseline and positive control. The muscle volume did not change in the vehicle-treated group ($1.5 \pm 0.389\%$, $p = 0.031$ compared with zero) and increased in nifedipine-treated group ($7.4 \pm 0.941\%$, $p = 0.041$ compared with the vehicle group). The correlation coefficient between integral $T_2$ and the volume changes were statistically significant ($r = 0.741, p = 0.033$; Fig. 3). Based on this strong correlation, we used the integral $T_2$ increase values as a surrogate for peripheral edema.

**Edema Assessment in SHR Skeletal Muscle at Equal Blood Pressure-Lowering Doses for Nifedipine or Mibefradil.** The average predrug mean MBP across all the SHRs was $141 \pm 3$ mm Hg. Nifedipine and mibefradil each decreased mean MBP in a dose-dependent manner with nifedipine being approximately 10 times more potent than mibefradil (Fig. 4A) The mean MBP decrease caused by 1 mg/kg nifedipine ($-77 \pm 4$ mm Hg; $p < 0.05$) was not statis-
edema was compared between the SHR and a normotensive rat model where antihypertensives have been shown to exert attenuated activity. As shown in Table 1, the BP-lowering effect of 1 mg/kg nifedipine was significantly less in the normotensive SD rat when compared with the same dose in the SHR. The MRI edema index was significantly less in the normotensive rat compared with the SHR.

**Table 1**

Comparison of hindleg edema and blood pressure lowering of 1 mg/kg nifedipine between the SHR and SD rat.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Hindleg Edema</th>
<th>Maximum MBP Lowering</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>% increase in integral T2</td>
<td>mm Hg</td>
</tr>
<tr>
<td>SHR</td>
<td>2606 ± 86 (n = 6)</td>
<td>−77 ± 4 (n = 6)</td>
</tr>
<tr>
<td>SD rat</td>
<td>1439 ± 129* (n = 3)</td>
<td>−19 ± 3* (n = 3)</td>
</tr>
</tbody>
</table>

*p < 0.05, Student’s t test; SHR vs. SD rat.

**Fig. 4.** Effects of nifedipine and mibefradil on SHR blood pressure and increase in percentage integral T2 (N × average ΔT2) as an MRI index of peripheral edema. A, MBP-lowering effects of nifedipine (0.01, 0.1, and 1 mg/kg) and mibefradil (0.1, 1, and 10 mg/kg) in the anesthetized SHR. Nifedipine has 10-fold greater potency in BP lowering than mibefradil. B, dose-response to nifedipine (0.01, 0.1, and 1 mg/kg) and mibefradil (0.1, 1, and 10 mg/kg) on SHR hindleg edema formation. Nifedipine has a 2.5-fold greater propensity to form edema than mibefradil. The data in both A and B are means ± S.E.M. *p < 0.05, significant difference from vehicle group.

Peripheral edema can be caused by either an increase in capillary hydrostatic pressure forcing water into the extracellular space or by increased extracellular osmotic pressure due to increased protein leakage through the capillary wall. To rule out that protein extravasation into the extracellular space of the SHR hindleg muscle was possibly the mechanism for the rat hindleg edema, immunostaining for albumin in the skeletal muscle formalin-fixed sections was used. As shown in Fig. 5, C and D, no albumin was present in the extracellular space of the muscle after a 30-min nifedipine (1 mg/kg) or vehicle i.v. infusion in the SHR. The 30-min infusion was used because MRI studies have shown that there was edema developed already at this time point.

Normotensive SD rats were also investigated as a control for the hypertension in the SHR, and no albumin immunostaining was observed in the SD rat after 1 mg/kg nifedipine compared with vehicle (Fig. 5, B and A, respectively). Immunostaining for albumin within vessels in the muscle sections acted as an internal positive control for the albumin antibody in both the SHR and SD rat tissue sections.

**αH (a T-Type Ca2+ Channel Subunit) and αC (an L-Type Ca2+ Channel Subunit) mRNA Expression in the Femoral Artery and Femoral Vein.** The mRNA expression levels of the T-type Ca2+ channel subunit, αH, and the L-type Ca2+ channel subunit, αC, from arterial and venous tissues were analyzed by the TaqMan quantitative RT-PCR technique. In the SHR femoral artery, the -fold change (from GAPDH housekeeping gene) in αC (Fig. 6A) was 5.3 ± 2.2, whereas the femoral vein αC (Fig. 6B) -fold change was 1.4 ± 0.4. The -fold change in the T-type Ca2+ channel mRNA expression was 1.1 ± 0.1 in the SHR femoral artery versus 1.2 ± 0.1 in the SHR femoral vein. The arterial L/T-type expression ratio is 4.2-fold compared with the venous expression ratio.

As a comparison, mRNA from femoral arteries and veins in the normotensive SD rat was evaluated to assess L- and T-type Ca2+ channel expression levels. As shown in Fig. 6A, the αC expression level was significantly reduced in the arterial compared with the venous L-type expression levels. However, the venous αC expression levels were not different between the SHR and normotensive SD rat. As for the T-type Ca2+ channel expression levels (i.e., αH mRNA), there were no significant differences between the arterial or venous tissues.
L-type Ca\(^{2+}\) channel expression is greater in the arterial tissue (consistent with a hydrostatic edema mechanism), and T-type expression is more prevalent in the veins, we thought that adding a T-type CCB might attenuate L-type-induced edema. To determine whether the mixed T- and L-type CCB, mibefradil, could attenuate the edema induced by the L-type CCB nifedipine, a combination i.v. infusion of nifedipine at 1 mg/kg plus varying doses of mibefradil (0, 0.3, or 3 mg/kg) was assessed for edema formation in the SHR hindleg muscle. Representative MRI \(\Delta T_2\) maps for the nifedipine and mibefradil combinations are shown in Fig. 7. Figure 7A is the 1 mg/kg nifedipine dose alone and shows the greatest increase in integral \(T_2\) (i.e., edema) compared with either the nifedipine plus 0.3 mg/kg mibefradil (Fig. 7B) or nifedipine plus 3 mg/kg mibefradil (Fig. 7C) combinations. Upon quantification of the increase in integral \(T_2\), a dose-dependent attenuation of the level of edema measured by MRI in the SHR hindleg muscle was observed as shown in Fig. 7D. Nifedipine at 1 mg/kg plus vehicle elicited an edema response of 2606 \pm 86\% , whereas nifedipine plus 0.3 mg/kg mibefradil and nifedipine plus 3 mg/kg mibefradil significantly reduced edema index (1725 \pm 306 and 1139 \pm 279\% , respectively). The maximal BP lowering of nifedipine alone, nifedipine plus 0.3 mg/kg mibefradil, and nifedipine plus 3 mg/kg mibefradil was \(-77 \pm 4, -75 \pm 3,\) and \(-100 \pm 7,\) respectively. Only the nifedipine plus 3 mg/kg mibefradil combination produced MBP lowering that was significantly different from the other treatment groups.

**Discussion**

L-type CCBs, although highly effective in lowering BP, also cause lower extremity edema. The incidence is dose-dependent as the result of vasodilation (Messerli, 2003). The edema probably develops by the mechanism of distal arteriolar dilation with capillary leakage common with many CCBs, including late generation agents amlodipine and isradipine. To meet the increasingly challenging BP guidelines, the physician is faced with either up-titrating the dose of the CCB or resorting to combination therapy with the addition of an angiotensin-converting enzyme inhibitor, angiotensin receptor blocker, and/or diuretic. The development of the nondihydropyridine CCBs, such as the mixed T- and L-type CCB mibefradil, has improved tolerability by producing less vaso-dilatory edema (Karch et al., 1997; Kobrin et al., 1997). However, predicting the level of edema caused by novel agents preclinically has plagued new compound development. We report the use of MRI to demonstrate significantly less edema formation with the mixed T- and L-type CCB, mibefradil, compared with the L-type CCB, nifedipine, at equal antihypertensive doses in the SHR model of hypertension. Edema, measured by the MRI parameter, \(T_2\), confirms the difference observed clinically in the incidence of edema caused by nifedipine and mibefradil.

Peripheral edema is clinically characterized by a diffuse swelling in the lower extremities and is the most frequent adverse effect reported by patients receiving CCBs. The reported incidence of swollen ankles has been the major measurement for edema due to CCB treatment. However, determining edema comes late in the drug discovery process and renders a large risk to development of safer CCBs. Objective methods for measuring edema involve the evaluation of foot-

**Fig. 5.** Immunostaining for albumin shows no protein in the rat hindleg muscle interstitial space. Albumin expression was determined by immunohistochemistry. Representative images of immunostaining for both formalin-fixed, paraffin-embedded normotensive Sprague-Dawley (A and B) and SHR (C and D) hindleg skeletal muscle. Arrows, specific albumin staining (dark-brown areas) inside blood vessels as positive staining control for antibody. Vehicle control tissues for both animals (A and C) as well as the edema-inducing L-type CCB, nifedipine tissues (B and D) show no albumin staining in the muscle. The albumin-specific antibody was visualized by diaminobenzidine chromogen; magnification, 20x.

**Fig. 6.** a\(1\)H (a T-type Ca\(^{2+}\) channel subunit) and a\(1\)C (a L-type Ca\(^{2+}\) channel subunit) mRNA expression in the femoral artery and femoral vein of the rat. For quantitative RT-PCR data, the relative levels (fold change) of the a\(1\)C (A) and a\(1\)H (B) mRNA expressions were normalized to the housekeeping gene coding for GAPDH ribosomal RNA. The data are means \(\pm\) S.E.M. with significance determined at the \(p < 0.05\) level using a one-way analysis of variance with mean comparison by Hsu’s MCB. *, significance for a\(1\)C gene expression compared with normotensive SD rat femoral artery, SHR femoral vein, and normotensive SD rat femoral vein.

**Effects of Mibefradil in Combination with Nifedipine-Induced Edema in SHR Skeletal Muscle.** Because
Ankle volume by water displacement. The changes in foot volume are greater with the less lipophilic CCBs nifedipine and amlodipine than with more lipophilic CCBs manidipine or lercanidipine (van Hamersvelt et al., 1996; Lund-Johansen et al., 2003). However, only large changes in foot-ankle volume can be detected. A more sensitive measurement of edema earlier in the preclinical and clinical evaluation would enable the discovery and development of safer antihypertensive agents.

The ability to assess edema by MRI currently exists in the hospital setting because most MRI practices are able to perform the T_2 mapping for detecting many pathological conditions, including muscle edema (Muy et al., 2000; Patten et al., 2003). Aabneh et al. (2005) have shown that the T_2 proton relaxations, including muscle edema (May et al., 2000; Patten et al., 2003), should be limited to 16 due to RF power deposition and subsequent heating of the subject, which may affect T_2 measurement significantly.

During method development (data not shown), we found that the number of refocusing (180°) RF pulses should be limited to <16 due to RF power deposition and subsequent heating of the subject, which may affect T_2 measurement significantly. We have observed a consistent change in T_2 values measured with a monoexponential fit after the induction of edema (from 31.2 ± 0.7 to 44.8 ± 1.0 s after 60-min infusion of 1 mg/kg nifedipine). To increase the dynamic range and sensitivity of the measurement, we combined both the intensity of the change in T_2 (ΔT_2) and the affected area (N, number of pixels with ΔT_2 > 10% over baseline) to derive integral T_2 parameter or MRI edema index. Although T_2 changes were not quite reaching the plateau 50 min after drug infusion (see Fig. 2), we chose to use predetermined time points to quantify the MRI edema index rather than wait for T_2 stabilization.

To increase the throughput and decrease the effect of anesthesia on edema, we decided to limit scanning to 60 min after drug infusions. To validate, at least partially, the use of T_2 mapping for detecting edema, we have measured the changes of muscle volume in response to the CCB treatment. These changes were very subtle and detectable only for two extreme cases: the vehicle- and nifedipine-treated groups. There was a significant correlation between changes in muscle volume and integral T_2 values, which confirms the specificity of T_2 mapping method to measure edema. However, the sensitivity of the integral T_2 is greater by several orders of magnitude. It should be noted that the change in volume of the muscle is the potential source of error in estimation of integral T_2 change because it is based on pixel-by-pixel comparisons. The pixels in the edematous images are slightly shifted compared with the baseline. However, the extent of the linear shift is very small because the volume of the muscle changed for only 7.4% in the extreme case. It should have not changed pixel-by-pixel comparisons significantly.

The next question was whether integral T_2 increase is sensitive enough to differentiate between the edema-causing dihydropyridine CCBs (nifedipine) and the mixed T- and L-type CCB, mibefradil. This study describes the novel utility...
of MRI in differentiating between compounds by measuring their ability to produce edema. A mechanistic limitation inherent in this study stems from the lack of a selective T-type Ca\textsuperscript{2+} CCB. Even though mibefradil has T-type Ca\textsuperscript{2+} channel-blocking activity, it also has L-type CCB activity that can confound data interpretations. However, our results indicate that the lessening of the L-type CCB activity and/or increasing the T-type CCB activity can reduce peripheral edema.

Edema formation is increased by nifedipine in the SHR hindleg muscle in a short period of time (i.e., <1 h). A possible mechanism for the increase in extracellular water in the skeletal muscle involves an increased capillary hydrostatic pressure rather than extravasation of serum protein because immunostaining for albumin in muscle was negative. The ability of mibefradil to attenuate edema induced by the nifedipine may indicate a differentiating mechanism in the vasodilatory effects of the two CCBs on precapillary arterioles and the postcapillary venules. This study suggests that acute water accumulation in skeletal muscle is a hemodynamic mechanism versus any vessel wall restructuring, i.e., albumin leakage. The mRNA results for α1H and 1C differential expression demonstrating a 4.2-fold increase in the arterial L-type mRNA expression ratio versus veins are consistent with the hypothesis that peripheral edema caused by CCBs results from selective arterial dilation and increased capillary hydrostatic pressure. The differential inhibitory effects between arteries and veins of various CCBs have been demonstrated where the L-type CCBs dilated arteries more than venules (Harris et al., 1980; Magnon et al., 1995; Ozawa et al., 2001). Even though the differential T- and L-type Ca\textsuperscript{2+} channel expression between the femoral artery and vein in this study is on major vessels, the results confirm the published differences of particularly the L-type Ca\textsuperscript{2+} channel involvement in dilating the precapillary side to a greater extent than the postcapillary vessels (Li and Schiffrin, 1996). To support the hypothesis that the greater the L-type Ca\textsuperscript{2+} channel present on the arterial side of the capillary the more edema that could result was tested in normotensive SD rats. These studies confirmed that less edema developed in the normotensive rat compared with the SHR at the same dose of nifedipine. These data plus the differential L-type Ca\textsuperscript{2+} channel mRNA expression provide strong support of the degree of differential L-type Ca\textsuperscript{2+} channel between the arterial and venous sides that can drive the level of edema. This study has shown that extracellular protein accumulation is not driving the mechanism of CCB-induced peripheral edema and that L-type differential expression between the arterial and venous sides may actually play a large role in driving the peripheral edema formation due to L-type CCBs.

General anesthesia may have confounding effects on animals' BP and response to hypertensive therapy. However, it was shown that isoflurane had the least effect on cardiovascular parameters, especially if the minimal levels of anesthesia were used (Dardai and Heavner, 1987). These levels are what we used in this article. Because cardiovascular parameters drive the edema response the minimal effects should be seen on edema formation using isoflurane.

In summary, this study reveals for the first time that the MRI T\textsubscript{2} mapping provides a highly sensitive biomarker of edema, which could be used for drug research. Due to relative simplicity, this method has the potential for clinical translatability. At equal BP-lowering doses, compounds with both T- and L-type Ca\textsuperscript{2+} channel-blocking activity have significantly less increase in integral T\textsubscript{2} or edema formation than compounds with selective L-type Ca\textsuperscript{2+} channel-blocking activity. In addition, mibefradil can dose dependently attenuate the peripheral edema induced by nifedipine. This study may indicate that mixed T- and L-type CCBs may equalize the hydrostatic pressure across the capillary bed by equally dilating arterioles and venules, reducing vasodilatory edema. These findings suggest that the design of novel CCBs should incorporate a mix of T- and L-type Ca\textsuperscript{2+} channel activity to reduce the risk of edema. Thus, the development of a mixed T- and L-type CCB could replace the edema-laden L-type CCBs as antihypertensives.

Acknowledgments

We thank David G. Taylor and Robert Leadley for scientific discussions and editorial assistance in preparing this manuscript.

References


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