Deletion of MLCK210 induces subtle changes in vascular reactivity but does not affect cardiac function

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MYOSIN LIGHT CHAIN KINASES (MLCK) are Ca2+/calmodulin-activated enzymes that trigger actomyosin interaction and initiate contraction in smooth muscle cells (SMC; see Ref. 14). Phosphorylation of MLCK induces the contraction in SMC, whereas it only potentiates the force and speed of contraction in cardiac and skeletal muscle cells (14). Importantly, MLCK also regulates diverse cellular functions involving the interaction of myosin and actin at the level of the cytoskeleton. For example, in the vascular endothelium, MLCK plays a role in cell migration, cell retraction, cell barrier regulation, transendothelial migration of neutrophils, and apoptosis (20, 25, 30).

The short isoform of MLCK (MLCK108) is ubiquitous in all adult tissues, with the highest amounts in SMC (1, 9, 13). On the contrary, the long isoform (MLCK210) is prominently expressed in embryonic SMC and in adult cells of nonmuscular lineage (10, 26). Of note, both isoforms are present in mature endothelial cells (4). These two MLCK isoforms differ in the length of their NH2-terminal tail domain, which contains amino acid sequence motifs associated with subcellular targeting or protein-protein interactions in the proteome (17, 23). This noncatalytic domain of MLCK is distinct from the kinase domain. Its role as a cellular organizer, providing integration among diverse cytoskeletal proteins, has been confirmed recently (16).

Although the importance of MLCK108 in muscle contraction is well described (reviewed in Ref. 24), the physiological role of MLCK210 is less known. Recently, in an MLCK210 knockout (KO) mouse model, Wainwright and colleagues (27) have shown a decreased susceptibility of the endothelium to injury after an endotoxin shock and have proposed that MLCK210 could be a therapeutic target to prevent lung injury. These data have been reproduced in wild-type (WT) mice in which MLCK210 has been blocked by a specific inhibitor. However, the cardiovascular effects of MLCK210 ablation or blockade have not been studied up to now.

Therefore, the current study was undertaken to investigate the effect of MLCK gene deletion on cardiovascular parameters in vivo and on vascular reactivity in vitro.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with institutional guidelines. Protocols were approved by the French Animal Care Committee in accordance with European regulations. MLCK210 KO mice and WT mice (15–21 wk old; strain C57-Black6/SV-129) were used. The KO mice were generated as previously described (27).

Electrocardiograms. Electrocardiograms were recorded using a telemetry system (Data Sciences, St. Paul, MN). An implantable radiofrequency transmitter was inserted (TA10ETA-F20, 3.6 g) at least 7 days before data collection, under anesthesia with ketamine (100 mg/kg ip) and medetomidine (50 μg/kg ip). The transmitter was implanted subcutaneously; the negative lead was positioned and sutured at the right shoulder, and the positive lead was sutured toward the right chest wall. The positive lead was sutured at the right shoulder, and the positive lead was sutured toward the right chest wall. The negative lead was sutured at the right shoulder, and the positive lead was sutured toward the right chest wall.
the lower left chest. A receiver was placed under the cage of each animal, and the data were recorded at 1,000 Hz. The ECG was measured continuously for 24 h. The R-R interval, P-R interval, Q-R-S interval, P wave duration, P amplitude, and T amplitude were determined automatically with the Emka ECG analysis software. All analyses of the Q-T interval were controlled by the same operator, and corrected Q-T interval for heart rate (Q-Tc) was calculated as Q-Tc = Q-T/√R-R/100, where R-R is the interval in seconds between two successive R waves.

**Arterial blood pressure.** In another set of animals, arterial blood pressure was recorded using a telemetry system (TA11-PA20, 3.4 g; Data Sciences). Briefly, a catheter was inserted in the common carotid artery under general anesthesia by ketamine (100 mg/kg ip) and medetomidine (50 μg/kg ip) and connected to the transmitter that was positioned subcutaneously in the mouse’s right flank. Mice were allowed 1 wk of recovery from surgery before any measurement was made. Mean arterial pressure and heart rate were recorded for 30 s every 15 min during 24 h. The mean ± SE for each 15 min over a 24-h period was reported.

**Echocardiographic Examination**

Transthoracic echocardiography was performed using an ATL-HDI 5500 ultrasound machine equipped with a 15-MHz imaging transducer in mice anesthetized with pentobarbital sodium (54 mg/kg ip). Measurements were made using the leading-edge method of the American Society of Echocardiography (11). Doppler cardiac output (CO) was calculated by the following formula: CO = π × D²/4 × IVTÁo, where D represents the diameter of the aortic left ventricular outflow tract and IVTÁo, the velocity-time integral in the left ventricular outflow tract.

**Vascular Reactivity Experiments**

**Aortic ring preparation.** Segments of 2 mm aorta, cleaned of fat and connective tissue, were mounted on a wire myograph, as previously described (21). Concentration-response curves were constructed by cumulative application of the thromboxane A2 analog 9,11-dideoxy-9α,11α-methanoepoxy PGF₂α (U-46619, 10 nM-1 μM) on vessels with functional endothelium in the absence and in presence of either the NO synthase inhibitor Nω-nitro-l-arginine (l-NNA, 100 μM) alone, 2) l-NNA plus the COX inhibitor indomethacin (10 μM), 3) l-NNA plus indomethacin in the presence of 15 mM KCl to inhibit also the endothelium-derived hyperpolarization factor (EDHF). The above procedure was performed to analyze the relative contribution of endothelial factors in inducing vasodilatation such as NO, COX products, and EDHF. The different components of the vasorelaxation were determined as follow: the NO-dependent dilation was calculated as the difference between the dilation without inhibitors and the dilation in presence of l-NNA. The dilation dependent to COX products was obtained as the difference between the dilation in the presence of l-NNA alone and the dilation in the presence of l-NNA plus indomethacin. The EDHF component was assessed in the presence of l-NNA plus indomethacin as the difference between the dilation in normal PSS and in a 15 mM KCl PSS.

In another set of experiments, the same protocol was conducted in MLCK WT and KO mice treated by an MLCK aminopyridine-based inhibitor, the MMZ-10 – 057. The latter was applied to confirm the results observed in MLCK null mice mesenteric arteries. MLCK inhibitor was administered as previously described (27) by intraperitoneal injection at the dose of 2.5 mg/kg mouse body wt 1 h before death.

**Real-Time Quantitative RT-PCR Experiments**

RNA was isolated from mouse mesenteric arteries and aorta using the RNAasy Micro Kit (Qiagen, Hilden, Germany). After mRNA extraction, 400 ng total RNA were reverse transcribed to single-stranded cDNA using the High-Capacity cDNA Archive Kit. Real-time RT-PCR was performed using an ABI Prism 7900 (Applied Biosystems, Foster City, CA). Primer sequences were targeted on exons 6, 8, 16, and 26 of MLCK using Primer Express software (Applied Biosystems). Each sample was performed in duplicate, and each reaction was performed two times. Relative quantification was performed by normalizing threshold cycles (Ct) values of each MLCK exon with Ct values of the ribosomal 18S housekeeping gene (ΔCt). Data were analyzed by comparing means of the ΔCt values in mesenteric arteries or aorta from WT and MLCK KO mice.

**Statistical Analysis**

Results are expressed as means ± SE of n experiments, where n represents the number of mice. Statistical evaluation was carried out using unpaired Student’s t-test or ANOVA for repeated measures, when appropriate. In all cases, a P value <0.05 was considered to be significant.

**RESULTS**

**Systolic Blood Pressure and Heart Rate in Conscious MLCK KO and WT Mice**

Telemetry records of blood pressure are represented in Fig. 1 and show no significant difference of mean arterial pressure between either strain.

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The electrocardiographic analysis demonstrated no significant differences between WT and MLCK KO in heart rate, atrioventricular conduction (P-R interval), and intraventricular conduction (Q-R-S duration; Table 1). Repolarization as evaluated by Q-T and Q-Tc duration was also similar. The P wave duration, representing the interatrial conduction, but not the P wave amplitude, was slightly but significantly shorter in KO mice than in WT.

**Echocardiographic Evaluation**

To detect potential cardiac structural differences between KO and WT mice, transthoracicechocardiography was performed. No significant differences between WT and MLCK210 KO mice (Table 2) were observed in terms of left ventricular end-diastolic diameter and left ventricular end-systolic diameter. Systolic function, as determined by left ventricular fractional shortening (FS), was normal in WT and KO mice despite the fact that the animals were anesthetized. Of note, no significant difference in FS between the two strains was observed. Heart rate during the echocardiography was not different between WT and KO mice. Calculated left ventricular mass and estimated Doppler CO were not significantly different between the two groups.

**Evaluation of Vascular Reactivity in MLCK KO mice**

Aorta. **CONTRACTION EXPERIMENTS.** Concentration-response curves to norepinephrine, phenylephrine, 5-HT, and the thromboxane agonist U-46619 of aortic rings with endothelium were not different in WT and KO mice (Fig. 2 and Table 3). The vasoconstriction effect of U-46619 was not affected by L-NAME or indomethacin in either WT or KO mice. Sensitivity to U-46619, expressed as the pD2 value, which represents −log of the half-maximally effective molar concentration, is shown in Table 3.

**RELAXATION EXPERIMENTS.** In endothelium-denuded arteries, ACh failed to produce any relaxant response (data not shown). However, ACh produced relaxation in a concentration-dependent manner in aortic segments with endothelium precontracted with U-46619 (Fig. 3A). The relaxant response to ACh was not significantly different between WT and KO mice. In the presence of the NO synthase inhibitor L-NAME, the response to ACh was abolished in both WT and KO mice (data not shown), suggesting that the endothelium-dependent response to the agonist was mediated mainly by the release of NO. The COX inhibitor indomethacin did not affect the relaxation to ACh in either strain (data not shown). Also, exposure of vessels to the NO donor SNAP elicited a concentration-dependent relaxation that was similar in mice from both genotypes (Fig. 3B).

**Mesenteric arteries. RESPONSE TO FLOW INCREASES IN WT AND KO MICE.** Flow increases induced a significant vasodilator response in SMA from both WT and KO mice (Table 4). However, the vasodilator response from KO mice was blunted compared with the WT (P < 0.001). Compared with the untreated WT, flow-mediated dilation was significantly reduced in SMA from WT mice treated with MMZ (P < 0.001). As a consequence, it was similar to the response observed in untreated KO mice. No variations in flow-mediated dilation were observed in SMA between treated and untreated KO mice (Fig. 4A).

**EFFECTS OF L-NNA ON RESPONSE TO FLOW INCREASES IN WT AND KO MICE.** Flow-induced vasodilation was significantly reduced after incubation with L-NNA in SMA from both WT and KO mice (Table 4). Similarly, L-NNA incubation caused a significant decrease in flow-induced vasodilation in SMA from WT and KO mice treated with MMZ-10−57. Of note, the response to L-NNA in untreated KO and in WT and KO mice

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**Table 1. Electrocardiograms parameters**

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 6)</th>
<th>KO (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>454.6 ± 53.6</td>
<td>430.0 ± 49.0</td>
</tr>
<tr>
<td>P, ms</td>
<td>16.97 ± 0.98</td>
<td>13.48 ± 0.74*</td>
</tr>
<tr>
<td>P, µV</td>
<td>0.042 ± 0.006</td>
<td>0.044 ± 0.008</td>
</tr>
<tr>
<td>Q-R-S, ms</td>
<td>10.71 ± 0.30</td>
<td>10.33 ± 0.21</td>
</tr>
<tr>
<td>P-R, ms</td>
<td>37.32 ± 2.78</td>
<td>37.71 ± 3.15</td>
</tr>
<tr>
<td>R-R, ms</td>
<td>139.7 ± 17.6</td>
<td>146.8 ± 18.9</td>
</tr>
<tr>
<td>Q-T, ms</td>
<td>39.62 ± 4.74</td>
<td>37.70 ± 3.69</td>
</tr>
<tr>
<td>Q-Tc, ms</td>
<td>33.07 ± 3.78</td>
<td>31.41 ± 2.87</td>
</tr>
<tr>
<td>T, µV</td>
<td>0.074 ± 0.01</td>
<td>0.076 ± 0.01</td>
</tr>
</tbody>
</table>

Results are given as means ± SE for n, no. of experiments. Electrocardiogram parameters recorded on conscious myosin light chain kinase long isofrom (MLCK210) knockout (KO) and wild-type (WT) mice telemetry. The electrocardiogram (ECG) was measured continuously during 24 h to determine heart rate (HR), P wave (P) duration and amplitude, Q-R-S complex duration (Q-R-S), P-R duration (PR), R-R duration (RR), Q-T duration (QT), corrected Q-T (QTc) duration and T wave amplitude (T). Q-Tc = Q-Tc/√RR/100. *P < 0.05, difference between KO and WT mice for P duration.
treated with MMZ-10–057 was significantly lower than the response in untreated WT (Fig. 4B).

EFFECTS OF INDOMETHACIN ON RESPONSE TO FLOW INCREASES IN WT AND KO MICE. During NO inhibition with L-NNA, incubation with indomethacin caused a significant decrease in the vasodilator response to flow in both WT and KO mice (Table 4). However, the reduction observed in KO animals was more pronounced than the one recorded in WT mice ($P < 0.0001$). In SMA from animals treated with MMZ-10–057, the effects of indomethacin on the vasodilator response to flow were blunted compared with SMA from WT ($P < 0.01$). MMZ-10–057 treatment did not modify the effect of indomethacin on flow-mediated dilation in arteries from KO mice (Fig. 4C).

EFFECTS OF KCL ON RESPONSE TO FLOW INCREASES IN WT AND KO MICE. During NO and COX inhibition with L-NNA and indomethacin, incubation with 15 mM KCl PSS induced similar reductions in the vasodilator response to flow in untreated WT and KO mice and in WT and KO animals treated with

Table 3. Vascular responses of aortic rings to U-46619

<table>
<thead>
<tr>
<th></th>
<th>$pD_2$</th>
<th>$E_{max}$, mN/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.69±0.02</td>
<td>4.21±0.05</td>
</tr>
<tr>
<td>KO</td>
<td>7.72±0.03</td>
<td>4.51±0.08</td>
</tr>
<tr>
<td>WT L-NAME</td>
<td>7.71±0.12</td>
<td>4.23±0.3</td>
</tr>
<tr>
<td>KO L-NAME</td>
<td>7.73±0.04</td>
<td>4.64±0.1</td>
</tr>
<tr>
<td>WT Indo</td>
<td>7.72±0.10</td>
<td>4.45±0.1</td>
</tr>
<tr>
<td>KO Indo</td>
<td>7.65±0.14</td>
<td>4.52±0.5</td>
</tr>
</tbody>
</table>

Sensitivity (expressed as $pD_2$) and maximal effect ($E_{max}$) of aortic rings from MLCK210 WT and KO mice to U-46619 in basal conditions and in the presence of the NO synthase inhibitor N$^\infty$-nitro-$l$-arginine methyl ester (L-NAME, 300 $\mu$M) or the cyclooxygenase inhibitor indomethacin (Indo, 10 $\mu$M).
Table 4. Vasodilation responses of mesenteric arteries to flow according to the applied inhibitors

<table>
<thead>
<tr>
<th>Level of Flow</th>
<th>Dilation to Flow, µm</th>
<th>15 µl/min</th>
<th>25 µl/min</th>
<th>40 µl/min</th>
<th>70 µl/min</th>
<th>92 µl/min</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Basal</td>
<td></td>
<td>7.61 ± 0.92</td>
<td>11.22 ± 0.84</td>
<td>12.88 ± 0.99</td>
<td>16.36 ± 1.05</td>
<td>18.06 ± 1.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WT L-NNA</td>
<td></td>
<td>3.47 ± 0.34</td>
<td>4.49 ± 0.64</td>
<td>5.30 ± 0.79</td>
<td>6.35 ± 0.35</td>
<td>7.42 ± 0.50</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WT L-NNA + Indo</td>
<td></td>
<td>1.40 ± 0.52</td>
<td>2.44 ± 0.80</td>
<td>3.42 ± 0.69</td>
<td>4.03 ± 0.64</td>
<td>4.93 ± 0.60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WT L-NNA + Indo + KCl</td>
<td></td>
<td>0.27 ± 0.07</td>
<td>0.52 ± 0.16</td>
<td>0.89 ± 0.27</td>
<td>1.15 ± 0.29</td>
<td>1.51 ± 0.27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KO Basal</td>
<td></td>
<td>8.12 ± 1.03</td>
<td>9.50 ± 1.26</td>
<td>11.19 ± 0.72</td>
<td>12.15 ± 0.96</td>
<td>12.95 ± 1.39</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KO L-NNA</td>
<td></td>
<td>5.50 ± 0.57</td>
<td>7.23 ± 0.68</td>
<td>7.69 ± 0.54</td>
<td>8.46 ± 0.41</td>
<td>8.92 ± 0.48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KO L-NNA + Indo</td>
<td></td>
<td>1.54 ± 0.30</td>
<td>2.08 ± 0.58</td>
<td>3.19 ± 0.52</td>
<td>3.74 ± 0.36</td>
<td>4.63 ± 0.53</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KO L-NNA + Indo + KCl</td>
<td></td>
<td>0.23 ± 0.09</td>
<td>0.61 ± 0.11</td>
<td>0.83 ± 0.13</td>
<td>1.14 ± 0.18</td>
<td>1.38 ± 0.21</td>
<td>&lt;0.0001</td>
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<tr>
<td>WT + MMZ Basal</td>
<td></td>
<td>7.05 ± 1.2</td>
<td>9.1 ± 0.89</td>
<td>10.40 ± 0.92</td>
<td>12.11 ± 0.49</td>
<td>14.05 ± 0.50</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WT + MMZ L-NNA</td>
<td></td>
<td>4.30 ± 1.63</td>
<td>5.90 ± 1.43</td>
<td>6.01 ± 0.81</td>
<td>8.02 ± 0.41</td>
<td>9.50 ± 0.48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WT + MMZ L-NNA + Indo</td>
<td></td>
<td>1.68 ± 0.59</td>
<td>2.36 ± 0.38</td>
<td>3.05 ± 0.28</td>
<td>3.82 ± 0.14</td>
<td>4.58 ± 0.43</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WT + MMZ L-NNA + Indo + KCl</td>
<td></td>
<td>0.53 ± 0.17</td>
<td>0.68 ± 0.21</td>
<td>0.82 ± 0.18</td>
<td>1.00 ± 0.20</td>
<td>1.22 ± 0.24</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KO + MMZ Basal</td>
<td></td>
<td>8.17 ± 0.72</td>
<td>9.95 ± 1.23</td>
<td>10.80 ± 0.51</td>
<td>12.13 ± 0.44</td>
<td>13.25 ± 0.54</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KO + MMZ L-NNA</td>
<td></td>
<td>5.07 ± 0.48</td>
<td>6.64 ± 0.82</td>
<td>7.64 ± 0.19</td>
<td>7.89 ± 0.86</td>
<td>9.18 ± 0.60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KO + MMZ L-NNA + Indo</td>
<td></td>
<td>1.17 ± 0.13</td>
<td>1.57 ± 0.15</td>
<td>2.20 ± 0.32</td>
<td>2.59 ± 0.39</td>
<td>4.30 ± 0.46</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KO + MMZ L-NNA + Indo + KCl</td>
<td></td>
<td>0.21 ± 0.07</td>
<td>0.37 ± 0.06</td>
<td>0.48 ± 0.08</td>
<td>0.77 ± 0.10</td>
<td>1.29 ± 0.44</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Results are given as means ± SE of 4–7 experiments. Vasodilation (in µm) to different levels of flow (15, 25, 40, 70, and 92 µl/min) of mesenteric arteries from MLCK210 WT, KO, WT treated with MMZ-10-057 (WT+MMZ), and KO treated with MMZ-10-057 (KO+MMZ) in basal conditions (basal), in the presence of Nω-nitro-arginine (L-NNA, 100 µM), L-NNA (100 µM) + Indo (10 µM), and in the presence of L-NNA (100 µM) + Indo (10 µM) in a physiological salt solution containing 15 mM KCl (KCl). P < 0.001, ANOVA for repeated measures. Repeated runs of flow-mediated vasodilation in small mesenteric arteries from WT mice showing no alteration of the response (time control: n = 3 experiments).

MMZ (Fig. 4D). This result indicates that the EDHF component of the vasodilation to flow is comparable in the four groups. PASSIVE DIAMETER AND MYOGENIC TONE OF SMA. In basal conditions, pressure-induced tone (myogenic tone) in SMA was similar between WT and KO mice. Treatment with MMZ-10–057 did not affect the changes in vascular diameter in response to intraluminal increases in pressure (Fig. 5).
DISCUSSION

The results of the present study indicate that deletion of MLCK210 does not affect physiological parameters in vivo, such as blood pressure, heart chamber size, contractility, or ECG electrophysiological characteristics. In contrast, it is shown here that MLCK210 plays a role in the release of endothelial factors (i.e., NO and COX products) in response to flow in SMA without affecting the response to vasoconstrictor or vasodilator agents within the aorta.

Cardiac morphological and electrophysiological parameters, measured by echocardiography and ECG telemetry, were similar between WT and KO mice, except for P duration, which was slightly shorter in the KO group. In humans, previous studies have suggested that altered P wave duration may reflect interatrial conduction disturbances independent of increases in atrial size, which could predispose to the initiation of atrial fibrillation (8). However, because the 24-h telemetry recordings did not exhibit either ventricular or supraventricular arrhythmia, shorter P wave duration in MLCK210 KO mice does not seem to have unlikely pathophysiological relevance. Taken together, these results do not support a significant role of MLCK210 in heart function under the experimental conditions used, although the presence of compensatory mechanism masking cardiac effects of this kinase cannot be excluded. Moreover, the anesthesia during echocardiographic experiments may have affected the reported measurements. However, we consider it unlikely, since left ventricular FS was not altered in either genotype.

In conductance vessels like the aorta, the study suggests that deletion of MLCK210 did not affect responses to different vasoconstrictor agonists acting on G protein-coupled receptors such as norepinephrine, phenylephrine, 5-HT, or thromboxane analog. Evidence in the literature supports the predominant role of the short isoform in vascular contraction regulation. Indeed, in cultured SMC, downregulation of the MLCK short isoform reduced (up to 80%) developed force dramatically, and further inhibition of the MLCK long isoform by transfection was not associated with an additional inhibition of contraction (5). Thus the results presented here provide a direct in vivo test of the hypothesis that MLCK210 does not play a significant role in SMC contraction.

In aortic rings, relaxation to ACh was unaffected in MLCK210 null mice compared with WT mice. In addition, responses to SNAP were not different between both strains. Taken together these results suggest that in conduit vessels, MLCK210 is not involved in endothelium-dependent or -independent vasodilation reactivity.

In SMA, shear stress-induced vasodilation was significantly impaired in MLCK210 KO mice. An involvement of MLCK in the response to flow vasodilation has been suggested by Ando et al. (2), who showed an upregulation of MLCK mRNA transcription in human cultured umbilical endothelial cells during exposure to shear stress. Modifications of endothelial intracellular Ca\(^{2+}\) could be involved in the role of MLCK in shear stress. Indeed, Watanabe et al. described a regulatory role of MLCK in Ca\(^{2+}\) entry and NO release in response to agonists (29) and to flow (28) that could be blocked by MLCK antisense and wortmannin in endothelium-cultured porcine aortic cells. However, wortmannin also blocks the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (15) and may act as a nonspecific inhibitor. Shear stress has been shown to be able to alter the cell cytoskeleton and to modify the shape of endothelial cells, which align in the direction of flow (7, 22). Proteins of the cytoskeleton are also involved in shear stress mechanotransduction (6, 7, 12). Similarly, it has been reported that MLCK is implicated in cytoskeletal spatial reorganization under shear stress stimulation in cultured pulmonary endothelial cells (3).
Therefore, MLCK210 might be implicated in shear stress mechanotransduction or regulation through its interaction with the cytoskeleton. The reduction in flow-induced responses in MLCK210 KO mice in SMA was associated with a decrease in the NO component of shear stress partially compensated by an enhanced indomethacin-sensitive dilation. The underlying molecular mechanisms leading to NO production from eNOS in response to flow remain unclear and controversial (5). Shear-induced production of NO involves Ca\(^{2+}\)-independent mechanisms, including mainly the phosphorylation of eNOS by a PI3K-Akt-Akt pathway and interactions with other proteins like heat shock protein-90, caveolin, and other Ser/Thr protein kinases, or tyrosine kinases (reviewed in Ref. 6). Our data do not permit estimation of the potential implication of MLCK210 among these mechanisms. The cause of the observed increase in COX products could be a direct interaction between MLCK210 and COX, a compensative mechanism to impaired NO component, or the result of an interaction between the NO and the COX pathways (19). We cannot distinguish among these possibilities based on the results reported here.

As stated above, MLCK210 deletion resulted in modifications in vasodilation responses in SMA but not in aorta. This discrepancy may result from differences in the type of endothelial stimulation (humoral Ach in one hand and physical stretch in the other hand). However, to rule out the hypothesis of the potential absence of expression of MLCK 210 in aortic rings of WT mice, RT-PCR experiments were performed and confirmed the presence of MLCK210 exon mRNA transcripts in both aorta and mesenteric arteries.

Shear stress-induced vasodilation was impaired in SMA from MLCK210 without an alteration of systemic blood pressure under basal conditions. Because CO, measured by echo-cardiography, was similar in both strains, it is likely that overall systemic resistances are not increased in KO mice. These results strengthen the hypothesis that, even though flow-dilation has a key role in the control of local blood flow, it is not necessarily and/or directly related to the basal level of systemic blood pressure (12, 18).

In addition to its catalytic kinase domain, evidence supports that MLCK210 is able to interact in the cell through specific binding with other proteins, like actin-cytoskeleton, via its NH\(_2\)-terminal extension (16). Hence the effects related to the deletion of MLCK210 might be the consequence of either the absence of its kinase activity or of the loss of structural binding with protein partners. Because the pharmacological inhibition of the catalytic domain of MLCK in WT mice reproduced the same pattern of decreased shear stress as MLCK210 gene deletion, it suggests that the kinase activity of MLCK is the main contributor to this response rather than direct interactions between MLCK and other proteins (16, 17, 23). Moreover, the relative rapid time course of the “knockout-like” effect of the inhibitor (1 h) may point out that the alterations seen in the KO are not the result of chronic adaptation and are mimicked by the acute loss of enzymatic activity.

In conclusion, our findings indicate that, in physiological conditions, MLCK210 plays a role through the endothelial cells, in the regulation of the vascular reactivity to fluid-flow stimulation in SMA. Nevertheless, the main cardiovascular parameters are not altered in MLCK210 KO mice.

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REFERENCES


