Mechanisms Underlying a Decrease in KCl-Induced Contraction after Long-Term Serum-Free Organ Culture of Rat Isolated Mesenteric Artery

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Organ culture of blood vessel is a useful technique to investigate the long-term effects of drugs. However, some functional changes may occur from freshly isolated vessel (Fresh). Mammalian/mechanistic target of rapamycin (mTOR) regulates smooth muscle contractility and Ca2+ mobilization. We thus investigated mechanisms of alteration in smooth muscle contractility after serum-free organ culture focusing on mTOR. Rat isolated mesenteric arteries were cultured for 5 days without (0% serum) or with rapamycin. In 0% serum, absolute contraction by KCl significantly decreased from Fresh, which was significantly rescued by rapamycin. In 0% serum, mTOR expression significantly increased from Fresh, which was significantly rescued by rapamycin. In 0% serum, expression of myocardin, a key regulator of smooth muscle differentiation markers, significantly decreased from Fresh, which was significantly rescued by rapamycin. However, in 0% serum, absolute contraction by caffeine significantly decreased from Fresh, which was significantly rescued by rapamycin. In conclusion, expression of mTOR increased during serum-free organ culture of rat isolated mesenteric artery for 5 days, which may be at least partly responsible for the decreased smooth muscle contractility perhaps due to the decrease in the stored Ca2+ in smooth muscle.

KEY WORDS: contractility, mammalian/mechanistic target of rapamycin, organ culture, vascular smooth muscle.

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some experiments, the endothelium was removed by rubbing the intimal surface with a flat face of a pair of forceps. Arterial rings were placed in 1 ml serum-free Dulbecco’s Modified Eagle Medium (DMEM) without or with 50 nM rapamycin supplemented with 1% penicillin-streptomycin. Because previous studies reported that rapamycin at 10–50 nM can regulate the differentiation of smooth muscle cells [5, 8, 9, 18], we chose 50 nM rapamycin in the present study. They were maintained at 37°C in an atmosphere of 95% air and 5% CO₂ for 5 days. The culture medium was replaced every other day. Animal care and treatment were conducted in conformity with the institutional guidelines of the Kitasato University.

Measurement of isometric contraction: The arterial rings were placed in normal physiological salt solution (PSS), which contained (mM): NaCl 136.9, KCl 5.4, CaCl₂ 1.5, MgCl₂ 1.0, NaHCO₃ 23.8 and glucose 5.5. Ethylenediaminetetraacetic acid (EDTA), 1 µM, was also added to remove the contaminating metal ions which catalyze oxidation of organic chemicals. The high K+ (72 mM) solution was prepared by replacing NaCl with equimolar KCl. These solutions were saturated with a 95% O₂-5%CO₂ mixture at 37°C and pH 7.4. Smooth muscle contractility was recorded isometrically with a force-displacement transducer (Nihon Kohden, Tokyo, Japan) as described previously [19–23, 32]. Each arterial ring was attached to a holder under a resting tension of 0.5 g. After equilibration for 30 min in a 3 ml organ bath, each ring was repeatedly exposed to high K+ solution until the responses became stable (60–90 min). Concentration-response curves were obtained by the cumulative application of KCl (5.4–75.4 mM).

Western blotting: Western blotting was performed as described previously [19, 29, 30, 32]. Protein lysates were obtained by homogenizing mesenteric artery with Triton-based lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄ and 1 µg/ml leupeptin). Equal amounts of
proteins (13–25 μg) were separated by SDS-PAGE (6–14%) and transferred to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, U.S.A.). After being blocked with 3% bovine serum albumin (for phosphorylation antibodies) or 0.5% skim milk (for others), membranes were incubated with primary antibodies (1:50–1:500 dilution) at 4°C overnight, and the membrane-bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 hr) and the EZ-ECL system (Biological industries, Kibbutz Beit haemek, Israel). The results were analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan). Protein loading pattern was checked by a Ponceau-S staining.

**Chemicals:** The chemicals used were as follows: DMEM (Wako, Osaka, Japan), penicillin-streptomycin (Invitrogen/GIBCO, Carlsbad, CA, U.S.A.), rapamycin, (Cayman Chemical, Ann Arbor, MI, U.S.A.), KCl (Kanto Chemical, Tokyo, Japan) and caffeine (Sigma-Aldrich, St. Louis, MO, U.S.A.). Antibody sources were as follows: total mTOR, phospho-mTOR (Ser2448) (Acris antibodies, San Diego, CA, U.S.A.), α-actin (Dako, Glostrup, Denmark), myocardin (Bioss, Woburn, MA, U.S.A.), calponin (Thermo Fisher Scientific, Yokohama, Japan), phospho-calcmodulin-dependent protein kinase (CaMK) II (Thr286) (Signalway Antibody, College Park, MD, U.S.A.), total CaMKII δ and SM22α/ transgelin (Genetex, Irvine, CA, U.S.A.).

**Statistics:** The results of the experiments were expressed as means ± S.E.M. Statistical evaluation of the data was performed by ANOVA followed by Bonferroni’s test. A value of P<0.05 was taken as statistically significant. EC50 (concentration at which the half maximal effect occurred) was calculated by a sigmoid curve fitting.

## RESULTS

**Effects of rapamycin on KCl-induced contraction in serum-free organ-cultured rat mesenteric artery:** We first examined whether KCl-induced contraction after serum-free organ culture for 5 days. In freshly isolated arteries (Fresh), KCl (5.4–75.4 mM) caused contraction in a concentration-dependent manner (Fig. 1A). In the arteries organ-cultured in a serum-free condition for 5 days (0% serum), KCl-induced maximal contraction significantly decreased from Fresh (Fig. 1A, contraction induced by 75.4 mM KCl; 2.38 ± 0.14 g/mg wet weight (w.w., n=21 for Fresh and 1.05 ± 0.16 g/mg w.w., n=7 for 0% serum, P<0.01). In serum-free organ-cultured arteries in the presence of 50 nM rapamycin for 5 days (Rapamycin), the decreased KCl-induced maximal contraction was significantly rescued (Fig. 1A, contraction induced by 75.4 mM KCl; 1.91 ± 0.29 g/mg w.w., n=7, P<0.05 vs. 0% serum). The normalized concentration-response curve in 0% serum was significantly shifted to the left compared with that in Fresh (Fig. 1B, EC50 (mM) for Fresh and 0% serum was 40.1 ± 1.4 and 20.1 ± 3.6, respectively, P<0.01). The normalized concentration-response curve in Rapamycin did not change compared with that in 0% serum (Fig. 1B, EC50 (mM); 19.3 ± 2.3).

**Effects of rapamycin on expression and phosphorylation of mTOR in serum-free organ-cultured rat mesenteric artery:** Next, we investigated whether mTOR expression changed after serum-free organ culture in the absence or presence of rapamycin by Western blotting. The expression of mTOR in 0% serum significantly increased from Fresh (Fig. 2A and 2B, n=10, P<0.01 vs. Fresh), which was significantly rescued in Rapamycin (n=10, P<0.01 vs. 0% serum). The relative level of phosphorylation of mTOR (Ser2448) did not change between Fresh, 0% serum and Rapamycin when normalized to the total expression (Fig. 2C).

**Effects of rapamycin on expression of myocardin, α-actin, calponin and SM22α in serum-free organ-cultured rat mesenteric artery:** Next, we hypothesized that the decrease in expression of smooth muscle contractile proteins may be responsible for the decreased contractility in 0% serum and that rapamycin treatment might rescue it. We thus investigated expression of myocardin, a key regulator of expression of smooth muscle contractile proteins as well as α-actin, SM22α and calponin. Compared with Fresh, expression of myocardin (Fig. 3A, n=9, P<0.01 vs. Fresh), SM22α (Fig. 3C, n=9, P<0.01 vs. Fresh) and calponin (Fig. 3D, n=14, P<0.01 vs. Fresh) significantly decreased in 0% serum. Expression of α-actin slightly decreased in 0% serum, which was not statistically significant (Fig. 3B, n=39). In Rapamycin, the expression of myocardin was significantly recovered (Fig. 3A, n=9, P<0.01 vs. 0% serum). However, the expression of all contractile proteins in Rapamycin did not change from 0% serum (Fig. 3B–3D, n=9–39). In 0% serum, phosphorylation of Akt2 (a functional regulator of myocardin) decreased from Fresh (n=6, P<0.05, data not shown). In Rapamycin, phosphorylation of Akt2 did not change from 0% serum (n=6).

**Effects of rapamycin on basal phosphorylation of CaMKII in serum-free organ-cultured rat mesenteric artery:** Since the expression of contractile proteins did not change between 0% serum and Rapamycin, we next focused on the functional regulator of key contractile protein. For this purpose, we examined the effects of rapamycin on expression and basal phosphorylation of CaMKII, which is one of the upstream regulators of myosin light chain. The CaMKII expression in 0% serum significantly increased from Fresh (Fig. 4A and 4B, n=6, P<0.05 vs. Fresh), which was not modulated by rapamycin (n=6). The relative phosphorylation level of CaMKII normalized to the total expression significantly increased in 0% serum (Fig. 4A and 4C, n=6, P<0.01 vs. Fresh), which was significantly rescued in Rapamycin (n=6, P<0.01 vs. 0% serum).

**Effects of rapamycin on caffeine-induced contraction in serum-free organ-cultured rat mesenteric artery:** The increased basal CaMKII phosphorylation in 0% serum may suggest an increase in the basal cytosolic Ca2+ level. It was reported that mTOR promoted inositol 1, 4, 5-trisphosphate receptor (IP3R)-mediated Ca2+ release [15, 17] from SR. Thus, we hypothesized that the increased mTOR expression in 0% serum may induce the basal Ca2+ release (leak) from SR, which in turn decreases the stored Ca2+ in SR. To test this, we next investigate the effects of rapamycin on an opener of SR ryanodine receptor, caffeine-induced contrac-
In Fresh, caffeine (20 mM) caused a transient contraction (Fig. 5A, n=12). In 0% serum, caffeine-induced contraction significantly decreased from Fresh (Fig. 5A, contraction induced by 20 mM caffeine: 0.41 ± 0.05 g/mg w.w., n=12 for Fresh and 0.23 ± 0.04 g/mg w.w., n=14 for 0% serum, \(P<0.05\)). In Rapamycin, the decreased caffeine-induced contraction was significantly rescued (Fig. 5A, contraction induced by 20 mM caffeine: 0.40 ± 0.05 g/mg w.w., n=14, \(P<0.05\) vs. 0% serum). We confirmed that the KCl (72 mM)-induced contraction was inhibited in the presence of caffeine (Fig. 5A, contraction induced by 20 mM caffeine: 0.40 ± 0.05 g/mg w.w., n=14, \(P<0.05\) vs. 0% serum). We confirmed that the KCl (72 mM)-induced contraction was inhibited in the presence of caffeine (Fig. 5B, n=4). Calcineurin may also regulate the Ca\(^{2+}\) release by forming the FKBP-ryanodine receptor or FKBP-IP\(_3\)R complex [2, 16]. It was reported that H\(_2\)O\(_2\)-induced activation of calcineurin inhibited phosphorylation of myosin light chain and caused a decrease in KCl-induced contraction, which was rescued by rapamycin [6]. In the present study, however, treatment with a calcineurin inhibitor, FK506, for 5 days did not affect the decreased KCl-induced contraction in 0% serum (data not shown).

**DISCUSSION**

In the present study, we showed that caffeine-induced contraction in 0% serum significantly decreased from Fresh, which was significantly rescued by rapamycin (Fig. 5A). It was reported that mTOR regulates intracellular Ca\(^{2+}\) mobilization [15, 17, 25]. The mTOR forms a complex with FK506-binding protein (FKBP) and IP\(_3\)R. It was reported in portal vein that IP\(_3\)R mediated Ca\(^{2+}\) release in cooperation with FKBP and mTOR and that rapamycin inhibited IP\(_3\)R-mediated Ca\(^{2+}\) release via the inhibition of mTOR [15, 17]. It was also reported that mTOR increased Ca\(^{2+}\)-releasing activity of type 2 IP\(_3\)R through phosphorylation in pancreatic adenocarcinoma AR4-2J cells [25]. Thus, it might be possible that the increase in mTOR expression in 0% serum would potentiate Ca\(^{2+}\)-release through IP\(_3\)R (Ca\(^{2+}\) leak), which may lead to the increased intracellular Ca\(^{2+}\) concentration, resulting in basal phosphorylation of CaMKII. In addition, it is suggested that the subsequent decrease in stored Ca\(^{2+}\) in SR may prevent the KCl-induced contraction in 0% serum. The data that KCl-induced contraction was prevented in the presence of caffeine (Fig. 5B) may support the concept. Calcineurin may also regulate Ca\(^{2+}\) release by forming the FKBP-ryanodine receptor or FKBP-IP\(_3\)R complex [2, 16].

**Fig. 3.** After rat mesenteric arteries were freshly isolated (Fresh) or cultured in serum-free medium without (0% serum) or with 50 nM rapamycin (Rapamycin) for 5 days, protein lysates were harvested. Expression of myocardin (A: n=9), α-actin (B: n=39), SM22α (C: n=9) and calponin (D: n=14) was determined by Western blotting and shown as fold-increase relative to Fresh. Results were expressed as means ± S.E.M. **: \(P<0.01\) vs. Fresh. ##: \(P<0.01\) vs. 0% serum.
not shown), suggesting that calcineurin may not be involved in the effects of rapamycin on smooth muscle contractility.

It was reported that mRNA of contractile protein decreased after organ culture of mice isolated aorta [34]. In the present study, we showed that the expression of smooth muscle differentiation markers including SM22α and calponin remains decreased in Rapamycin, even though the expression of myocardin (a key regulator of differentiation markers) was recovered in Rapamycin. It was reported that Akt activation may mediate translocation of forkhead box O4 (FoxO4) to the cytoplasm and promote expression of smooth muscle differentiation markers by activating serum response factor (SRF)-myocardin complex [12]. In addition, it was reported that rapamycin inhibited mTORC1/p70 S6 kinase-induced serine phosphorylation of IRS-1 and promoted smooth muscle differentiation by potentiating IGF-1-induced Akt2 activation [18]. Thus, we explored the phosphorylation of Akt2. In 0% serum, phosphorylation of Akt2 decreased from Fresh, which was not modulated by rapamycin (data not shown). These data may explain that the decrease in the expression of differentiation markers in Rapamycin may be attributable to the decreased phosphorylation of Akt2. It is also suggested that SRF-myocardin complex might be inactive due to FoxO4 binding even though the myocardin expression was recovered in Rapamycin.

mTOR exists in two different complexes, mTORC1 and 2. The two complexes have different sensitivity to rapamycin. mTORC1 is acutely inhibited by rapamycin, while long-term treatment with rapamycin inhibits mTORC2 in some types of cells. Since we treated rapamycin for a long period of time, it might be possible that rapamycin affects not only mTORC1 but also mTORC2 in the present study. To support this, we confirmed in 0% serum that phosphorylation of Akt1 (Ser473), a known substrate of mTORC2 [33], increased from Fresh (n=4, P<0.01 vs. Fresh, data not shown), which was rescued in Rapamycin (n=4, P<0.01 vs. 0% serum).

In conclusion, the present study demonstrated that expres-

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Fig. 4. After rat mesenteric arteries were freshly isolated (Fresh) or cultured in serum-free medium without (0% serum) or with 50 nM rapamycin (Rapamycin) for 5 days, protein lysates were harvested. Representative images were shown in (A). Expression (B) and phosphorylation (C) of calmodulin-dependent protein kinase (CaMK) II (n=6) were determined by Western blotting and shown as fold-increase relative to 0% serum. Results were expressed as means ± S.E.M. *, **: P<0.05, 0.01 vs. 0% serum. ##: P<0.01 vs. Fresh.

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Fig. 5. Caffeine (20 mM)-induced transient contraction in endothelium-denuded rat isolated mesenteric arteries; freshly isolated (Fresh, n=12) or cultured in serum-free medium without (0% serum, n=14) or with 50 nM rapamycin (Rapamycin, n=14) for 5 days (A). Contraction was expressed as an absolute value (g/mg tissue w.w.). Results were expressed as means ± S.E.M. *: P<0.05 vs. Fresh. #: P<0.05 vs. 0% serum. Representative recording of contraction induced by KCl (72 mM) in the absence or presence of caffeine (20 mM) in endothelium-denuded freshly isolated rat mesenteric arteries (B, n=4). PSS: physiological salt solution.
Fig. 6. Summary of the present results. Expression of mTOR increased during serum-free organ culture of rat isolated mesenteric artery for 5 days, which may lead to the decrease in KCl-induced contraction presumably via the decrease in the stored Ca$^{2+}$ in SR (Fig. 6). Addition of rapamycin is useful, because it rescues the decreased contractility in serum-free organ-cultured mesenteric artery. Nonetheless, further studies are required in order to better preserve the contractile function of organ-cultured blood vessels.

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