FULL PAPER *Pharmacology*

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

Tomoka MORITA[1)], Muneyoshi OKADA[1)] and Hideyuki YAMAWAKI[1)]*

[1)]*Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Kitasato University, Aomori 034–8628, Japan*

(Received 10 January 2014/Accepted 14 March 2014/Published online in J-STAGE 1 April 2014)

ABSTRACT. Organ culture of blood vessel is a better 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 differentiation and Ca^{2+} 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, the decrease in expression of contractile proteins, including SM22α and calponin, was not changed by rapamycin. Basal phosphorylation of calmodulin-dependent protein kinase II significantly increased in 0% serum, which was significantly rescued by rapamycin. 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 Ca^{2+} in smooth muscle.

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

doi: 10.1292/jvms.14-0022; *J. Vet. Med. Sci.* 76(7): 963–969, 2014

Organ culture of blood vessels is a useful technique to investigate the long-term effects of drugs and/or physiologically active substances, because tissue architecture and function are well preserved in the system [24]. It has been considered that organ culture in a 'serum-free condition' is the best way to maintain differentiated cell function of isolated blood vessel [19, 20]. However, compared with freshly isolated vessel, some functional changes, such as decreased contractility, increased sensitivity to agonists and changes in receptor expression, may occur during a serum-free organ culture, which limits the terms of this technique [3, 4, 13, 14, 20, 27, 28, 31].

Mammalian/mechanistic target of rapamycin (mTOR) is atypical serine/threonine protein kinase and exists in two different complexes, termed mTOR complex (mTORC) 1 and 2. The two complexes have different sensitivity to rapamycin. mTORC1 is acutely inhibited by rapamycin, while mTORC2 is resistant to rapamycin in most cells. Of note, it has been recently demonstrated that long-term treatment with rapamycin inhibits mTORC2 in some types of cells [1, 7, 11, 26]. It was reported that mTOR regulates differentiation of various types of cells and that inhibition of mTOR by rapamycin in combination with insulin-like growth factor (IGF)-1 stimulation promotes vascular smooth muscle differentiation via modulating p70 S6 kinase/insulin receptor substrate (IRS)-1/Akt2 pathway [18]. In addition, it was reported that mTOR regulates intracellular Ca^{2+} mobilization from sarcoplasmic reticulum (SR) in smooth muscle [15, 17]. The mTORC1 integrates at least five major signals, including growth factor, stress, energy status and amino acid [10]. While the upstream mechanisms of mTORC2 still remain to be uncovered, it was recently reported that growth factors and insulin activate it in a phosphoinositide 3-kinase (PI3K)-dependent manner [7]. We hypothesized that mTOR may be at least in part responsible for functional changes after serum-free organ culture. The aim of the present study was thus to investigate the mechanisms of alteration in smooth muscle contractility after long-term serum-free organ culture, specifically focusing on mTOR. We have found that 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.

MATERIALS AND METHODS

Tissue preparation and organ culture procedure: Organ culture of rat isolated mesenteric artery was performed as described previously [19, 20]. In brief, male Wistar rats (6–14-week-old) were anesthetized with urethane (1.5 g/kg, i.p.) and euthanized by exsanguination. The main branch of superior mesenteric artery was isolated under sterile conditions. After removal of fat and adventitia, the mesenteric artery was cut into rings (1-mm in diameter) for organ culture and measurement of isometric contraction. In

*CORRESPONDENCE TO: YAMAWAKI, H., Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Kitasato University, Higashi 23 bancho 35–1, Towada, Aomori 034–8628, Japan.
e-mail: yamawaki@vmas.kitasato-u.ac.jp

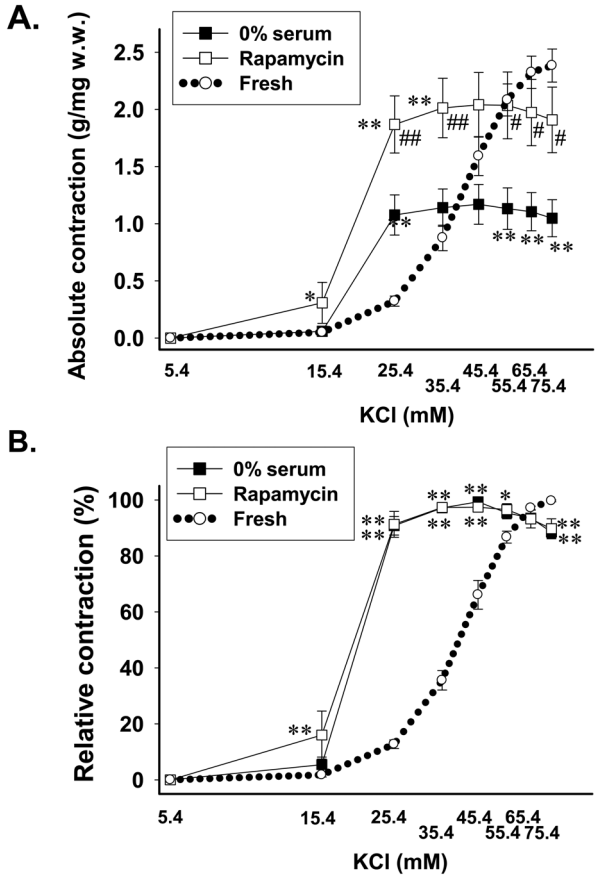

Fig. 1. Concentration-contraction relationship for KCl (5.4–75.4 mM) in rat mesenteric arteries; freshly isolated (Fresh, open circle, n=21) or cultured in serum-free medium without (0% serum, closed square, n=7) or with 50 nM rapamycin (Rapamycin, open square, n=7) for 5 days. KCl was cumulatively applied. Contraction was expressed as an absolute value (g/mg tissue wet weight (w.w.)) (A) or normalized to the maximal response (B). 100% represents the maximal response. Results were expressed as means ± S.E.M. *, **: $P<0.05$, $P<0.01$ vs. Fresh. $^{\#,\#\#}$: $P<0.05$, $P<0.01$ vs. 0% serum.

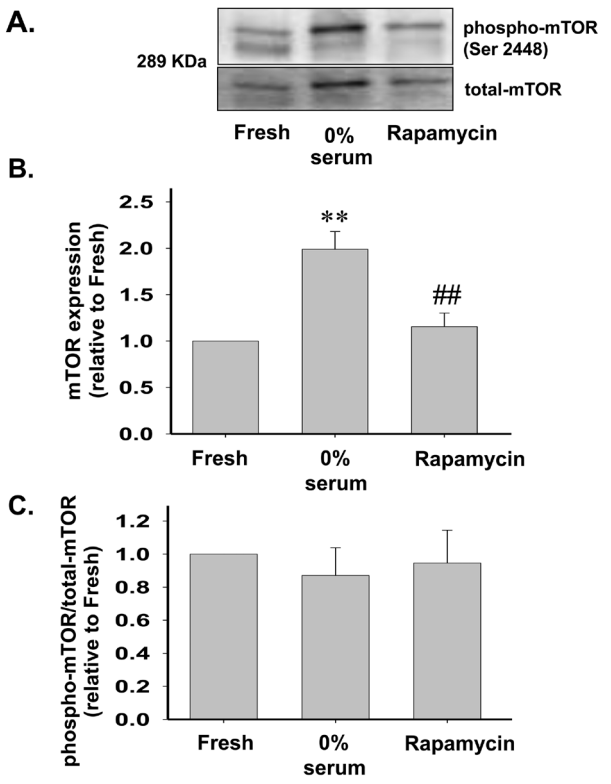

Fig. 2. After rat mesenteric arteries were freshly isolated (Fresh) or cultured in serum-free medium without (0% serum) or with 50 nM rapamycin (Rapamycin) for 5 days, protein lysates were harvested. Representative images were shown in (A). Expression (B) and phosphorylation (C) of mammalian/mechanistic target of rapamycin (mTOR) were determined by Western blotting and shown as fold-increase relative to Fresh (n=10). Results were expressed as means ± S.E.M. **: $P<0.01$ vs. Fresh. $^{\#\#}$: $P<0.01$ vs. 0% serum.

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 m*l* 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% CO_2 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, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8 and glucose 5.5. Ethylendiaminetetraacetic 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% O_2-5%CO_2 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 m*l* 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 Na_3VO_4 and 1 μg/m*l* 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 Beithaemek, 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-streptomycine (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.), calopnin (Thermo Fisher Scientific, Yokohama, Japan), phospho-calmodulin-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. EC_{50} (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 the change in 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, EC_{50} (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, EC_{50} (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), SM 22α (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 Ca^{2+} level. It was reported that mTOR promoted inositol 1, 4, 5-trisphosphate receptor (IP_3R)-mediated Ca^{2+} release [15, 17] from SR. Thus, we hypothesized that the increased mTOR expression in 0% serum may induce the basal Ca^{2+} release (leak) from SR, which in turn decreases the stored Ca^{2+} in SR. To test this, we next investigate the effects of rapamycin on an opener of SR ryanodine receptor, caffeine-induced contrac-

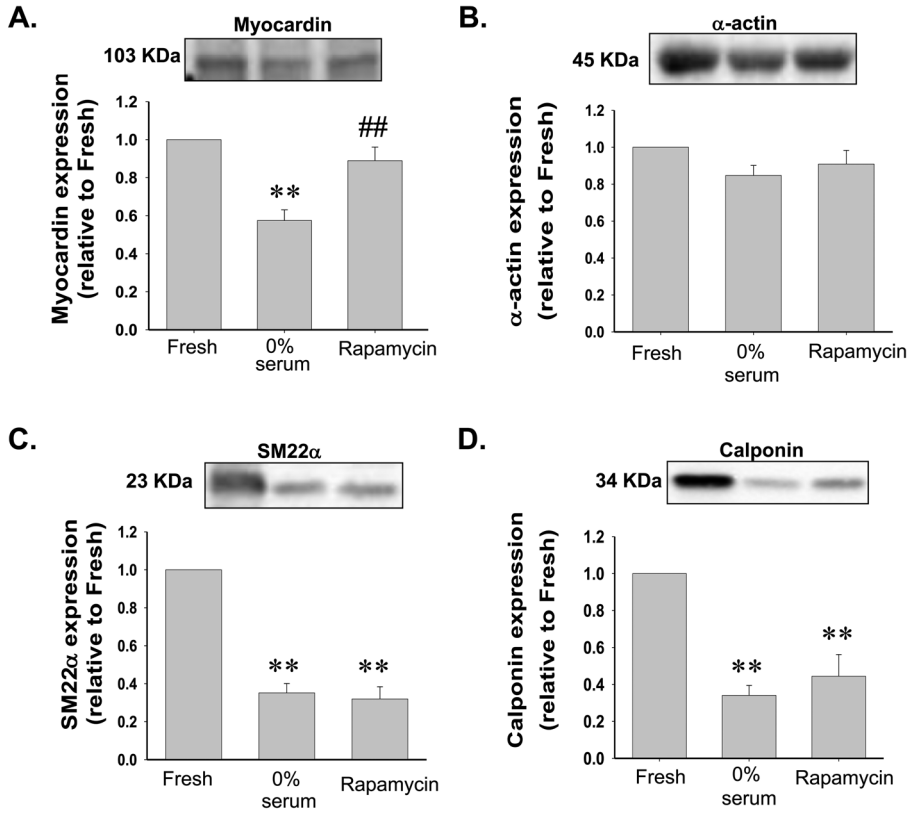

Fig. 3. After rat mesenteric arteries were freshly isolated (Fresh) or cultured in serum-free medium without (0% serum) or with 50 nM rapamycin (Rapamycin) for 5 days, protein lysates were harvested. Expression of myocardin (A: n=9), α-actin (B: n=39), SM22α (C: n=9) and calponin (D: n=14) was determined by Western blotting and shown as fold-increase relative to Fresh. Results were expressed as means ± S.E.M. **: P<0.01vs. Fresh. ##: P<0.01 vs. 0% serum.

tion. 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 5B, n=4). Calcineurin may also regulate the Ca^{2+} release from SR [2, 16]. However, treatment with a calcineurin inhibitor, FK506 (1–10 μM), for 5 days did not affect the decreased KCl-induced contraction in 0% serum (n=4, 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_3R. It was reported in portal vein that IP_3R mediated Ca^{2+} release in cooperation with FKBP and mTOR and that rapamycin inhibited IP_3R-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_3R 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_3R (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_3R complex [2, 16]. It was reported that H_2O_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

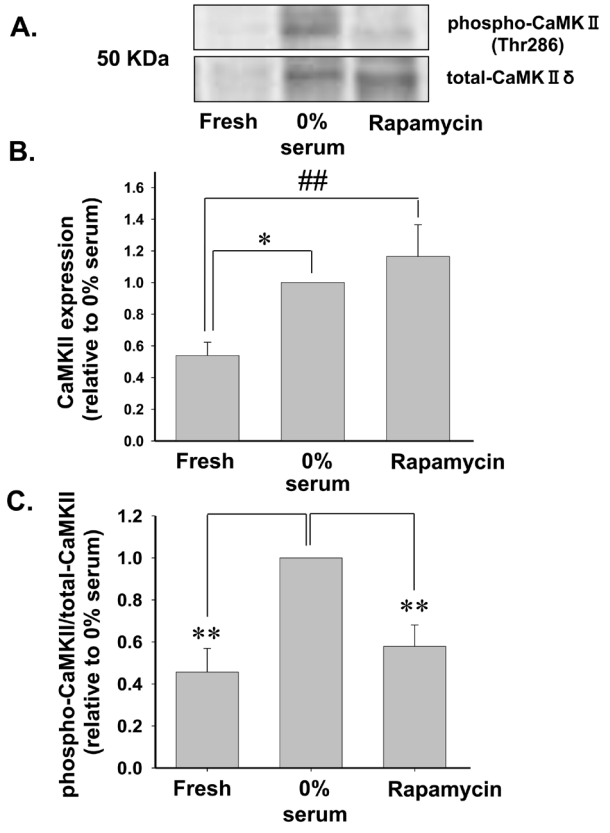

Fig. 4. After rat mesenteric arteries were freshly isolated (Fresh) or cultured in serum-free medium without (0% serum) or with 50 nM rapamycin (Rapamycin) for 5 days, protein lysates were harvested. Representative images were shown in (A). Expression (B) and phosphorylation (C) of calmodulin-dependent 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.

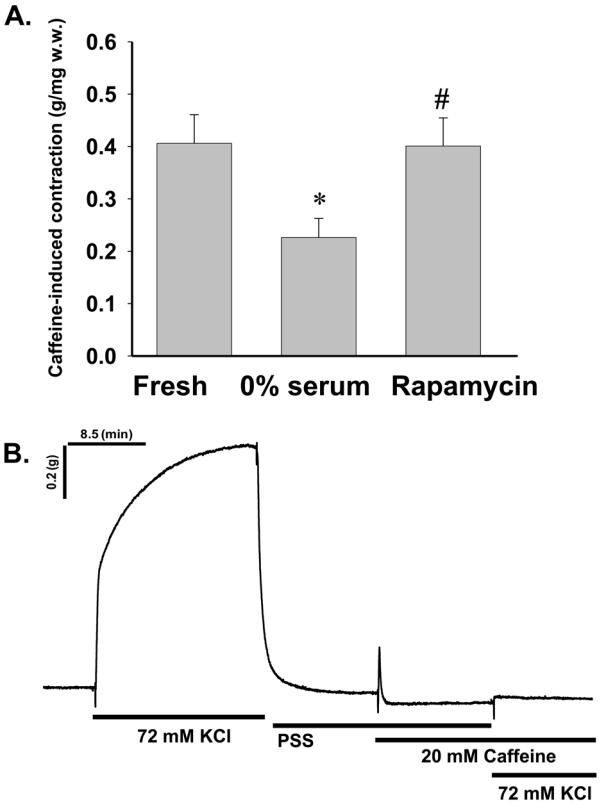

Fig. 5. Caffeine (20 mM)-induced transient contraction in endothelium-denuded rat isolated mesenteric arteries; freshly isolated (Fresh, n=12) or cultured in serum-free medium without (0% serum, n=14) or with 50 nM rapamycin (Rapamycin, n=14) for 5 days (A). Contraction was expressed as an absolute value (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.

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-

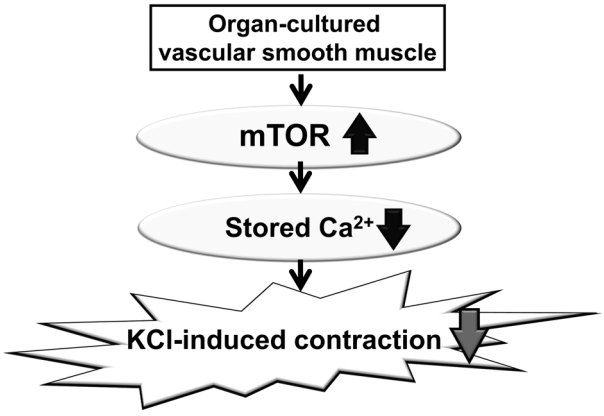

Fig. 6. Summary of the present results. Expression of mTOR increased during serum-free organ culture of rat isolated mesenteric artery for 5 days, which may be at least partly responsible for the decreased smooth muscle contractility perhaps due to the decrease in the stored Ca^{2+} of sarcoplasmic reticulum in smooth muscle.

sion of mTOR increases 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.

ACKNOWLEDGMENT. This study was supported in part by Grants for Scientific Research from the Japan Society for the Promotion of Science.

REFERENCES

1. Barilli, A., Visigalli, R., Sala, R., Gazzola, G. C., Parolari, A., Tremoli, E., Bonomini, S., Simon, A., Closs, E. I., Dall'Asta, V. and Bussolati, O. 2008. In human endothelial cells rapamycin causes mTORC2 inhibition and impairs cell viability and function. *Cardiovasc. Res.* **78**: 563–571. [Medline] [CrossRef]
2. Bultynck, G., Vermassen, E., Szlufcik, K., De Smet, P., Fissore, R. A., Callewaert, G., Missiaen, L., De Smedt, H. and Parys, J. B. 2003. Calcineurin and intracellular Ca^{2+}-release channels: regulation or association? *Biochem. Biophys. Res. Commun.* **311**: 1181–1193. [Medline] [CrossRef]
3. Cao, Y. X., He, L. C., Xu, C. B., Luo, G. G. and Edvinsson, L. 2005. Enhanced transcription of contractile 5-hydroxytryptamine 2A receptors via extracellular signal-regulated kinase 1/2 after organ culture of rat mesenteric artery. *Basic Clin. Pharmacol. Toxicol.* **96**: 282–288. [Medline] [CrossRef]
4. Cao, Y. X., Xu, C. B., Luo, G. G. and Edvinsson, L. 2006. Up-regulation of alpha1A-adrenoceptors in rat mesenteric artery involves intracellular signal pathways. *Basic Clin. Pharmacol. Toxicol.* **98**: 61–67. [Medline] [CrossRef]
5. Furgeson, S. B., Simpson, P. A., Park, I., Vanputten, V., Horita, H., Kontos, C. D., Nemenoff, R. A. and Weiser-Evans, M. C. 2010. Inactivation of the tumour suppressor, PTEN, in smooth muscle promotes a pro-inflammatory phenotype and enhances neointima formation. *Cardiovasc. Res.* **86**: 274–282. [Medline] [CrossRef]
6. Gao, G., Li, J. J., Li, Y., Li, D., Wang, Y., Wang, L., Tang, X. D., Walsh, M. P., Gui, Y. and Zheng, X. L. 2011. Rapamycin inhibits hydrogen peroxide-induced loss of vascular contractility. *Am. J. Physiol. Heart Circ. Physiol.* **300**: H1583–H1594. [Medline] [CrossRef]
7. Goncharova, E. A. 2013. mTOR and vascular remodeling in lung diseases: current challenges and therapeutic prospects. *FASEB J.* **27**: 1796–1807. [Medline] [CrossRef]
8. Halayko, A. J., Kartha, S., Stelmack, G. L., McConville, J., Tam, J., Camoretti-Mercado, B., Forsythe, S. M., Hershenson, M. B. and Solway, J. 2004. Phophatidylinositol-3 kinase/mammalian target of rapamycin/p70S6K regulates contractile protein accumulation in airway myocyte differentiation. *Am. J. Respir. Cell Mol. Biol.* **31**: 266–275. [Medline] [CrossRef]
9. Houssaini, A., Abid, S., Mouraret, N., Wan, F., Rideau, D., Saker, M., Marcos, E., Tissot, C. M., Dubois-Rande, J. L., Amsellem, V. and Adnot, S. 2013. Rapamycin reverses pulmonary artery smooth muscle cell proliferation in pulmonary hypertension. *Am. J. Respir. Cell Mol. Biol.* **48**: 568–577. [Medline] [CrossRef]
10. Laplante, M. and Sabatini, D. M. 2012. mTOR signaling in growth control and disease. *Cell* **149**: 274–293. [Medline] [CrossRef]
11. Li, W., Petrimpol, M., Molle, K. D., Hall, M. N., Battegay, E. J. and Humar, R. 2007. Hypoxia-induced endothelial proliferation requires both mTORC1 and mTORC2. *Circ. Res.* **100**: 79–87. [Medline] [CrossRef]
12. Liu, Z. P., Wang, Z., Yanagisawa, H. and Olson, E. N. 2005. Phenotypic modulation of smooth muscle cells through interaction of Foxo4 and myocardin. *Dev. Cell* **9**: 261–270. [Medline] [CrossRef]
13. Luo, G., Jamali, R., Cao, Y. X., Edvinsson, L. and Xu, C. B. 2006. Vascular endothelin ET(B) receptor-mediated contraction requires phosphorylation of ERK1/2 proteins. *Eur. J. Pharmacol.* **538**: 124–131. [Medline] [CrossRef]
14. Luo, G., Xu, C. B., Cao, Y. X. and Edvinsson, L. 2004. Transcriptional up-regulation in expression of 5-hydroxytryptamine2A and transcriptional down-regulation of angiotensin II type 1 receptors during organ culture of rat mesenteric artery. *Basic Clin. Pharmacol. Toxicol.* **95**: 280–287. [Medline] [CrossRef]
15. MacMillan, D., Currie, S., Bradley, K. N., Muir, T. C. and McCarron, J. G. 2005. In smooth muscle, FK506-binding protein modulates IP3 receptor-evoked Ca^{2+} release by mTOR and calcineurin. *J. Cell Sci.* **118**: 5443–5451. [Medline] [CrossRef]
16. MacMillan, D., Currie, S. and McCarron, J. G. 2008. FK506-binding protein (FKBP12) regulates ryanodine receptor-evoked Ca^{2+} release in colonic but not aortic smooth muscle. *Cell Calcium* **43**: 539–549. [Medline] [CrossRef]
17. MacMillan, D. and McCarron, J. G. 2009. Regulation by FK506 and rapamycin of Ca^{2+} release from the sarcoplasmic reticulum in vascular smooth muscle: the role of FK506 binding proteins and mTOR. *Br. J. Pharmacol.* **158**: 1112–1120. [Medline] [CrossRef]
18. Martin, K. A., Merenick, B. L., Ding, M., Fetalvero, K. M., Rzucidlo, E. M., Kozul, C. D., Brown, D. J., Chiu, H. Y., Shyu, M., Drapeau, B. L., Wagner, R. J. and Powell, R. J. 2007. Rapamycin promotes vascular smooth muscle cell differentiation through insulin receptor substrate-1/phosphatidylinositol 3-kinase/Akt2 feedback signaling. *J. Biol. Chem.* **282**: 36112–36120. [Medline] [CrossRef]
19. Morita, T., Okada, M., Hara, Y. and Yamawaki, H. 2011.

Mechanisms underlying impairment of endothlium-dependent relaxation by fetal bovine serum in organ-cultured rat mesenteric artery. *Eur. J. Pharmacol.* **668**: 401–406. [Medline] [CrossRef]
20. Morita, T., Yamawaki, H., Okada, M. and Hara, Y. 2010. Contractile characteristics of rat mesenteric artery after organ culture. *J. Vet. Med. Sci.* **72**: 1621–1627. [Medline] [CrossRef]
21. Mukohda, M., Yamawaki, H., Okada, M. and Hara, Y. 2010. Methylglyoxal augments angiotensin II-induced contraction in rat isolated carotid artery. *J. Pharmacol. Sci.* **114**: 390–398. [Medline] [CrossRef]
22. Mukohda, M., Yamawaki, H., Okada, M. and Hara, Y. 2010. Methylglyoxal enhances sodium nitroprusside-induced relaxation in rat aorta. *J. Pharmacol. Sci.* **112**: 176–183. [Medline] [CrossRef]
23. Nomura, H., Yamawaki, H., Mukohda, M., Okada, M. and Hara, Y. 2008. Mechanisms underlying pioglitazone-mediated relaxation in isolated blood vessel. *J. Pharmacol. Sci.* **108**: 258–265. [Medline] [CrossRef]
24. Ozaki, H. and Karaki, H. 2002. Organ culture as a useful method for studying the biology of blood vessels and other smooth muscle tissues. *Jpn. J. Pharmacol.* **89**: 93–100. [Medline] [CrossRef]
25. Régimbald-Dumas, Y., Fregeau, M. O. and Guillemette, G. 2011. Mammalian target of rapamycin (mTOR) phosphorylates inositol 1,4,5-trisphosphate receptor type 2 and increases its Ca^{2+} release activity. *Cell. Signal.* **23**: 71–79. [Medline] [CrossRef]
26. Sarbassov, D. D., Ali, S. M., Sengupta, S., Sheen, J. H., Hsu, P. P., Bagley, A. F., Markhard, A. L. and Sabatini, D. M. 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol. Cell* **22**: 159–168. [Medline] [CrossRef]
27. Tai, K., Vandenberg, G., Hamaide, M. C., Wibo, M. and Morel, N. 2009. Effect of organ culture on noradrenaline- evoked contraction, calcium signalling and TRPC expression in rat mesenteric artery. *J. Vasc. Res.* **46**: 353–364. [Medline] [CrossRef]
28. Uddman, E., Henriksson, M., Eskesen, K. and Edvinsson, L. 2003. Role of mitogen-activated protein kinases in endothelin ETB receptor up-regulation after organ culture of rat mesenteric artery. *Eur. J. Pharmacol.* **482**: 39–47. [Medline] [CrossRef]
29. Usui, T., Yamawaki, H., Kamibayashi, M., Okada, M. and Hara, Y. 2010. CV-159, a unique dihydropyridine derivative, prevents TNF-induced inflammatory responses in human umbilical vein endothelial cells. *J. Pharmacol. Sci.* **113**: 182–191. [Medline] [CrossRef]
30. Usui, T., Yamawaki, H., Kamibayashi, M., Okada, M. and Hara, Y. 2010. Mechanisms underlying the anti-inflammatory effects of the Ca^{2+}/calmodulin antagonist CV-159 in cultured vascular smooth muscle cells. *J. Pharmacol. Sci.* **113**: 214–223. [Medline] [CrossRef]
31. Yamawaki, H., Sato, K., Hori, M., Ozaki, H., Nakamura, S., Nakayama, H., Doi, K. and Karaki, H. 2000. Morphological and functional changes of rabbit mesenteric artery cultured with fetal bovine serum. *Life Sci.* **67**: 807–820. [Medline] [CrossRef]
32. Yamawaki, H., Tsubaki, N., Mukohda, M., Okada, M. and Hara, Y. 2010. Omentin, a novel adipokine, induces vasodilation in rat isolated blood vessels. *Biochem. Biophys. Res. Commun.* **393**: 668–672. [Medline] [CrossRef]
33. Zhang, Z., Zhang, G., Xu, X., Su, W. and Yu, B. 2012. mTOR-rictor is the Ser473 kinase for AKT1 in mouse one-cell stage embryos. *Mol. Cell. Biochem.* **361**: 249–257. [Medline] [CrossRef]
34. Zheng, J. P., Ju, D., Shen, J., Yang, M. and Li, L. 2010. Disruption of actin cytoskeleton mediates loss of tensile stress induced early phenotypic modulation of vascular smooth muscle cells in organ culture. *Exp. Mol. Pathol.* **88**: 52–57. [Medline] [CrossRef]