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Treatment of Normal Skeletal Muscle with FK506 or Rapamycin Results in Halothane-induced Muscle Contracture

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Background: FKBP12 is a protein that is closely associated with the ryanodine receptor type 1 of skeletal muscle and modulates Ca^{2+} release by the channel. The immunosuppressants FK506 and rapamycin both bind to FKBP12 and in turn dissociate the protein from the ryanodine receptor. By treating healthy human skeletal muscle strips with FK506 or rapamycin and then subjecting the strips to the caffeine-halothane contracture test, this study determined that FK506 and rapamycin alter the sensitivity of the muscle strip to halothane, caffeine, or both.

Methods: Skeletal muscle strips from 10 healthy persons were incubated in Krebs medium equilibrated with a 95% oxygen and 5% carbon dioxide mixture, which contained either 12 μM FK506 ($n = 8$) or 12 μM rapamycin ($n = 6$), for 15 min at 37°C. The strips were subjected to the caffeine-halothane contracture test for malignant hyperthermia according to the European Malignant Hyperthermia Group protocol.

Results: Treatment of normal skeletal muscle strips with FK506 and rapamycin resulted in halothane-induced contractures of 0.44 ± 0.16 g and 0.6 ± 0.49 g, respectively, at 2% halothane.

Conclusions: The results obtained show that pre-exposure of healthy skeletal muscle strips to either FK506 or rapamycin is sufficient to give rise to halothane-induced contractures. This is

most likely caused by destabilization of Ca^{2+} release by the ryanodine receptor as a result of the dissociation of FKBP12. This finding suggests that a mutation in FKBP12 or changes in its capacity to bind to the ryanodine receptor could alter the halothane sensitivity of the skeletal muscle ryanodine receptor and thereby predispose the person to malignant hyperthermia. (Key words: Caffeine; cyclosporin A; FKBP12; malignant hyperthermia; ryanodine receptor.)

THE 12-kd FK506 binding protein (FKBP12) has been shown to be tightly associated with the calcium release channel-ryanodine receptor type 1 (RYR1)¹ of skeletal muscle and the inositol- P_3 receptor.² FKBP12 modulates and stabilizes calcium release by these ion channels,^{2,3} a function that is independent of FKBP12's cis-trans prolyl isomerase activity.⁴ Removal of FKBP12 from RYR1 in terminal cisternae vesicles decreased the rate of Ca^{2+} uptake, and treatment of single channels decreased the open probability after caffeine activation and increased the mean open time for outward Ca^{2+} currents.^{1,3} FKBP12 can be dissociated from RYR1 by the immunosuppressive drugs FK506 and rapamycin,¹ both of which initially bind FKBP12 with high specificity, although their subsequent immunosuppressive effects are *via* different pathways.⁵ The rapamycin-FKBP12 complex inhibits the FKBP-rapamycin associated protein. The FK506-FKBP12 complex inhibits calcineurin, a Ca^{2+} -dependent serine-threonine phosphatase, in a manner similar to the cyclosporin A-cyclophilin A complex.⁶ Calcineurin has also been shown to be physiologically associated with RYR1 and the inositol- P_3 receptor and regulates the phosphorylation status of the inositol- P_3 receptor leading to changes in Ca^{2+} -sensitive regulation of inositol- P_3 -mediated Ca^{2+} flux.^{7,8}

Studies investigating the physiologic effects of FK506 and rapamycin have revealed that both drugs enhance the caffeine sensitivity of whole rat soleus muscle, which causes increased caffeine-induced developed tension. This is mediated by removal of FKBP12 from RYR1.³

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Lamb and Stephenson⁹ showed that exposure of skinned rat skeletal muscle fibers to FK506 and rapamycin results in a progressive and irreversible loss of excitation-induced Ca^{2+} release after repeated depolarizations without affecting the caffeine activation of RYR1. This finding indicates that FKBP12 may play a role in normal excitation-contraction coupling in skeletal muscle.

Alterations in the regulation of calcium levels in skeletal muscle in response to potent inhalational anesthetics such as halothane are fundamental to the malignant hyperthermia (MH) reaction.¹⁰ Linkage of MH to the RYR1 gene locus at chromosome 19q13.1 has been found,¹¹ and several mutations in the gene have been described. However, not all MH families are linked to the RYR1 locus,^{12,13} and the search for other potential gene candidates in MH continues. An area of skeletal muscle calcium release that may provide suitable candidates for MH is in proteins that modulate calcium release by the RYR1, such as FKBP12. For this reason we have used the drugs FK506 and rapamycin to manipulate the RYR1-FKBP12 interaction in healthy skeletal muscle and have used the caffeine-halothane contracture test to determine whether the treated muscle behaves in a manner similar to MH-susceptible skeletal muscle.

Materials and Methods

This study was approved by the Committee for Research on Human Subjects of the University of Witwatersrand. Muscle biopsies were obtained under local anesthesia from the vastus lateralis or quadriceps muscle of the participants for the purpose of routine testing for MH in our laboratory. The biopsy material was immediately placed in Krebs buffer equilibrated with 95% oxygen and 5% carbon dioxide before being transported to the laboratory. Strips of skeletal muscle were screened for MH using the *in vitro* caffeine-halothane contracture test according to the European Malignant Hyperthermia Group protocol.¹⁴ Briefly, strips of skeletal muscle 15–25 mm in length and 2–3 mm thick were placed in a tissue bath at 37°C. The bath was perfused with Krebs' medium and oxygenated with 95% oxygen and 5% carbon dioxide. The strips were attached to a tension transducer (type Q11; Hottinger Baldwin Messtechnik GMBH, Darmstadt, Germany) and placed under a tension of approximately 2 g. The strips were electrically stimulated with a 1-ms stimulus at 0.25 Hz such that the induced twitch tension was between 1 and 6 g. Malignant hyperthermia status was determined by the expo-

sure of at least three pairs of strips to incremental concentrations of caffeine (1 mM, 2 mM, 4 mM, 8 mM, and 16 mM in Krebs medium) or halothane (0.5%, 1%, 2%, and 4%). A fresh pair of previously untested strips from 10 persons (aged 5–58 yr) who had negative test results were then preincubated in Krebs' medium containing 12 μM of either FK506 (Fujisawa Pharmaceutical Co., Osaka, Japan; eight pairs of strips from eight healthy persons), rapamycin (Sigma Chemical Co., St. Louis, MO; six pairs of strips from six healthy persons), or cyclosporin A (Sandoz, Basel, Switzerland; four pairs of strips from four healthy persons). Incubation was at 37°C for 15 min with constant aeration using 95% oxygen and 5% carbon dioxide. FK506, rapamycin, and cyclosporin A were dissolved in absolute ethanol at a stock concentration of 1 mg/ml. Preincubated muscle strips were exposed to caffeine or halothane according to the protocol previously described for routine testing. During testing, no FK506, rapamycin, or cyclosporin A was added to the tissue bath or the perfusion medium. Testing of the exposed strips was completed within 2 h of when the samples arrived in the laboratory. In addition, three strips from three healthy persons were preincubated with 12 μM FK506 and tested by exposure to halothane according to the normal protocol but without electrical stimulation. In this case, the viability of the muscle strips was determined by a short period of electrical stimulation (1 ms, 0.25 Hz) before and after the test. Ethanol-treated control strips (four pairs of strips from four persons; 1% ethanol in Krebs medium, 37°C for 15 min) were also tested on the same apparatus with the standard protocol.

Results

Normal skeletal muscle shows no change in baseline tension when exposed to halothane (figs. 1A and 2). After treatment of normal skeletal muscle strips with FK506, exposure of the strips to increasing concentrations of halothane resulted in a progressive increase in baseline tension (an increase in mean baseline tension at 2% halothane of 0.44 ± 0.16 g; figs. 1A and 3). Treatment with rapamycin resulted in a similar pattern, with a mean increase in baseline tension at 2% halothane of 0.6 ± 0.49 g (figs. 1A and 4). All strips that were treated with FK506 or rapamycin showed contractures of at least 0.2 g at 2% halothane. In contrast, neither FK506 nor rapamycin significantly altered the response of healthy skeletal muscle to caffeine when compared with the untreated strips (figs. 1B and 2–4).

FK506 AND RAPAMYCIN CAUSE HALOTHANE-INDUCED CONTRACTURES IN SKELETAL MUSCLE

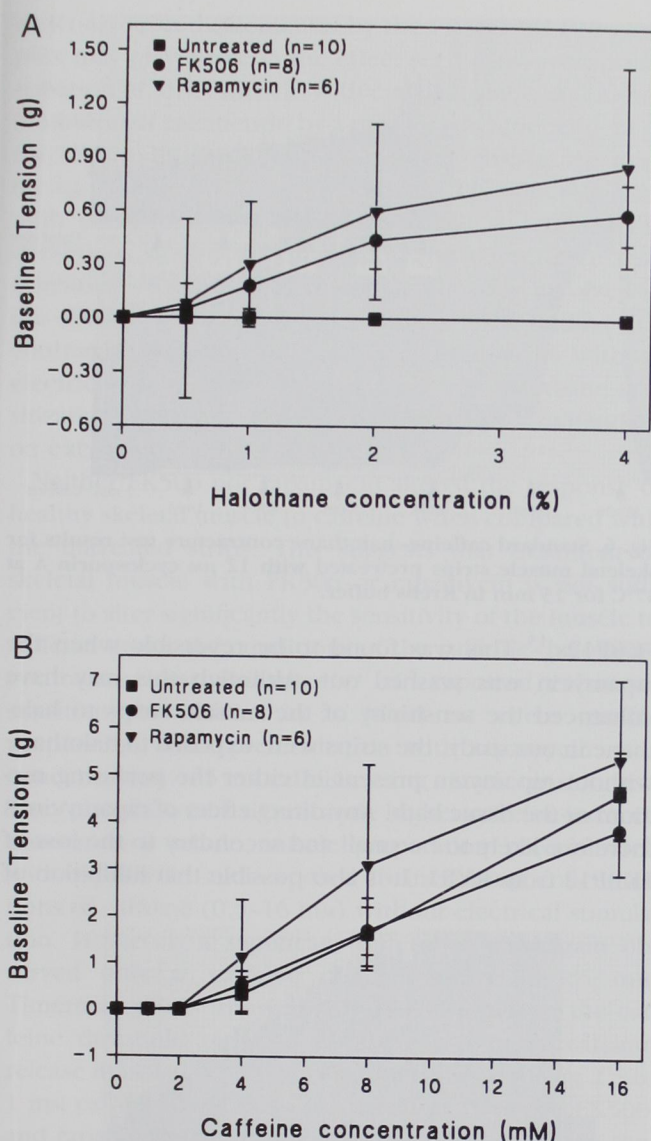


Fig. 1. Summary of increases in baseline tension (mean \pm SD) obtained in response to increased concentrations of halothane (A) or caffeine (B) for untreated skeletal muscle strips and for those treated with 12 μ M FK506 and 12 μ M rapamycin.

To determine whether the induction of contractures depended on electrical stimulation of the muscle strip, three additional strips from three persons were tested for halothane sensitivity but without electrical stimulation (fig. 5). The mean increase of baseline tension at 2% halothane was 0.43 ± 0.05 g for the three strips tested. This result indicates that 12 μ M FK506 can sensitize healthy skeletal muscle to halothane without electrical stimulation. Preincubation of healthy skeletal muscle strips with 12 μ M cyclosporin A (fig. 6) or 1% ethanol

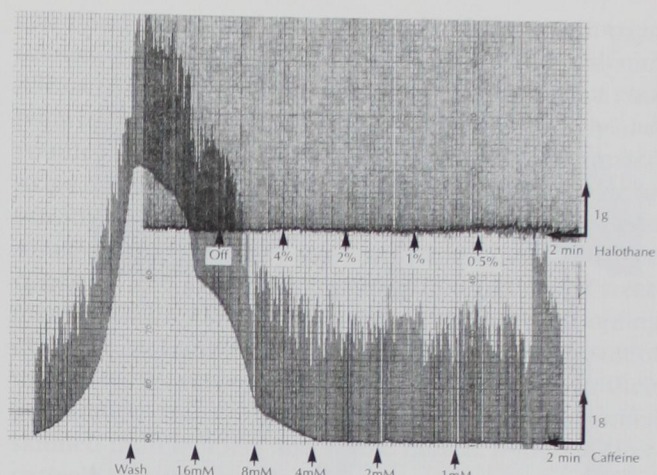


Fig. 2. Standard caffeine-halothane contracture test results for untreated skeletal muscle strips.

(fig. 7) did not sensitize the muscle strips to either caffeine or halothane.

Discussion

The results obtained in this study show that prior treatment of healthy skeletal muscle strips with FK506 or rapamycin results in a halothane-induced increase in baseline tension that is not seen in untreated muscle. The response seen is most likely due to stripping of

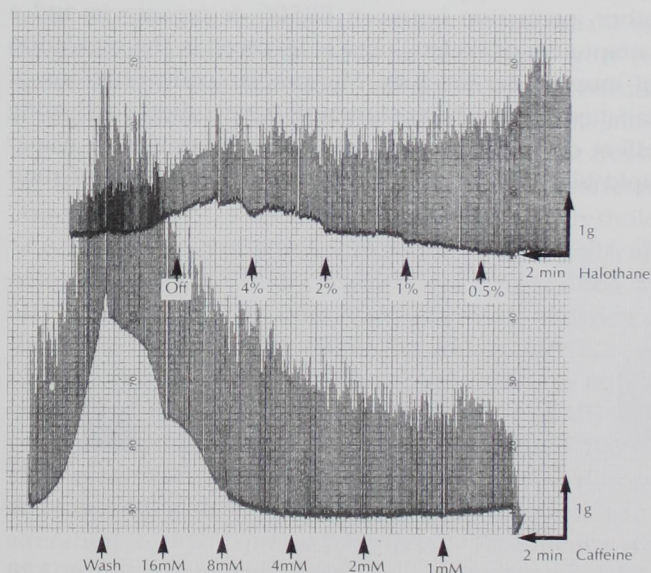


Fig. 3. Standard caffeine-halothane contracture test results for skeletal muscle strips, from the same person shown in figure 2, that were pretreated with 12 μ M FK506 at 37°C for 15 min in Krebs buffer.

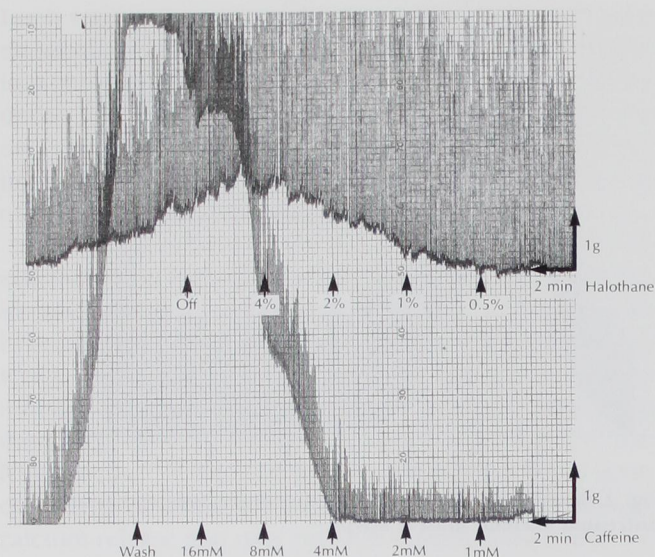


Fig. 4. Standard caffeine-halothane contracture test results for skeletal muscle strips, from the same person shown in figure 2, that were pretreated with 12 μ M rapamycin at 37°C for 15 min in Krebs buffer.

FKBP12 from the skeletal muscle ryanodine receptor by FK506 and rapamycin, sensitization of the channel to halothane, and destabilization of Ca^{2+} release. This in turn sensitizes the skeletal muscle strips to halothane and gives rise to halothane-induced contractures during the caffeine-halothane contracture test. It could be argued that the effect observed may be a result of some other interaction between FK506 or rapamycin and a component of skeletal muscle involved in the regulation of intracellular calcium. Micromolar concentrations of rapamycin have been shown to have a direct activating effect on RYR1 in terminal cisternae vesicles in planar lipid bilayers that had been stripped of >95% of their



Fig. 5. A muscle strip treated with 12 μ M FK506 and exposed to halothane according to the normal protocol but without electrical stimulation. Areas showing contractions at the start and end of the test demonstrate the viability of the muscle.

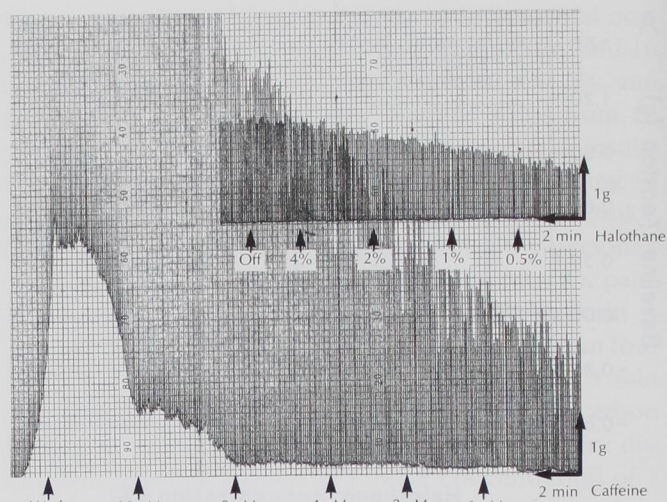


Fig. 6. Standard caffeine-halothane contracture test results for skeletal muscle strips pretreated with 12 μ M cyclosporin A at 37°C for 15 min in Krebs buffer.

FKBP12s.¹⁵ This was found to be reversible when the rapamycin was washed out. Although this may have influenced the sensitivity of the muscle strips to halothane in our study, the strips were exposed to halothane without rapamycin present in either the perfusing medium or the tissue bath. Any direct effect of rapamycin is therefore likely to be small and secondary to the loss of FKBP12 from RYR1. It is also possible that inhibition of

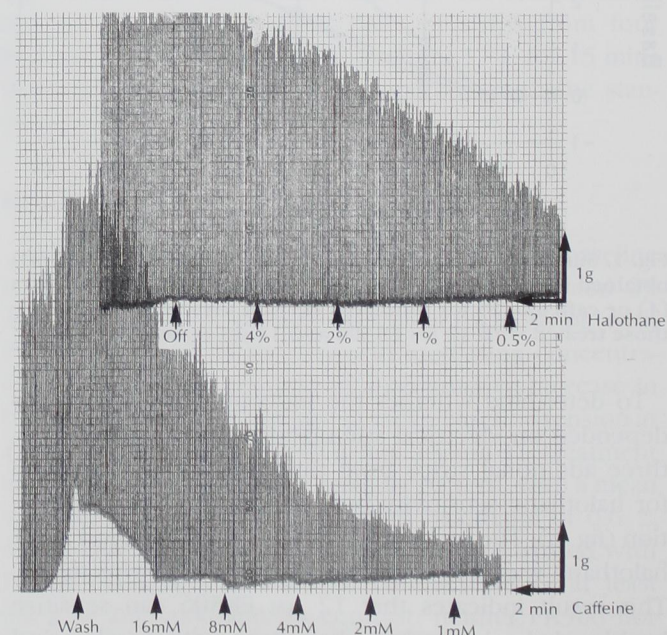


Fig. 7. Caffeine-halothane contracture test results for skeletal muscle strips, from the same person shown in figure 2, that were pretreated with 1% ethanol in Krebs medium.

FK506 AND RAPAMYCIN CAUSE HALOTHANE-INDUCED CONTRACTURES IN SKELETAL MUSCLE

RyR1-associated calcineurin by the FK506-FKBP12 complex may play a role in the effect seen. However, preincubation of MHN muscle with cyclosporin A, and hence inhibition of calcineurin by a mechanism independent of the FK506-FKBP12 complex, did not sensitize the muscle to either caffeine or halothane. This indicates that, with the protocol used in this study and under the conditions of the caffeine-halothane contracture test, inhibition of calcineurin is not significantly involved in the sensitization of RyR1 to halothane. The finding that contractures can be induced in muscle strips without electrical stimulation (fig. 5) shows that halothane sensitization occurs by a mechanism that is not dependent on excitation.

Neither FK506 nor rapamycin altered the response of healthy skeletal muscle to caffeine when compared with the untreated strips. This suggests that treatment of skeletal muscle with FK506 or rapamycin is not sufficient to alter significantly the sensitivity of the muscle to caffeine under the conditions of the caffeine-halothane contracture test. Brillantes *et al.*³ reported enhanced sensitivity to caffeine in an intact rat soleus muscle preparation, after preincubation with rapamycin, at a concentration of 5 mM caffeine using a static tension test. They also showed a significantly higher caffeine-induced tension in a whole muscle preparation preincubated with rapamycin and exposed to increasing concentrations of caffeine (0.5–16 mM) without electrical stimulation. However, a significant difference was again observed only at caffeine concentrations of ≥ 5 mM. Timerman *et al.*¹ showed that FK-506 reduced the caffeine threshold required for caffeine-induced calcium release in isolated terminal cisternae vesicles from 2.5 to 1 mM caffeine. It is possible, therefore, that the FK506- and rapamycin-treated strips in our study are more sensitive to caffeine. This increased sensitivity may be reflected in an increase in caffeine-induced contractures at concentrations of ≥ 4 mM caffeine or in a more rapid development of maximum contractures at the various caffeine concentrations used. However, given the small sample size of our study and the variability in the amplitude of the increase in caffeine-induced contractures between individual muscle strips of the same participant and between participants, it was not possible to obtain any meaningful data in this regard in this study.

As can be seen in figures 3 and 4, some strips showed a gradual loss of excitation-contraction coupling during the course of the halothane test after treatment with FK506 and rapamycin. Lamb and Stephenson⁹ have reported that repeated depolarizations of skinned rat skel-

etal muscle fibers in the presence of FK506 or rapamycin caused irreversible loss of excitation-induced calcium release. The gradual loss of excitation-contraction coupling seen in some of the treated strips may be accounted for by this finding. However, several treated strips showed no loss of coupling while still showing sensitivity to halothane. The gradual loss of coupling in some strips, therefore, may be a consequence of the condition and age of the muscle strips and not of treatment with FK506 or rapamycin. The loss of coupling observed by Lamb and Stephenson⁹ may be as a result of the constant presence of FK506 and rapamycin in their preparation, or it may reflect a difference between skinned muscle fibers and whole muscle bundles. Alternatively, a species difference between rat and human muscle may account for the apparent discrepancy of these results.

The findings we report here support the idea that FKBP12 is an important physiologic modulator of Ca^{2+} release by the skeletal muscle RyR1. This study, and others, have shown that treatment of skeletal muscle with agents that affect the interaction of FKBP12 with RyR1 resulted in an increased sensitivity of that muscle to pharmacologic stimulators of RyR1 Ca^{2+} release such as caffeine and halothane. However, the exact mechanism by which FKBP12 modulates RyR1 and the precise location of its binding site on the receptor are still unclear.

The finding that treatment of healthy skeletal muscle with FK506 and rapamycin results in halothane-induced contractures may also be important with respect to MH. The European Malignant Hyperthermia Group protocol states that the threshold for diagnosing a person as MH susceptible is a sustained increase of at least 0.2 g in baseline tension at a caffeine concentration of ≤ 1.5 mM, and a halothane threshold of 0.2 g increase of baseline tension at $\leq 2\%$ (vol/vol) halothane.¹⁴ A second classification is described as MH equivocal and is defined as individuals showing a threshold response to either halothane (MHE_H) or caffeine but not to both. Although classified as equivocal, these persons are clinically regarded as MH susceptible. In this study, all strips treated with FK506 or rapamycin showed an increase in baseline tension of at least 0.2 g at 2% halothane with no significant alteration in the response to caffeine. Treatment with FK506 and rapamycin, therefore, can produce a MHE_H phenotype in healthy muscle, with and without electrical stimulation, using current protocols to identify MH-positive persons. Assuming that dissociation of

FKBP12 from the RYR1 is responsible for our observations, it would be expected that mutations within FKBP12, or at its binding site on RYR1, which prevent proper association of the two proteins, would also produce an MHE_H test result for the caffeine-halothane contracture test. In our laboratory, approximately 15% of tested persons are categorized as MHE, with most being MHE_H. Therefore we now screen these and other MH-positive persons for mutations in the gene for FKBP12.

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