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Effect of Arg145Gly Mutation in Human Cardiac Troponin I on the ATPase Activity of Cardiac Myofibrils[1]

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In order to determine the functional consequences of the Arg145Gly mutation in troponin I found in familial hypertrophic cardiomyopathy, human cardiac troponin I and its mutant were expressed in *Escherichia coli* and purified, and then their effects on the ATPase activity of porcine cardiac myofibrillar preparations from which both troponins C and I had been depleted were examined. Both the wild-type and mutant troponin Is suppressed the ATPase activity of the troponin C·I–depleted myofibrils, but the maximum inhibition caused by mutant troponin I was weaker than that by wild-type troponin I. In the Ca^{2+}-activation profile of the myofibrillar ATPase activity after reconstitution with both troponins I and C, the Ca^{2+}-sensitivity with mutant troponin I was higher than that with wild-type troponin I, whereas the maximum level of the ATPase activity with mutant troponin I was lower than that with wild-type troponin I. These findings strongly suggest that the Arg145Gly mutation in human cardiac troponin I modulates the Ca^{2+}-regulation of contraction by impairing the interaction of troponin I with both actin-tropomyosin and troponin C.

Key words: ATPase, familial hypertrophic cardiomyopathy, myofibrils, troponin I.

The contraction of vertebrate striated muscle is regulated by Ca^{2+} through specific regulatory proteins, troponin (Tn), and tropomyosin. Tn is a complex of three subunits: Ca^{2+}-binding subunit, troponin C (TnC), inhibitory subunit, troponin I (TnI), and tropomyosin binding subunit, troponin T (TnT). The inhibitory action of TnI represents the inhibitory activity of Tn-tropomyosin in the absence of Ca^{2+} and the inhibition by TnI is released by TnC regardless of the Ca^{2+}-concentration. The full Ca^{2+}-sensitivity of the contraction, *i.e.*, inhibition in the absence of Ca^{2+} and removal of inhibition with low activation in the presence of Ca^{2+}, is recovered only in the presence of TnT. All three components of Tn are thus required for the Ca^{2+}-regulation of the contraction (*1*).

Familial hypertrophic cardiomyopathy (HCM) is an autosomal dominant cardiac disease caused by mutations in cardiac sarcomeric proteins including β-myosin heavy chain, myosin light chains, α-cardiac actin, α-tropomyosin, myosin binding protein C, TnT, and TnI (*2–9*). In the case of HCM due to mutations in TnI, six mutations have been identified (*4*). The present study was undertaken to examine the functional consequence of one (Arg145Gly) of the HCM-associated mutations of TnI. For this purpose, a recombinant TnI mutant was expressed in *E. coli* and purified, and then the effect of the mutation on the ATPase activity of myofibrils prepared from porcine cardiac muscle was examined. The inhibitory ability of TnI was found to be reduced by the Arg145Gly mutation. The increase in the Ca^{2+}-sensitivity and the suppression of the maximum activity were also demonstrated to be caused by this mutation of TnI.

The cloning and mutagenesis of human cardiac Tn cDNAs (TnC, TnI, and TnT) were carried out as described previously (*10*, *11*). Briefly, human cardiac Tn cDNAs were cloned by RT-PCR from human heart mRNA and the obtained wild-type Tn cDNAs were then constructed into a pET-3d vector for expression. To obtain mutant Arg145Gly TnI, mutagenesis was carried out by PCR. The result of the mutation in TnI cDNA was confirmed by cDNA sequencing. The wild type Tn and mutant TnI were expressed in *E. coli* BL21(DE3), and then purified using an FPLC system as described previously (*11*) (Fig. 1). The myofibrils were prepared from porcine cardiac left ventricular muscle and stored in a solution containing 50% glycerol, 100 mM KCl, 20 mM MOPS (pH 7.0), and 0.5% 2-ME at –20°C, as described previously (*11*). TnC·I–depleted myofibrils were prepared from the myofibrils according to the previously reported method with a slight modification (*12*, *13*). The myofibrils (600 μg/ml) were incubated in a solution containing 20 mM MOPS/KOH (pH 6.2), 265 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 0.5 mM 2-ME, and 50 μg/ml recombinant human cardiac TnT at 25°C for 1 h, with shaking, and then centrifuged at 10,000 rpm (TMP-11 rotor, TOMY) for 3 min at 4 °C. The sediment was washed once with a solution containing 0.5% Brij-58, 20 mM MOPS (pH 7.0), 265 mM

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Abbreviations: HCM, familial hypertrophic cardiomyopathy; Tn, troponin; TnC, troponin C; TnI, troponin I; TnT, troponin T; TnC·I, troponin C·I; MOPS, 3-(N-morpholino)propanesulfonic acid; pCa, –log[Ca^{2+}]; 2-ME, 2-mercaptoethanol.

KCl, 5 mM $MgCl_2$, and 0.5 mM 2-ME, and then twice with a solution containing 60 mM KCl and 1 mM $NaHCO_3$. The final precipitate was suspended in a small amount of a solution containing 60 mM KCl and 1 mM $NaHCO_3$. The reaction mixture (150 μl) for the ATPase assay consisted of 90 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS (pH 7.0), 1 mM Ca^{2+}-EGTA, 4 mM ATP, and 40 μg TnC·I–depleted myofibrils. The reaction was started by adding ATP at 25°C and was terminated by adding 1.0 ml of a mixture of 50% acetone, 2.5 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ and 1.25 N H_2SO_4. The contents were mixed carefully and 100 μl of 1 M citric acid was added to the tubes, and then the resultant yellow color was measured at OD_{355} (*14*). The Ca^{2+}-concentration in the reaction mixture was determined as described previously (*15*).

The bacterially expressed and purified human cardiac wild-type and Arg145Gly mutant TnIs exhibited almost identical mobility on a 15% SDS-PAGE gel (Fig. 1). Figure 2 shows the effects of these two recombinant TnIs on the ATPase activity of TnC·I–depleted myofibrils. Both TnIs suppressed the ATPase activity in a dose-dependent manner. No significant difference in the amount of TnI required for 50% inhibition (IC_{50}) was detected between the wild-type TnI (2.1 ± 0.2 μg/ml) and Arg145Gly TnI mutant (2.5 ± 0.1 μg/ml). However, the maximum inhibition caused by Arg145Gly TnI (20.3 ± 1.1 nmol P_i/mg/min) was significantly weaker than that caused by the wild-type TnI (12.7 ± 1.7 nmol P_i/mg/min).

Previous studies on rabbit skeletal TnI with 178 amino acid residues demonstrated that most of the inhibitory activity of this protein resides in a small region of residues, 96–116, called the inhibitory region (*16*). Investigation with synthetic peptides also indicated that the C-terminal portion (residues 105–114) is important for the inhibitory action of the inhibitory region of TnI (*17*). Recent work involving deletion mutants of skeletal TnI also showed that the C-terminal portion of the inhibitory region (residues 105–115) is much more critical for the inhibitory activity of TnI than the N-terminal portion (residues 96–104) (*18*). The sequence of the inhibitory region in rabbit skeletal TnI is mostly preserved in the region of residues 128–148 in human cardiac TnI, as follows.

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                    96                  100                 105
rabbit skeletal  Asn-Gln-Lys-Leu-Arg-Asp-Leu-Arg-Gly-Lys-Phe-Lys-
                          110                 113                 116
                 Arg-Pro-Pro-Leu-Arg-Arg-Val-Arg-Met
                    128
human cardiac    Thr-Gln-Lys-Ile-Arg-Asp-Leu-Arg-Gly-Lys-Phe-Lys-
                 Arg-Pro-Thr-Leu-Arg-Arg-Val-Arg-Ile
                          145                 148
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The Arg145 residue of human cardiac TnI, which corresponds to the Arg113 residue of rabbit skeletal TnI, is situated in the C-terminal portion of the inhibitory region. The present paper demonstrated that the Arg145Gly missense mutation resulted in a definite decrease in the inhibitory ability of TnI, although the amount of TnI necessary for 50% inhibition was not affected by this mutation in TnI. This finding strongly indicates that the Arg145 residue plays a critical role in the inhibitory activity of TnI and that the Arg145Gly mutation reduces the inhibitory activity of TnI by impairing the interaction with actin-tropomyosin.

Figure 3 shows the effect of Ca^{2+} on the ATPase activity of TnC·I–depleted myofibrils after reconstitution with TnI and TnC. At a low Ca^{2+}-concentration, the suppressed level of the ATPase activity with the mutant TnI was slightly higher than that with the wild-type TnI. This tendency was

a b

Fig. 1. **SDS-gel electrophoretic pattern of the recombinant human cardiac troponin I.** a) wild-type. b) Arg145Gly mutant. SDS-PAGE was performed according to the procedure of Laemmli (*23*) with an acrylamide concentration of 15%.

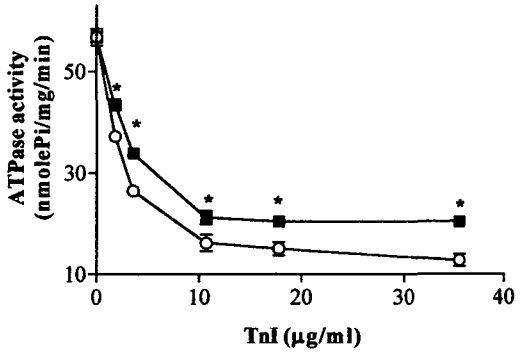

Fig. 2. **The effect of Arg145Gly TnI on the ATPase activity of TnC·I–depleted myofibrils.** Myofibrils were treated with recombinant human cardiac TnT (50 μg/ml) for 1 h at 25°C to remove endogenous TnC and I, and then reconstituted with various amounts of wild-type (○) or Arg145Gly (■) TnI for 1 h at 4°C. The ATPase activity of the treated myofibrils was measured in the absence of Ca^{2+} (pCa 7.05). The data are the means ± SE for five experiments performed in duplicate. (*) $p < 0.05$ compared with wild-type TnI-reconstituted myofibrils.

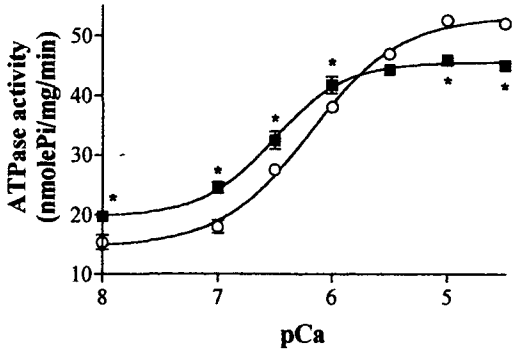

Fig. 3. **The effect of Arg145Gly TnI on the Ca^{2+}-activated myofibrillar ATPase activity.** Myofibrils were treated with recombinant human cardiac TnT (50 μg/ml) for 1 h at 25°C to remove endogenous TnC and I, and then reconstituted with wild-type (○) or Arg145Gly (■) TnI (17.8 μg/ml) and recombinant human cardiac TnC (13.1 μg/ml) for 1 h at 4°C. The Ca^{2+}-activated ATPase activity of the reconstituted myofibrils was measured at pH 7.0. The results are the means ± SE for four experiments performed in duplicate. (*) $p < 0.05$ compared with wild-type TnI-reconstituted myofibrils.

in accord with the data on the inhibitory action of TnI (without TnC) shown in Fig. 2. The maximum ATPase activity after reconstitution with the mutant TnI (44.2 ± 0.6 nmol P_i/mg/min) was reduced by about 20% compared with the maximum activity after reconstitution with the wild-type TnI (52.0 ± 1.0 nmol P_i/mg/min). This result suggests that the Arg145Gly mutation also impairs the interaction of the inhibitory region of TnI with TnC. The pCa at half-maximum activation for the ATPase activity was significantly higher for the myofibrils reconstituted with the mutant TnI (6.49 ± 0.04) than those reconstituted with the wild-type TnI (6.20 ± 0.02).

A series of studies on the effects of mutations in TnT associated with HCM have indicated that the increase in the Ca^{2+}-sensitivity of force development and/or ATPase activity is related to the pathogenesis of HCM (*10–11, 19–22*). In the present study, the Arg145Gly mutation in TnI was also demonstrated to cause an increase in the Ca^{2+}-sensitivity of the myofibrillar ATPase activity. These findings suggest that Ca^{2+} sensitization may be a common effect of HCM-associated Tn mutations. Further intensive studies are needed to clarify all the features leading to the pathogenesis of HCM.

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