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Ⅳ. 附録

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REVIEW

Biochemical and Physiological Regulation of Cardiac Myocyte Contraction by Cardiac-Specific Myosin Light Chain Kinase

Osamu Tsukamoto, MD, PhD; Masafumi Kitakaze, MD, PhD

Cardiac-specific myosin light chain kinase (cMLCK) is the kinase predominantly responsible for the maintenance of the basal level of phosphorylation of cardiac myosin light chain 2 (MLC2), which it phosphorylates at Ser-15. This phosphorylation repels the myosin heads from the thick myosin filament and moves them toward the thin actin filament. Unlike smooth muscle cells, MLC2 phosphorylation in striated muscle cells appears to be a positive modulator of Ca^{2+} sensitivity that shifts the Ca^{2+}-force relationship toward the left and increases the maximal force response and thus does not initiate muscle contraction. Recent studies have revealed an increasing number of details of the biochemical, physiological, and pathophysiological characteristics of cMLCK. The combination of recent technological advances and the discovery of a novel class of biologically active nonstandard peptides will hopefully translate into the development of drugs for the treatment of heart diseases. (*Circ J* 2013; **77:** 2218–2225)

Key Words: Ca^{2+}/calmodulin; Muscle contraction; Myosin light chain kinase; Regulatory myosin light chain

Actin and myosin are essential for the generation of contractile force. In 1954, Huxley and Hanson proposed the "sliding filament theory", which states that contractile force is generated through slippage of the cross-bridges between actin and myosin filaments[1] and that purified actin and myosin are not sufficient for active movement. In 1962, intracellular calcium ($[Ca^{2+}]i$) was identified as acting a trigger of muscle contraction.[2] Although force generation can be achieved with only myosin and actin, additional proteins are required for its regulation. In smooth muscle, an increase in $[Ca^{2+}]i$ induces the binding of Ca^{2+}/calmodulin to smooth muscle-specific myosin light chain kinase (smMLCK), which leads to the phosphorylation of cardiac myosin light chain 2 (MLC2) in thick myosin filaments, activation of the ATPase of myosin heads and initiation of muscle contraction.[3] Thus, smooth muscle cells use smMLCK as a $[Ca^{2+}]i$ sensor and initiator of muscle contraction[4] (**Figure 1**). In contrast, troponin is specifically expressed in skeletal and cardiac muscle cells and works as a sensing protein of $[Ca^{2+}]i$.[5] In contrast to smooth muscle cells, the initiation of striated muscle contraction is regulated primarily by the troponin-tropomyosin complex in thin actin filaments and not through MLC2 phosphorylation. The binding of Ca^{2+} to troponin C leads to relief of the inhibition of the binding of cross-bridges to thin actin filaments, and this relief triggers striated muscle contraction (**Figure 1**). However, endogenous expression of troponin proteins in smooth muscle cells was reported recently,[6] although the physiological role of the troponin complex in the regulation of smooth muscle contraction still remains unknown.

Following the discovery of smMLCK and skeletal muscle MLCK (skMLCK), a third MLCK, named cardiac MLCK (cMLCK), was identified in 2007[7] and is expressed exclusively in cardiac myocytes. However, what is the role of MLCK in striated muscle cells? Because both skeletal and cardiac muscle cells express specific MLCKs (ie, skMLCK and cMLCK, respectively), MLCKs might play specific physiological roles in each cell type.

Intracellular Ca^{2+} Homeostasis in Muscle Tissues

To set the stage for our discussion of MLCKs, it is necessary to understand the intracellular Ca^{2+} homeostasis in muscle tissues, because Ca^{2+} is a trigger of MLCK activation and the responses to $[Ca^{2+}]i$ differs among the 3 types of muscle cell: smooth muscle, skeletal muscle, and cardiac muscle (**Figure 1**). Local $[Ca^{2+}]i$ transients (Ca^{2+} sparks), which arise from the coordinated opening of a cluster of ryanodine-sensitive Ca^{2+} release channels, are necessary for effective Ca^{2+} signaling in all 3 types of muscle cell, although the rates of Ca^{2+} transients and contraction vary considerably in the different muscle cells. In general, however, these rates are slower in smooth muscle cells than in either skeletal or cardiac muscle cells. Smooth muscle cells gradually contract according to this slow increase in the level of $[Ca^{2+}]i$.[8] In skeletal muscle cells, the activation of the voltage-gated Ca^{2+} channel in the transverse tubule mem-

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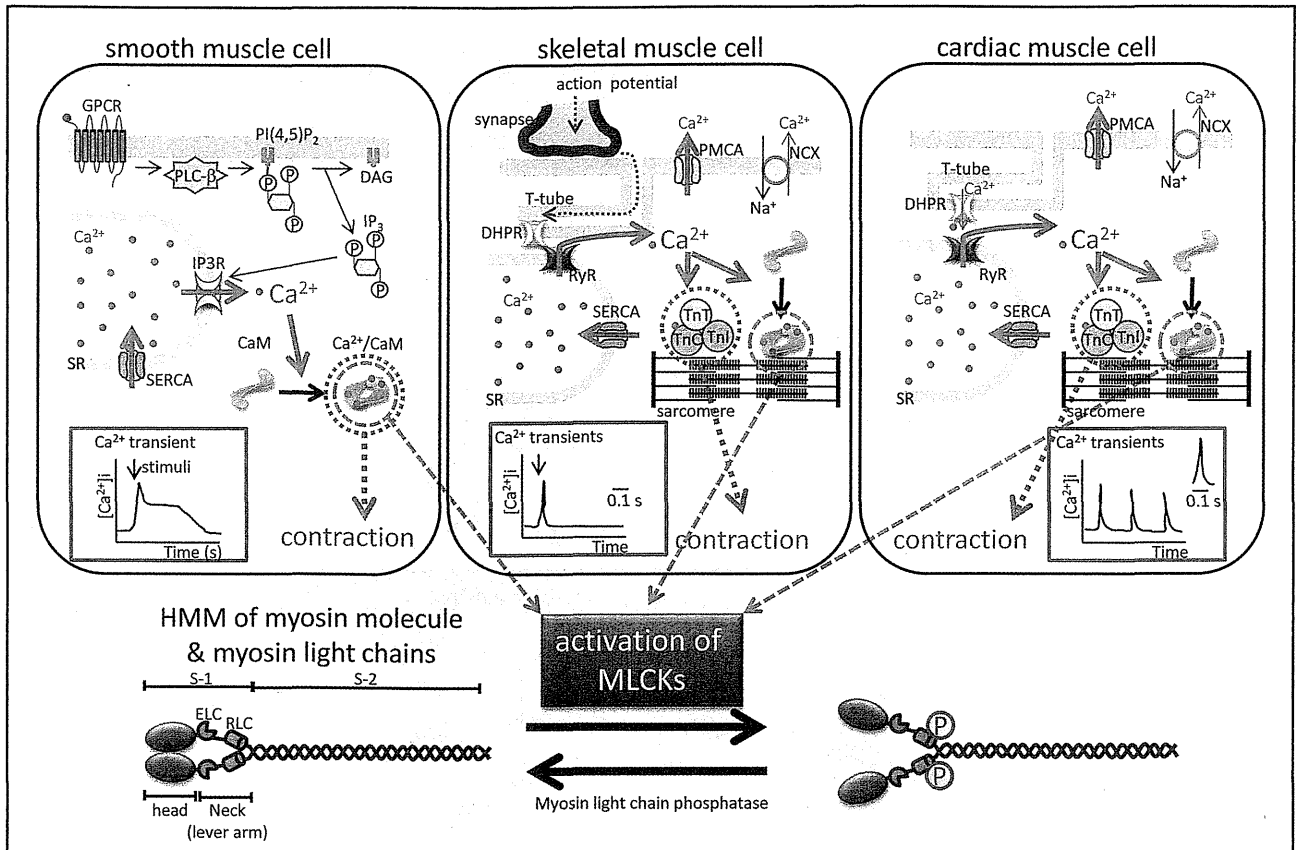

Figure 1. Intracellular calcium dynamics and muscle contraction. CaM, calmodulin; DAG, diacylglycerol; DHPR, dihydropyridine receptor; ELC, essential myosin light chain; GPCR, G protein-coupled receptor; IP3, inositol trisphosphate; IP3R, IP3 receptor; MLCK, myosin light chain kinase; NCX, sodium-calcium exchanger; PLC-b, phospholipase C beta; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; RLC, regulatory myosin light chain; RyR, ryanodine receptor; TnC, troponin C; TnI, troponin I; TnT, troponin T; T-tube, transverse tubules. For explanations, see text.

branes directly opens the ryanodine-sensitive Ca^{2+} release receptor on the sarcoplasmic reticulum (SR), which results in a rapid increase in $[Ca^{2+}]i$. In cardiac muscle cells, electrostimulation from the sinus node activates the voltage-dependent cation channel and thus increases the $[Ca^{2+}]i$ level, which induces a rapid and large release of Ca^{2+} from the SR, known as "Ca^{2+}-induced Ca^{2+} release". Importantly, the duration of the increased $[Ca^{2+}]i$ level in striated muscle cells is very short because the released Ca^{2+} is rapidly recaptured in the SR. In cardiac myocytes, electrostimulation increases $[Ca^{2+}]i$ from 100 nmol/L to a few hundred nmol/L (~1 μmol/L), which returns to the basal level within 0.1 s.

We now attempt to show why striated muscle cells do not use MLCK as a sensor of $[Ca^{2+}]i$ and an initiator of muscle contraction. The slow kinase reactions of MLCKs are not suitable as a $[Ca^{2+}]i$ sensor in striated muscles,[4] in which rapid and transient increase in the $[Ca^{2+}]i$ occurs. Instead, using a non-enzyme signal from the binding of Ca^{2+} to troponin, these cells contract promptly in response to the increased $[Ca^{2+}]i$. Thus, the increase in $[Ca^{2+}]i$ during muscle contraction exhibits distinct molecular kinetics in the 3 types of muscle, and these kinetics are related to the different physiological mechanisms that regulate contraction in these muscles.

Mechanism of MLCK Activation by Ca^{2+}/Calmodulin Binding

An increase in $[Ca^{2+}]i$ can enhance MLCK activity approximately 1,000-fold through binding to calmodulin. Ca^{2+}/calmodulin is the most important regulator of MLCKs. MLCK is catalytically inactive in the absence of Ca^{2+}/calmodulin because the autoinhibitory sequence of MLCK blocks the access of the substrate to the catalytic core (**Figure 2**).[9] The binding of Ca^{2+}/calmodulin to the calmodulin-binding domain displaces the autoinhibitory sequence from the surface of the catalytic core, which results in exposure of the catalytic site of the kinase and thus provides access to the N-terminus of MLC2.[9] MLCK is maximally activated by Ca^{2+}/calmodulin at a molar ratio of 1:1 with a dissociation constant of 1 nmol/L.[10]

Biochemistry of MLCKs

smMLCK

smMLCK is encoded by the single-copy MYLK1 gene, which expresses 3 transcripts in a cell-specific manner related to alternative initiation sites: non-muscle isoform (longer form), smooth muscle isoform (shorter form), and telokin.[11] Normally, smooth muscle expresses the shorter form of smMLCK. The smooth muscle isoform contains 3 DFRxxL motifs, a

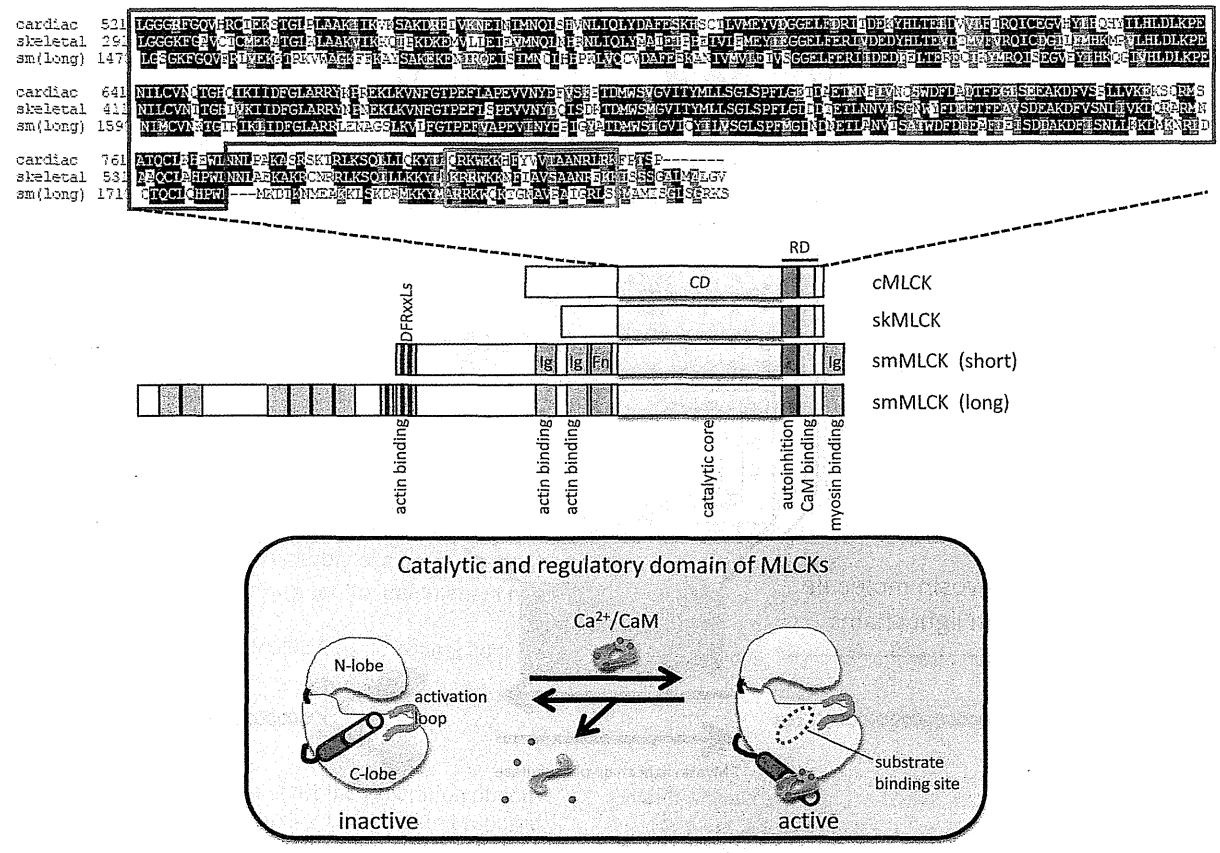

Figure 2. Structural and functional elements in myosin light chain kinase (MLCK) proteins. C, cardiac; CaM, calmodulin; CD, catalytic domain; Fn, fibronectin domain; Ig, immunoglobulin domain; RD, regulatory domain; sk, skeletal; sm, smooth muscle;. For explanations, see text.

proline-rich repeat, 3 immunoglobulin (Ig) modules, 1 fibronectin (Fn) module, and 1 kinase domain with a catalytic core and a regulatory segment. The 3 DFRxxL motifs at the N-terminus of smMLCK, which are not present in either of the striated muscle MLCKs, bind to thin actin filaments[10] through an extension of the catalytic core toward the myosin thick filaments for the phosphorylation of the smooth muscle MLC2 at Ser-19 (**Figure 2**).[12] smMLCK-induced Ser-19 phosphorylation activates myosin ATPase and initiates muscle contraction.[3] Interestingly, different kinases are also known to phosphorylate smMLC2. Rho-associated coiled-coil forming kinase (ROCK) phosphorylates smMLC2 at Ser-19 to regulate the assembly of stress fibers.[13] Protein kinase C (PKC) phosphorylates Ser-1/Ser-2/Thr-9, which inhibits myosin ATPase activity.[14] In contrast, several protein kinases, including PKA, PKC, CaMKII, and PAK, are reported to phosphorylate serine residues in the calmodulin-binding sequence in the regulatory domain in vitro, which results in a 10-fold increase in K_{cam}.[15] On the other hand, the phosphorylation of smMLCK at Thr-40 and Thr-43 by extracellular signal-regulated kinase (ERK) increases V_{max} without changing K_{CaM}.[15] ATP, which is the other substrate of MLCK, can bind to the MLCK catalytic core regardless of the positioning of the autoinhibitory sequence.[12] K_m for ATP is approximately 50–150 μmol/L.[12] The concentrations of smMLCK and its substrate, smMLC2, are approximately 4 μmol/L and 30–40 μmol/L, respectively.[8]

skMLCK

skMLCK is encoded by the MYLK2 gene and is predominantly expressed in skeletal muscle, although it was originally cloned from cardiac muscle.[16] skMLCK is reported to weakly bind to myofilaments,[10] likely because it lacks the actin binding domain that is found in smMLCK (**Figure 2**). skMLCK phosphorylates skeletal muscle MLC2 (skMLC2) at Ser-15.[17] In contrast to smMLCK, which phosphorylates only smooth muscle MLC2 efficiently, skMLCK can phosphorylate other MLC2s found in cardiac and smooth muscles that exhibit similar catalytic properties.[17] Previous physiological studies have demonstrated that the extent of MLC2 phosphorylation in skeletal muscle increases from 0–10% to 40–60% depending on the frequency of muscle stimulation.[17]

cMLCK

cMLCK is encoded by the MYLK3 gene and expressed exclusively in the heart, both the atria and ventricles.[18] cMLCK is structurally related to both skMLCK and smMLCK and contains a conserved kinase domain at its C-terminus that exhibits 58% identity with skMLCK and 44% identity with smMLCK.[7,18] However, the N-terminus of cMLCK lacks homologies to known proteins, including other MLCKs, which indicates that cMLCK may play some specific functional roles (**Figure 2**). Immunostaining of endogenous cMLCK in cardiac myocytes has shown a diffuse positive staining pattern in

Table. Biochemical Characteristics of Protein Kinases in the Heart

Kinase	Gene	Substrate	K_m (μmol/L)	V_{max} (μmol·min^{-1}·mg^{-1})	V_{max}/K_m
Cardiac MLCK	MYLK3	MLC2v	4.3±1.5	0.26±0.06	0.06
Skeletal MLCK	MYLK2	Skeletal MLC2	4.3±0.5	40±1.7	9.3
Smooth muscle MLCK	MYLK1	Smooth muscle MLC2	8.3±1.4	28±5.8	3.5
ZIPK	DAPK3	MLC2v	15.2±2.0	0.89±0.05	0.06
		Smooth muscle MLC2	1.8±0.3	0.42±0.03	0.23

MLCK, myosin light chain kinase; MLC2v, ventricular myosin regulatory chain-2; ZIPK, zipper-interacting protein kinase.

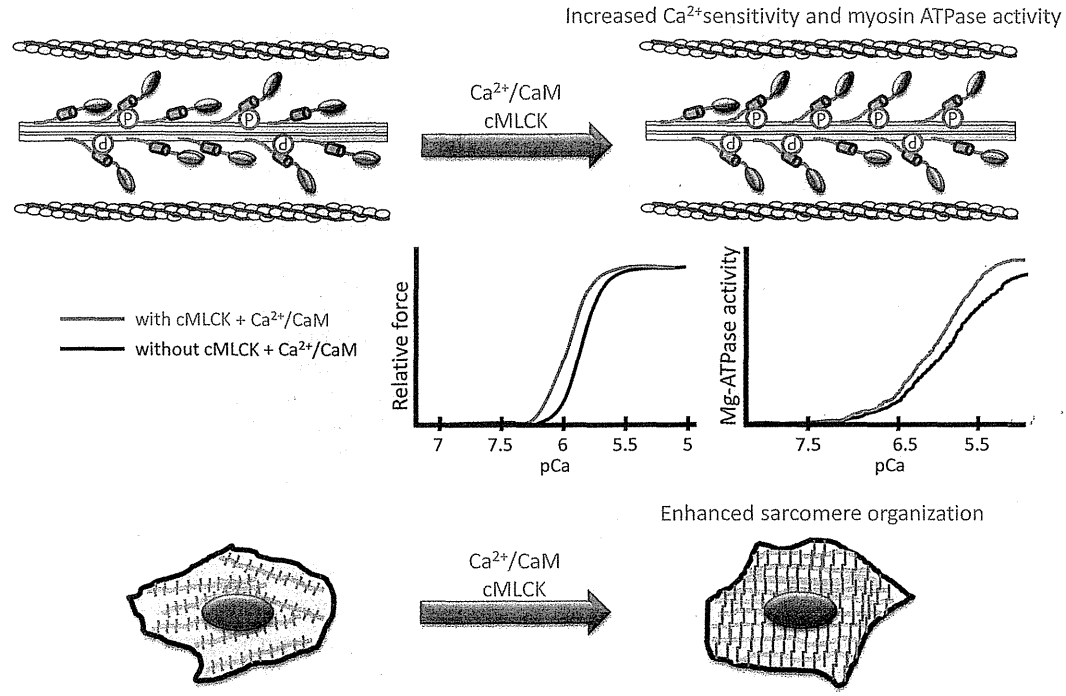

Figure 3. Physiological and biochemical effects of cardiac myosin light chain 2 (MLC2) phosphorylation by cardiac myosin light chain kinase (cMLCK) on cardiac muscle contraction. cMLCK-induced MLC2 phosphorylation enhances the contraction of cardiac myocytes probably via a combination of increased Ca^{2+} sensitization, myosin Mg-ATPase activity, and sarcomere structural organization. CaM, calmodulin. For explanations, see text.

the cytoplasm with a striated staining pattern in the cell periphery.[18] Interestingly, the striated MLCK staining colocalized with actin but not with its substrate.[18] Chan et al reported the independence of cMLCK activity from Ca^{2+}/calmodulin,[18] which was an unexpected result, because cMLCK also contains both autoinhibitory and calmodulin-binding sequences, similar to the other 2 types of MLCKs, and binds to calmodulin with high affinity in a Ca^{2+}-dependent manner.[7,19] There are conflicting results concerning the Ca^{2+}/calmodulin dependency of cMLCK activity,[7,19] and thus requires further examination.

The **Table** demonstrates the estimated kinetic constants of each MLCK determined by Lineweaver-Burk plots.[18,20–22] cMLCK has a high affinity for its substrate, similar to skMLCK and smMLCK. However, the catalytic efficiency of cMLCK, which is indicated by the V_{max}/K_m ratio, is lower than that of skMLCK and smMLCK. Thus, the maximal specific kinase activity of cMLCK is much lower than that of smMLCK and skMLCK. However, the low specific activity of cMLCK results in a slow turnover of phosphate in MLC2 (t1/2=250 min), with an MLC2 basal phosphorylation of approximately 0.2–0.4 mol of phosphate/mol of MLC2 under basal conditions,[23–26] which indicates that the kinase activity of cMLCK may be a primary limiting factor of MLC2 phosphorylation.[19]

Structural Changes of Myosin Head Induced by MLC2 Phosphorylation in Striated Muscles

Muscle myosin is the molecular motor in the thick filament of the sarcomere and is composed of 1 pair of myosin heavy chains (MHC) and 2 pairs of myosin light chains (MLC): essential MLC and regulatory MLC (ie, MLC2) (**Figure 1**). Both of the MLCs wrap around the neck region of the MHC. MLC2 is positioned at the S1–S2 junction of the MHC through its binding to a 35-amino-acid IQ motif on the MHC.[27] The MLC2

contains a highly conserved serine that is phosphorylatable by MLCK and plays an important role in the activation and modulation of myosin by fine-tuning the motion of the neck region of the MHC.[10] The MLC2 also contains a Ca^{2+}/Mg^{2+} binding site at its N-terminus from Asp-37 to Asp-48, located in the first helix-loop-helix motif, and the binding of divalent cation alters the structural and contractile properties.[28,29] The neck region of the myosin head has been proposed to act as a lever arm. The phosphorylation of MLC2 at Ser-15 results in the addition of a negative charge to the N-terminal region of MLC2, which induces the myosin head to swing out from a position close to the thick filament's backbone toward the actin filament, and this structural change increases the rate through which the myosin-actin interaction occurs and promotes force generation at a given level of Ca^{2+} (**Figures 1,3**).[30,31] Interestingly, several mutations around the phosphorylatable Ser-15 and the Ca^{2+} binding site in MLC2 have been found in patients with familial hypertrophic cardiomyopathy.[32–34]

PKs and Protein Phosphatase Regulation of MLC2 Phosphorylation in the Heart

There are 2 types of cardiac MLC2: a ventricular myosin light chain-2 (MLC2v) and an atrium-specific form (MLC2a).[35] All 3 MLCKs are expressed in the heart. However, the amount of skMLCK in the heart is too low to maintain cardiac MLC2 phosphorylation,[36] and ablation of skMLCK has no effect on MLC2 phosphorylation in cardiac muscle,[36] which indicates that skMLCK does not play a major role in MLC2v phosphorylation. The expression of smMLCK in the heart is also 10- to 20-fold lower than that of cMLCK,[18] and cardiac MLC2 is not a good substrate for smMLCK.[36] smMLCK may phosphorylate non-muscle cytoplasmic myosin II-B and plays an important role in the heart.[37] Thus, there are currently 2 candidate kinases for MLC2 phosphorylation in cardiac myocytes: cMLCK[7,18] and zipper-interacting PK (ZIPK).[38] ZIPK is a Ca^{2+}-independent serine/threonine kinase that has been implicated in apoptosis and is ubiquitously expressed, including in adult and neonatal cardiac myocytes.[39] In permeabilized smooth muscle cells, constitutively active ZIPK initiates contraction through the phosphorylation of smooth muscle MLC2.[40] Cardiac MLC2 was also identified as a biochemically favorable substrate for ZIPK through an unbiased substrate search with purified ZIPK on heart homogenates and was phosphorylated at Ser-15 in vivo and in vitro by ZIPK.[38] In fact, V_{max} for cardiac MLC2 is 2-fold greater than that obtained for smooth muscle MLC2 (887±47 and 415±49 $nmol \cdot min^{-1} \cdot mg^{-1}$, respectively), whereas the K_m of cardiac MLC2 is higher than that of smooth muscle MLC2 (15.0±2.0 and 1.8±0.3 μmol/L, respectively).[38] However, ZIPK is considered not to be involved in the basal phosphorylation of cardiac MLC2 in vivo,[41] although knockdown of ZIPK in cardiac myocytes by siRNA decreased the extent of cardiac regulatory MLC phosphorylation by 34%.[38] The physiological role of ZIPK-induced cardiac MLC2 phosphorylation remains unknown.

It is now well established that cMLCK is the predominant cardiac MLC2 kinase responsible for basal phosphorylation in vivo, because the ablation of cMLCK almost completely abolishes the phosphorylation of MLC2v.[7,18,25,41] Ding et al demonstrated that a partial reduction in the amount of cMLCK protein in cMLCK hetero knockout mice ($cMLCK^{+/neo}$) resulted in a partial reduction in MLC2 phosphorylation in both ventricular and atrial muscles, and this reduction in MLC2 phosphorylation was proportional to the cMLCK expression level.[41] In addition, the overexpression of cMLCK increases MLC2v phosphorylation in a dose-dependent manner and, conversely, knockdown of cMLCK by RNAi decreases MCL2v phosphorylation in vitro.[18] Scruggs et al identified 3 distinct charge variants of endogenous MLC2v in vivo in the mouse: unphosphorylated, singly phosphorylated, and doubly phosphorylated at Ser-14/Ser-15.[42] In contrast, Ser-14 in murine MLC2v is replaced by Asn in human MLC2. However, human MLC2 also has 3 distinct charge variants in vivo: unphosphorylated, singly phosphorylated at Ser-15, and deamidated Asn-14/phosphorylated Ser-15.[42] Interestingly, the deamination of Asn to Asp can create a negative charge similar to that obtained through phosphorylation.[26] In addition, there is a spatial gradient of MLC2v phosphorylation through the ventricular wall: relatively low in the inner layer and high in the outer layer.[16,43] This gradient, which may be caused by reduced activity of the phosphatase in the outer layer,[44,45] may be important for normalizing wall stress and contributes to efficient contraction of the whole heart.[16]

The level of MLC2v phosphorylation is maintained relatively constant by the appropriate balance between phosphorylation by cMLCK and dephosphorylation by phosphatase in the physiologically constant beating heart. The dephosphorylation of MLC2v is mediated mainly by catalytic subunit of type 1 phosphatase (PP1c-δ) in concert with myosin phosphatase target subunit 2 (MYPT2).[46] In contrast, MLC2 phosphorylation by MLCK is transient in both smooth and skeletal muscle cells, although they have different MLC2 phosphorylation dynamics.[19] MLC2 phosphorylation is rapidly dephosphorylated by robust myosin light chain phosphatase activity in smooth muscle cells, whereas MLC2 phosphorylation is prolonged and slowly dephosphorylated by the low myosin light chain phosphatase activity in skeletal muscle cells.

Physiological Regulators of cMLCK Expression and Activity

The expression of cMLCK is highly regulated by the cardiac homeobox protein Nkx2-5 in neonatal cardiac myocytes.[18] cMLCK mRNA increases during development to adult stages and persists in the aged heart, whereas its protein level decreases in the aged heart, which suggests an alternation in post-transcriptional regulation.[18] Exercise has been reported to increase cMLCK expression and MLC2v phosphorylation.[25] Catalytic activity may be regulated by phosphorylation through upstream kinases.[47] cMLCK has several potential phosphorylation sites for other kinases, such as PKA and PKC,[18] although more studies are required to clarify the details of this mechanism.

Hypertrophic agonists, such as α1- or β1-adrenergic stimulation[18,48,49] and angiotensin II, induce MLC2v phosphorylation through MLCK activation in both cultured cardiac myocytes and the adult heart in vivo.[24] Other neurohumoral stimulators, such as endothelin[50] and prostanoid F receptors,[51] have also been reported to increase MLC2 phosphorylation and induce a prominent increase in the contractile force in the heart. Neuregulin, which activates ErbB receptor tyrosine kinase, has been reported to enhance the expression of cMLCK with a concomitant increase in MLC2v phosphorylation and improved cardiac performance after myocardial infarction in rats.[52]

Role of MLC2 Phosphorylation in Sarcomere Organization and Heart Development

The transfection of cardiac myocytes with skMLCK has resulted in the phosphorylation of MLC2v and led to a highly

organized sarcomeric pattern without induction of other hypertrophic phenotypes, such as the induction of fetal genes and an increase in cell size.[24] Furthermore, the dominant-negative kinase-inactive form of MLCK completely prevents sarcomere organization in response to angiotensin II,[24] which suggests that MLCK activation is necessary and sufficient to induce sarcomere organization (**Figure 3**). Consistent with these findings, the adenovirus-mediated overexpression of cMLCK in cardiac myocytes promotes sarcomere organization characterized by straight, thick, striated actin bundles,[18] whereas re-establishment of the phenylephrine-induced sarcomere structure is inhibited by pretreatment with RNAi against cMLCK.[7] Interestingly, the reduced cMLCK expression induced by the antisense morpholino causes severely impaired heart development in zebrafish and histological analysis showed that the structure of the sarcomere was poorly developed compared with control zebrafish.[7] However, studies with transgenic and knockout mice have shown that MLC2v phosphorylation is not critical for cardiogenesis in the mammalian system,[18,41,53] although it is necessary for the optimal contractile performance of the heart.

Physiological Role of MLC2 Phosphorylation in the Heart

The degree of MLC2 phosphorylation is known to play a critical role in the determination of the Ca^{2+}-sensitive cross-bridge transition in skeletal muscle.[54] In permeable cardiac muscle fibers, MLC2 phosphorylation induced by MLCK increases the Ca^{2+} sensitivity, which manifests as a leftward shift in the force-Ca^{2+} relationship, and MLC2 dephosphorylation by phosphatase decreases the Ca^{2+} sensitivity (ie, dephosphorylation resulted in Ca^{2+} desensitization).[55,56] MLC2 phosphorylation by MLCK is also associated with enhanced Ca^{2+}-stimulated myosin Mg-ATPase activity in rat cardiac myofibrils (**Figure 3**).[57] In the rat heart, MLC2v phosphorylation increases in response to an increase in the beat frequency and/or left ventricular pressure by exercise or inotropic agents, which may help augment the peak left ventricular pressure.[58,59] At the cellular level, adenovirus-mediated overexpression of cMLCK potentiates the amplitude of the contraction of cardiac myocytes and the kinetics of contraction and relaxation without changing the $[Ca^{2+}]i$ transients.[18]

Several studies have also addressed the role of MLC2 phosphorylation in the heart using genetically modified mice. Huang et al[60] generated transgenic mice expressing skMLCK specifically in cardiac myocytes (TG-skMLCK). These TG-skMLCK mice demonstrated marked increases in the phosphorylation of both cardiac MLC2 and cytoplasmic non-muscle MLC2 in the heart without significant cardiac hypertrophy or structural abnormalities up to 6 months of age, which indicates that increased cardiac MLC2 phosphorylation per se does not cause cardiac hypertrophy.[60] Interestingly, the hypertrophic cardiac response to exercise and isoproterenol treatment was attenuated in TG-skMLCK mice,[26] which supports the hypothesis that the phosphorylation of cardiac MLC2 may inhibit physiological and pathophysiological hypertrophic responses through enhanced contractile performance and efficiency. Sanbe et al[53] created transgenic mice (TG-MLC2v(P-)) in which 3 potentially phosphorylatable serines (Ser-14/Ser-15/Ser-19) in the MLC2v (ventricular regulatory myosin light chain) were mutated to alanine. After MLCK treatment, the isolated ventricular fibers from the non-transgenic control mice showed increased Mg-ATPase activity and Ca^{2+} sensitivity, as indicated by a leftward shift in the force-Ca^{2+} curve, whereas the fibers from the TG-MLC2v(P-) mice did not exhibit these increases.[53] This suppressed performance of muscle fibers with nonphosphorable MLC2v is consistent with the demonstrated effects of MLC2 phosphorylation in skeletal muscle.[36] Scruggs et al[48] examined the role of regulatory myosin light chain 2 phosphorylation in the ejection of the hearts of TG-MLC2v(P-) mice by measuring the systolic mechanics under basal conditions and in response to adrenergic stimulation. The TG-MLC2v(P-) mice demonstrated depressed contractility, decreased maximal left ventricular power development, and a decrease in the time-to-peak elastance during ejection under basal conditions.[48] Interestingly, the TG-MLC2v(P-) mice exhibited a blunting of the positive inotropic response to β1-adrenergic stimulation.[48] Because cMLCK has multiple PKA consensus sequences in its unique N-terminus region,[26] and β1-adrenergic stimulation increased MLC2v Ser-15 phosphorylation in hearts of non-transgenic control mice[48], there might be a possible relationship between β1-adrenergic signaling and MLC2v phosphorylation. The TG-MLC2v(P-) mice developed cardiac hypertrophy at 3–4 months of age, most likely because of a compensatory hypertrophic growth response to diminished contractile performance.[53] cMLCK-knockout mice ($cMLCK^{neo/neo}$) also demonstrated the critical role of cMLCK in normal physiological cardiac function, with decreased cardiac performance and the induction of cardiac hypertrophy at 4–5 months of age.[41] In contrast, transgenic mice overexpressing MYPT2 specifically in cardiac myocytes demonstrated enhanced expression of MYPT2 with a concomitant increase in the level of endogenous PP1c-δ, which resulted in a reduction of the level of in vivo MLC2v phosphorylation in the heart associated with a decrease in the myofilament response to Ca^{2+} and a decreased left ventricular contractility.[61] These findings are consistent with the evidence obtained from TG-MLC2v(P-) mice and cMLCK-knockout mice as mentioned earlier.

Surprisingly, Warren et al recently reported an inverse relationship between cMLCK expression and systolic pressure: cMLCK expression is higher in the right ventricular myocardium than the left ventricular myocardium.[25] Because the cMLCK expression in the left ventricle was markedly down-regulated as a result of pressure overload, those authors speculated that increased mechanical stress reduces the net expression of cMLCK.[25]

cMLCK in Heart Diseases

cMLCK was first identified through the integrated cDNA expression analysis of failing human myocardium, which showed that the cMLCK mRNA expression levels correlated well with the pulmonary arterial pressure of patients with heart failure.[7] Decreased phosphorylation of MLC2 was also reported in some patients with heart failure.[62,63] In animal models of myocardial infarction, the expression level of cMLCK in the heart has been found to be reduced.[18,52] In addition, pressure overload also led to a marked reduction in cMLCK and phosphorylated MLC2v in the heart 1 week after thoracic aortic constriction surgery.[25] Interestingly, the reduction in the cMLCK protein level in pressure-overloaded hearts was mediated by upregulation of the ubiquitin-proteasome degradation system.[25] The specific overexpression of cMLCK in cardiac myocytes attenuated the phenotype of the pressure overload-induced heart failure,[25] and this finding suggests a protective role of cMLCK against cardiac stress.

Induced pluripotent stem cell-derived cardiac myocytes (iPSC-CMs) are expected to become a new cell therapy for

heart diseases and iPSC-CMs express cardiac-specific proteins similar to neonatal cardiac myocytes.[64] However, so far there are no reports of investigations into cMLCK and MLC2 phosphorylation in iPSC-CMs.

New Approach to Finding Potential Inhibitors or Activators of Kinases

Recently, Suga et al developed a new technology to discover "natural product-like" nonstandard peptides against various therapeutic targets, and this technology, which is known as the RaPID (Random non-strand Peptides Integrated Discovery) system, comprises a FIT (Flexible in vivo Translation) system coupled with an mRNA display.[65] Using this system, Suga et al successfully isolated anti-E6AP macrocyclic *N*-methyl-peptides, one of which had high affinity (K_d=0.60 nmol/L) against a target E6AP and strongly suppressed its polyubiquitination ability against p53.[66] Because the targets of the RaPID system are not limited, this technology can be applied to the discovery of kinase activators or inhibitors.

Conclusion

We are now attempting to find a new potential activator or inhibitor of cMLCK, which will hopefully either provide new insights to cMLCK or be successfully applied in the clinical setting.

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Conflict of Interest: The authors have no conflicts of interest to declare.

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Letter to the Editor

Decreased serum brain-derived neurotrophic factor levels are correlated with exercise intolerance in patients with heart failure

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Exercise intolerance is a major clinical manifestation, and closely related to poor prognosis in heart failure (HF) patients [1]. Various factors, especially abnormalities in the skeletal muscle, have been shown to be involved in exercise intolerance [2,3]. However, its mechanism in HF has not been elucidated.

Brain-derived neurotrophic factor (BDNF), originally discovered in the brain as a member of neurotrophin family, has been demonstrated to regulate various neurotrophic functions including neuroregeneration, neuroprotection, and synaptic plasticity [4]. Serum BDNF levels have been shown to be decreased in patients with major depression [5]. On the other hand, BDNF is produced in skeletal muscle cells in response to contraction in association with enhancing fat oxidation [6], and serum BDNF levels increase by exercise training [7]. These findings raise the possibility that BDNF may be involved in lowered exercise capacity in HF patients. In the present study, we thus determined whether serum BDNF levels were decreased in HF patients compared to normal subjects and correlated with clinical variables including exercise capacity, glucose and fat metabolisms, cardiac function, and depressive symptoms.

Forty three consecutive patients with HF (33 men, 57.4 ± 14.8 years, left ventricular ejection fraction (LVEF) 33.2 ± 11.8%) and 27 age-matched healthy individuals as controls (23 men, 52.2 ± 8.8 years, LVEF 61.0 ± 5.8%) were studied. Three HF patients were New York Heart Association functional class I, 28 patients class II, and 12 patients class III. The etiologies of HF were ischemic heart disease in 12 and others in 31 patients. Informed consent was obtained from all subjects and the study protocol, conformed to the ethical guidelines of the Declaration of Helsinki, was approved by the ethics committee of Hokkaido University Hospital. Blood samples were collected and serum BDNF levels were determined by an enzyme immunoassay kit (R&D System, Inc., Minneapolis, USA). Platelets, total cholesterol, triglycerides, fasting blood glucose, HbA1c, and B-type natriuretic peptide (BNP) were also measured. The estimated glomerular filtration rate (eGFR) and the homeostasis model assessment of insulin resistance (HOMA-IR) index were calculated. Whole-body fat weight was measured by an air displacement plethysmography. LV end-diastolic (LVEDD) and LVEF were measured by transthoracic echocardiography. Cardiopulmonary exercise testing was performed to determine oxygen uptake (VO_2) and aerobic threshold (AT) using an ergometer with ramp protocol. Depressive symptoms, anxiety, HF-specific quality of life were assessed by Beck Depression Inventory-II (BDI-II), Patient Health Questionnaire (PHQ-9), State-Trait Anxiety Inventory (STAI), and Minnesota Living with Heart Failure Questionnaire (MLHFQ). Based on the previous study by Karege et al. [5], the needed numbers of study subjects were calculated to be 40 for HF and 26 for control ($\alpha = 0.05$, $\beta = 0.2$ and allocation ratio = 1.5 (HF/control)). Data are expressed as means ± SD. Student's t test was used to compare continuous variables. Linear regression analysis was used to determine the correlation between two variables. Multivariate linear regression analysis, including variables with a p value < 0.05 in the univariate model or clinical parameters, was performed to identify the independent variables associated with serum BDNF. All analyses were performed using JMP 9.0 (SAS Institute, Cary, NC, USA) and p-values < 0.05 were considered statistically significant.

Serum BDNF levels were significantly lower in HF patients compared to control subjects (17.5 ± 4.9 vs. 24.6 ± 5.8 ng/mL, $P < 0.001$) (Fig. 1A). By univariate analysis, there was a significant positive correlation between serum BDNF levels and peak VO_2 ($r = 0.553$, $P < 0.001$)

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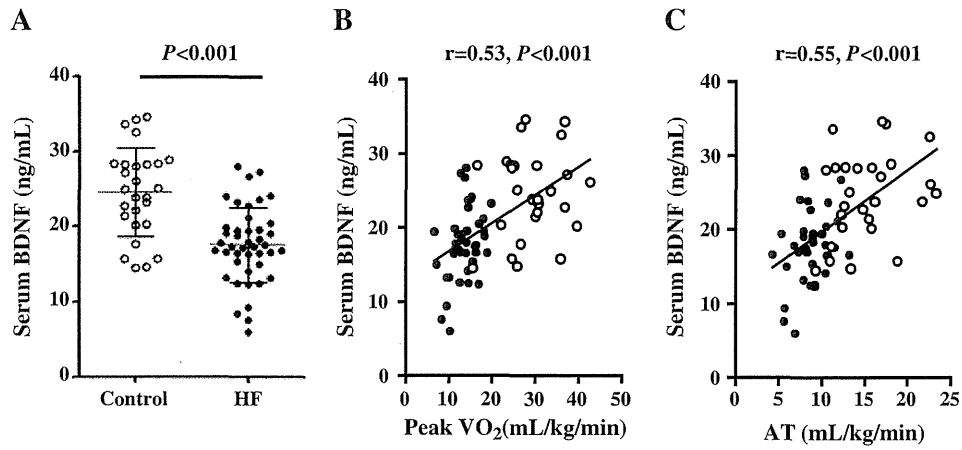

Fig. 1. (A) Serum BDNF levels in control subjects (**open circles**, $n = 27$) and patients with HF (**closed circles**, $n = 43$). Correlation between serum BDNF levels and peak VO_2 (B) and AT (C) in normal subjects (**open circles**, $n = 27$) and in patients with HF (**closed circles**, $n = 43$) BDNF, brain-derived neurotrophic factor; HF, heart failure; VO_2, oxygen uptake; AT, anaerobic threshold.

or AT ($r = 0.554$, $P < 0.001$) among all study subjects (Fig. 1B, C). Peak VO_2, AT, and triglyceride were also significantly correlated with serum BDNF in HF patients, but not age, body mass index, fat weight, LVEDD, LVEF, eGFR, HbA1c, HOMA-IR, and plasma BNP (Table 1). There was no significant correlation between serum BDNF levels and psychological status including BDI-II scores, PHQ-9 scores, STAI scores, or MLFHQ (Table 1). By multivariate analysis, peak VO_2 (β-coefficient = 5.03, 95%CI [1.64, 8.42], $P = 0.004$) and triglyceride (β-coefficient = 3.99, 95%CI [0.56, 7.42], $P = 0.023$) were identified as independent determinants of serum BDNF levels (Table 1).

BDNF is shown to be produced in the skeletal muscle, and increased by muscle contraction to enhance fat oxidation in an AMPK-dependent fashion, which can regulate glucose and fat metabolism [6,8]. Furthermore, serum BDNF levels and its expression in the skeletal muscle can be enhanced by exercise training [7,9]. Therefore, these findings suggest that the decrease in BDNF levels may be due to the physical inactivity in HF patients and that reduced BDNF could impair the metabolism of the skeletal muscle and lead to exercise intolerance associated with HF (Fig. 1, Table 1). Alternatively, a beneficial effect of exercise training on HF might be due to BDNF release from the skeletal muscle. There was also positive correlation between serum BDNF and triglyceride among HF patients (Table 1). In contrast, there was no significant correlation between serum BDNF levels and other parameters reflecting glucose and fat metabolism (Table 1). These results suggest that BDNF may play a partial role in metabolic derangements [8]. Further studies are necessary to better understand the pathophysiology underlying this causal relationship between serum BDNF level and exercise intolerance or metabolic abnormalities in HF.

BDNF has been shown to be linked to the pathophysiology of psychiatric disorders, and serum BDNF levels were decreased in patients with major depression [5]. We thus investigated the association between serum BDNF and depressive symptom score in HF patients. However, there was no significant correlation between them (Table 1). This discrepancy might be due to small number of HF patients with significant depressive symptoms or due to the difference of severity of depression in the present study, suggesting that decreased serum BDNF levels in HF

Table 1
Univariate and multivariate analyses of clinical variables for correlates with serum BDNF in patients with HF.

Variable	Univariate		Multivariate		
	Correlation coefficient	*P* value	β-coefficient	95%CI	*P* value
Age, years	0.023	0.882	3.07	−0.05–6.20	0.054
Gender (male = 0)	0.210	0.818	0.01	−1.67–1.70	0.985
BMI, kg/m^2	0.065	0.677	1.15	−2.06–4.38	0.470
Fat weight, kg	0.024	0.844			
LVEDD, mm	0.130	0.405			
LVEF, %	0.023	0.883	−2.31	−5.37–0.75	0.134
Peak VO_2, mL/kg/min	0.372	0.016	5.03	1.64–8.42	0.004
AT, mL/kg/min	0.324	0.043			
eGFR, mL/min^{-1}/1.73 m^{-2}	0.182	0.241			
Platelet, $\times 10^5/\mu L$	0.200	0.197			
Total cholesterol, mg/dL	0.300	0.050			
Triglyceride, mg/dL	0.455	0.002	3.99	0.56–7.42	0.023
Blood glucose, mg/dL	0.058	0.708			
HbA1c, %	0.182	0.241			
HOMA-IR	−0.040	0.974			
Plasma BNP, pg/mL	−0.138	0.377			
BDI-II score	−0.067	0.672			
PHQ-9 score	0.078	0.620			
STAI-state	−0.119	0.450			
STAI-trait	0.042	0.790			
MLHFQ	−0.058	0.712			

Male and female were assigned values of 0 and 1, respectively. BDNF, brain-derived neurotrophic factor; HF, heart failure; CI, confidence interval; BMI, body mass index; LV, left ventricular; EDD, end-diastolic diameter; EF, ejection fraction; VO_2, oxygen uptake; AT, anaerobic threshold; eGFR, estimated glomerular filtration rate; HOMA-IR, the homeostasis model assessment of insulin resistance; BNP, brain natriuretic peptide; BDI, Beck Depression Inventory; PHQ-9, Patient Health Questionnaire 9; STAI, State-Trait Anxiety Inventory; MLHFQ, Minnesota Living with Heart Failure Questionnaire.

patients may not reflect brain BDNF levels. Previous study has demonstrated that systemic ablation of BDNF induces the exacerbation of cardiac dysfunction after myocardial infarction [10]. However, the present study did not demonstrate the correlation between serum BDNF levels and cardiac function.

In conclusion, serum BDNF levels correlated with exercise capacity in HF, suggesting that serum BDNF could be a useful marker for predicting exercise capacity and the long-term outcomes of HF patients.

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The Impact of Renal Tubular Damage, as Assessed by Urinary β2–Microglobulin–Creatinine Ratio, on Cardiac Prognosis in Patients With Chronic Heart FailureClinical Perspective

Yoichiro Otaki, Tetsu Watanabe, Tetsuro Shishido, Hiroki Takahashi, Akira Funayama, Taro Narumi, Shinpei Kadowaki, Hiromasa Hasegawa, Shintaro Honda, Shunsuke Netsu, Mitsunori Ishino, Takanori Arimoto, Takehiko Miyashita, Takuya Miyamoto, Tsuneo Konta and Isao Kubota

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Original Article

The Impact of Renal Tubular Damage, as Assessed by Urinary β_2-Microglobulin–Creatinine Ratio, on Cardiac Prognosis in Patients With Chronic Heart Failure

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Background—Renal dysfunction was reported to be closely associated with clinical outcomes in patients with chronic heart failure (CHF). Renal tubulointerstitial damage has been shown to be an important factor in the development of renal dysfunction as well as glomerular damage. However, the impact of renal tubular damage on clinical outcomes in patients with CHF remains to be determined.

Methods and Results—Urinary β_2-microglobulin–creatinine ratio was measured in 315 patients with CHF. Renal tubular damage was defined as a urinary β_2-microglobulin–creatinine ratio ≥300 μg/g, as previously reported. Patients were prospectively followed up for a median period of 1097 days. There were 91 cardiac events, including 16 cardiac deaths and 75 rehospitalizations for worsening heart failure. Log_{10} urinary β_2-microglobulin–creatinine ratio was increased with worsening New York Heart Association functional class. Multivariate analysis revealed that renal tubular damage was an independent predictor of cardiac events. Kaplan–Meier analysis demonstrated that the rate of cardiac events was higher in patients with renal tubular damage compared with those without it. Patients were divided into 4 groups according to the presence of chronic kidney disease and renal tubular damage. The Cox proportional hazard analysis revealed that comorbidity of chronic kidney disease and renal tubular damage was associated with the highest risk for cardiac events compared with other groups.

Conclusions—Renal tubular damage was related to the severity of heart failure and was associated with poor outcomes in patients with CHF. Renal tubular damage could add clinical information to chronic kidney disease in patients with CHF. ***(Circ Heart Fail.* 2013;6:662-668.)**

Key Words: heart failure ■ renal tubular damage ■ urinary β2-microglobulin

Despite the reduction in mortality with advances in treatment, comorbidity of renal dysfunction is still indicative of an extremely poor prognosis in patients with chronic heart failure (CHF).[1,2] Therefore, evaluation of renal function for risk stratification of patients with CHF continues to be important.

Clinical Perspective on p 668

Renal tubule cells have diverse regulatory and endocrine functions. Renal tubule cells play a pivotal role in modulating acid base balance, active vitamin D synthesis, and reabsorption of sodium, water, and bicarbonate.[3] We have reported that renal tubular damage (RTD) is common and is a risk factor for deterioration of renal function in the general population, suggesting that there is an association between RTD and early abnormality in renal function.[4] Furthermore, some reports have indicated that in a population living in a cadmium-polluted region, severe RTD is related to future cardiovascular disease, as well as glomerular damage.[5,6]

Urinary β_2-microglobulin is a low molecular weight protein, produced by all cells expressing major histocompatibility complex class I antigen.[7] It is readily filtered through the glomerulus and almost completely reabsorbed by the proximal tubules.[8] When proximal tubule cells are damaged, an increase in excretion of urinary β_2-microglobulin results from impaired reabsorption in the proximal tubule. Therefore, urinary β_2-microglobulin is a reliable marker of RTD.[9] Excretion of urinary β_2-microglobulin was reported to be enhanced in patients with CHF as well as urinary albumin and protein.[10] We reported that urinary β_2-microglobulin–creatinine ratio

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