Sex-dependent, zinc-induced dephosphorylation of phospholamban by tissue-nonspecific alkaline phosphatase in the cardiac sarcomere

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Wang Y, Bishop NM, Taatjes DJ, Narisawa S, Millán JL, Palmer BM. Sex-dependent, zinc-induced dephosphorylation of phospholamban by tissue-nonspecific alkaline phosphatase in the cardiac sarcomere. Am J Physiol Heart Circ Physiol 307: H933–H938, 2014. First published July 11, 2014; doi:10.1152/ajpheart.00374.2014.—We have previously reported that Zn2⁺ infused into the coronary arteries of isolated rat hearts leads to the potent dephosphorylation of phospholamban (PLB) as well as a noticeable but less potent dephosphorylation of the ryanodine receptor 2. We hypothesized in the present study that a Zn2⁺-activated phosphatase is located in the vicinity of the sarcoplasmic reticulum (SR) where PLB and ryanodine receptor 2 reside. We report here the novel finding of tissue-nonspecific alkaline phosphatase (TNAP), a zinc-dependent enzyme, localized to the SR in the cardiac sarcomere of mouse myocardium. TNAP activity was enhanced by injection of Zn acetate into a tail vein before harvesting the heart and imaged using electron microscopy of electron dense deposits indicative of the hydrolysis of exogenous β-glycerophosphate. TNAP activity was observed localized to the ends of the Z-line corresponding to SR and was qualitatively more visible in myocardium of males compared with females. Correspondingly, PLB phosphorylation status was potently reduced in myocardium of males injected with Zn acetate, whereas there was no apparent effect of Zn acetate injection on PLB phosphorylation in females. Surprisingly, Western blot analysis of TNAP content suggested a significantly lower TNAP content in males compared with females. These data suggest that TNAP plays a role in governing the phosphorylation status of calcium handling proteins in the SR. Furthermore, the content and activity of TNAP are differentially regulated between the sexes and thus may account for some sex differences in cardiopathologies associated with calcium handling.

The phosphorylation of PLB at sites S166 by protein kinase A (PKA) and T17 by calmodulin-dependent protein kinase-II disinhibits sarcoplasmic reticulum Ca2⁺ ATPase 2a. Protein phosphatase-1 has so far been identified as the primary phosphatase to dephosphorylate both of these sites (17). The balance between kinase and phosphatase activities proves to be particularly important in governing the rate of Ca2⁺ removal and associated diastolic function over a range of performance demands on the myocardium signaled in part by adrenergic drive (14).

We have previously reported that PLB and RyR2 are dephosphorylated after the infusion of 50 μM Zn2⁺ into the coronary beds of isolated rat hearts (32). A Zn2⁺-induced dephosphorylation of these SR proteins suggests that a phosphatase has been activated or a kinase deactivated by Zn2⁺. In the current study, we hypothesized that a zinc-dependent phosphatase, namely tissue-nonspecific alkaline phosphatase (TNAP), is at least partially responsible. TNAP is bound to cellular membranes of several different organ systems including endothelial cells lining blood vessels (22). Its placement within the cardiomyocyte has been previously reported using confocal microscopy, although its localization could not be discerned because of resolution limitations (20). Previously published electron micrographs (EMs) of TNAP activity suggest its absence from the cardiac sarcomere (22). In the present study, we employed EMs to image TNAP activity in the cardiac sarcomere of mice that had been injected with Zn acetate, which according to our hypothesis would be expected to enhance TNAP activity and facilitate its detection. We demonstrate here that TNAP activity is found in the cardiac sarcomere and is localized in or near the SR with higher Zn2⁺-induced activity in males compared with females. In contrast, TNAP total content appears lower in males compared with females. These data suggest a novel mediator of SR Ca2⁺ handling that is sensitive to Zn2⁺-availability and differs in content and regulation between the sexes.

METHODS

Solutions. Chemicals and reagents were obtained from Sigma (St. Louis, MO) unless otherwise noted. Krebs-Henseleit solution consisted of (in mM) 108.8 NaCl, 4.0 KCl, 1.4 MgSO4, 1.4 KH2PO4, 25.0 NaHCO3, 11.1 glucose, 10.0 sodium pyruvate, and 2.0 CaCl2 (pH 7.4). Relaxing solution consisted of (in mM) 60 methylsulfate sodium salt, 0.12 CaCl2, 5 EGTA, 20 N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, 5.4 ATP sodium salt, 7.5 MgCl2, and 35 phosphocreatine and 300 U/ml creatine kinase (pH 7) to achieve pCa 8, 5 MgATP, 1 Mg2⁺, and 200 ionic strength as calculated by solving equations describing ionic equilibria (9).

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Mouse tissue preparation. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee at the University of Vermont. Black Swiss mice aged 10–20 wk were anesthetized with isoflurane (5% in O2). A 100-µl saline bolus, in some cases containing 100 µM Zn acetate, was injected into the tail vein. Left ventricles (LVs) were harvested 8 min later. Papillary muscles dissected in Krebs-Henseleit buffer and placed into Karnovsky’s solution for electron microscopy. LVs were flash frozen in liquid nitrogen, stored at −80°C and used later for Western blot analysis.

Electron microscopy. LV papillary muscle samples were fixed in 2.5% glutaraldehyde/1.0% paraformaldehyde buffer (pH 7.2) for 60 min at 4°C, rinsed three times with cacodylate buffer, and then rinsed three times in 0.1 M Tris-buffered saline (pH 8). TNAP activity was revealed by incubating samples in 1 mM β-glycerophosphate, 2 mM CaCl2, 0.00015% Triton X-100, and 5% sucrose in Tris-buffered saline for 2 h at 37°C. Negative controls consisted of omitting β-glycerophosphate or CaCl2 or by addition of 20 mM levamisole (31742 Fluka). All samples were then dehydrated with a series of ethyl alcohols and infiltrated and embedded in Spurr’s epoxy resin. Thin sections were prepared with a diamond knife, retrieved onto 150 mesh nickel grids, and contrasted with alcoholic uranyl acetate and lead citrate. Samples were imaged with a JEOL 1400 transmission electron microscope (JEOL USA, Danvers, MA) operating at 80 kV, and images were acquired with an AMT XR611 CCD camera.

TNAP dephosphorylates PLB. LVs from two male mice without tail injection were harvested, homogenized in relaxing solution with 0.05 mg/ml saponin, and digested overnight at 4°C. Samples were centrifuged, and the supernatant was exposed to 100 U/ml PKA (P-2645) for 2 h at 4°C and then to 10 µg/ml PKA inhibitor (P-0300). Separate samples were then prepared with different combinations of 0.45 nM recombinant TNAP (13) + 100 nM Zn acetate, 30 µM MLS-0038949 (EMD Millipore, 613810), and 20 µM N,N,N′,N′-tetrais(2-pyridylmethyl)ethane-1,2-diamine (TPEN). Western blot analysis. LVs were homogenized in relaxing solution, run on 18% Tris-HCl gels (Bio-Rad), transferred to nitrocellulose, and blotted using primary antibody for phosphorylated PLB-S16T17 (No. 8496, Cell Signaling). Blot was incubated for 2 h at room temperature. Washes were followed by using Tris-buffered saline (No. 170-6435, Bio-Rad) with 0.05% Tween-20 (No. 161-0781). Blot was incubated with anti-rabbit IgG (H&L) horseradish peroxidase conjugate (Ref. No. W4011, Promega) and visualization was done by VersaDoc Imagine system (Bio-Rad). Western blot analysis for GAPDH content was done with anti-rabbit IgG (H&L) horseradish peroxidase conjugate (Ref. No. W4021, Promega). GAPDH density was detected using anti-tissue nonspecific alkaline phosphatase (No. LS-B6663, LSBio), and two different protein loads of 1.8 and 3 µg were used to verify linear range of densitometry before quantification.

RESULTS

TNAP acts on PLB as substrate. Motivated by the potent Zn2+-induced dephosphorylation of PLB-S16T17 and RyR2-S2808 in the myocardium of male rats previously reported (32), we tested whether TNAP can target PLB for hydrolysis and whether TNAP activity is sensitive to Zn2+ availability. With the use of mouse myocardial homogenate in vitro, PLB-S16T17 was prephosphorylated by PKA and then dephosphorylated by TNAP in the presence of Zn2+ (Fig. 1A). TNAP was potently inhibited by 30 µM MLS-0038949 (11), and chelation of Zn2+ similarly led to reduced TNAP activity as evidence by the movement of an elevated PLB-S16T17 phosphorylation status in the presence of TPEN (Fig. 1A). Although not quantified, these data demonstrate that PLB is a putative substrate for TNAP and that TNAP activity on PLB is sensitive to Zn2+ availability.

Zn2+-induced dephosphorylation of PLB. The injection of Zn acetate into the tail vein resulted in the nearly complete dephosphorylation of PLB-S16T17 in male mouse myocardium (Fig. 1B). This potent dephosphorylation of PLB by Zn acetate in the males is in agreement with that reported for Zn acetate infusion into the coronary beds of isolated male rat hearts (32). There was no apparent effect of Zn acetate injection on PLB-S16T17 phosphorylation status in the females (Fig. 1B).

Zn2+-induced activation of TNAP. TNAP localization in cardiac tissue was visualized by EM imaging of the hydrolysis product of TNAP enzyme acting on the exogenous substrate β-glycerophosphate. Figure 2A illustrates the electron dense areas at the ends of the sarcomere Z-line, which corresponds to SR structures in striated muscle after bolus injection of Zn acetate into a male mouse tail vein. Without injection of Zn acetate, the electron dense areas corresponding to TNAP activity in the male myocardium were visible but not as distinct (Fig. 2B). TNAP activity in or near the SR, including SR surrounding mitochondria (8), was also detected in female mouse cardiac sarcomere after Zn acetate injection (Fig. 2C) although not as dense as in the male. TNAP activity was not visually apparent in the female mouse cardiac sarcomere without Zn acetate injection (Fig. 2D), which is consistent with previous negative results in female rat myocardium (22). These images showing sex differences in TNAP activity in or near the cardiac SR after enhancement by Zn acetate are consistent with the sex differences observed with Zn2+-induced dephosphorylation of PLB-S16T17.
Figure 2E illustrates the absence of TNAP activity detected in male cardiac sarcomere upon removal of β-glycerophosphate as substrate and therefore provides a control for the imaging method. A similar lack of staining was observed when CeCl₃ was removed from the process (not shown). Figure 2F demonstrates knockdown of TNAP activity in male myocardium with the addition of the inhibitor levamisole to the process, thus providing a further control for TNAP activity.

TNAP activity in endothelial cells within cardiac tissues has been previously reported (22) and in our study was most apparent after injection of Zn acetate. Figure 3, A and B, illustrate the localization of TNAP activity in the caveolae of endothelial cells that surround blood vessels in both male and female mouse myocardium, respectively. Figure 3C illustrates the loss of staining when CeCl₃ is removed from the process, and Fig. 3D illustrates the inhibition of TNAP activity by levamisole.
**DISCUSSION**

We provide here compelling evidence for the presence of TNAP in the cardiac SR, the activation of cardiac TNAP by Zn\(^{2+}\) availability in vivo, and a substantial sex difference in cardiac TNAP content (low in male/high in female) that does not account for the sex difference in Zn\(^{2+}\)-induced activation of TNAP in vivo (high in male/low in female). Because TNAP is able to bring down PLB phosphorylation status with Zn\(^{2+}\), TNAP would be expected to play a role in the regulation of SR Ca\(^{2+}\) uptake due to Zn\(^{2+}\) availability. Although not tested here, RyR2 is another SR calcium regulatory protein whose phosphorylation is likely affected by cardiac TNAP activation by Zn\(^{2+}\) as previously reported (32). Sex differences in the Zn\(^{2+}\)-induced dephosphorylation of PLB and activation of TNAP suggest a limited sensitivity to Zn\(^{2+}\) regulation in females and possibly a high susceptibility to a loss of this mechanism during zinc deficiency in males.

Zinc deficiency in the general population and particularly in individuals at risk of cardiac dysfunction is more pervasive than generally appreciated. Zinc deficiency (defined as <10.7 \(\mu\)M Zn\(^{2+}\) in blood plasma) occurs in \~15% of nonhospitalized aging persons in a Western society (6) and with greater occurrence in non-Western societies (27). Zinc deficiency accompanies all incidences of congestive heart failure detected in one cohort of octogenarians (26) and correlates significantly with the incidence and severity of ischemic cardiomyopathy (29), idiopathic dilated cardiomyopathy (23, 28, 30), and hypertrophic cardiomyopathy (28). Medications for hypertension and heart failure, including diuretics; angiotensin-converting enzyme inhibitors; and angiotensin II receptor blockers elevate zinc clearance in the urine and result in a systemic loss of serum zinc (12). The data we provide in the current study imply that a reduced activation of TNAP due to even subclinical zinc deficiency may play a role in the maintenance of a heightened phosphorylation status of PLB and possibly also RyR2, especially in patients taking commonly prescribed cardiac medications.

An elevated phosphorylation status of PLB-S\(^{166}\)T\(^{17}\) as may occur with zinc deficiency would be expected to enhance SR Ca\(^{2+}\) uptake rate. This particular consequence of zinc deficiency would theoretically improve diastolic function and inhibit progression to heart failure (5, 25) but would also elevate SR Ca\(^{2+}\) load. An elevated phosphorylation status of RyR2 associated with zinc deficiency, on the other hand, could have profound negative effects on cardiac function. Phosphorylation of RyR2-S\(^{2808}\), for example, has been implicated as reducing RyR2 affinity for FK506 binding protein, 12.6 kDa molecular mass, which plays an important role in reducing RyR2 open probability and SR Ca\(^{2+}\) leak (10, 18). Phosphorylation of RyR2-S\(^{2814}\) also elevates RyR2 open probability and can especially raise the risk of arrhythmia and cardiac arrest (31). The net effect of zinc deficiency on TNAP-dependent functions of SR calcium handling proteins, at least in males, would be an elevated SR Ca\(^{2+}\) load and leak, respectively. Considering that extracellular Zn\(^{2+}\) inhibits the L-type calcium channel inward current (1, 32) and reduces SR Ca\(^{2+}\) load (32), one consequence of zinc deficiency would be a substantial elevation in cardiomyocyte Ca\(^{2+}\) content mediated in part through the downregulation of TNAP.
Given that TNAP content is lower in male hearts compared with female hearts, sex hormones likely play a role in TNAP expression and/or accumulation in the myocardium and other tissues such as endothelial cells. TNAP activation by Zn²⁺ may likewise be regulated differently between the sexes. The influx of extracellular Zn²⁺ into the cardiomyocyte requires membrane depolarization (2) and occurs at a rate proportional to depolarization frequency (24). The primary influx pathway has been shown to be the L-type calcium channel (1), which affects membrane depolarization (2) and occurs at a rate proportional to tissue-nonspecific alkaline phosphatase. This work was supported by National Institutes of Health Grants HL-086902 (to B. M. Palmer) and DE-12889 (to J. L. Millán).

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