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Insulin-dependent rescue from cardiogenic shock is not mediated by phospholamban phosphorylation

NAA-ADJELEY ABLORH¹, FLORENTIN NITU¹, KRISTIN ENGBRETSSEN², DAVID D. THOMAS¹, and JOEL S. HOLGER²

¹Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN, USA

²Department of Emergency Medicine, Regions Hospital, University of Minnesota, St. Paul, MN, USA

Abstract

Introduction—We used immunoblots to determine whether inotropic and lusitropic effects of high-dose insulin (HDI) in cardiogenic shock, induced by a β -blocker (BB) or a calcium channel blocker (CCB), are mediated by phosphorylation of phospholamban (PLB). PLB is a membrane protein that regulates calcium uptake into the sarcoplasmic reticulum (SR) by inhibition of the cardiac calcium pump (SERCA2a). Phosphorylation of PLB relieves SERCA inhibition, thus enhancing diastolic relaxation and preload.

Methods—Our Institutional Animal Care and Use Committee approved this research. Swine myocardia from six groups were flash frozen immediately upon death or sacrifice. Groups 1–6 received: (1) no medications, (2) HDI and glucose only, (3) toxic propranolol infusions and saline resuscitation, (4) toxic propranolol infusions and HDI resuscitation, (5) toxic verapamil infusions and saline resuscitation, and (6) toxic verapamil infusions and HDI resuscitation. Groups 3–6 were resuscitated for 4 h. Tissue samples from all six groups were analyzed by quantitative immunoblots, using antibodies to both unphosphorylated PLB (uPLB) and phosphorylated PLB (pPLB), to determine the total PLB content and the fraction of PLB phosphorylated.

Results—There were no differences in either pPLB or total PLB in cardiac tissue among any of the six groups. However, infusion of a pig with the β -adrenergic agonist, isoproterenol, produced enhanced PLB phosphorylation.

Conclusion—The mechanism by which HDI produces its inotropic and lusitropic effects in CCB- and BB-induced cardiovascular toxicity, resulting in resuscitation, is not due to changes in phosphorylation of PLB or a change in the total PLB in the SR.

Keywords

Phospholamban; High-dose insulin; Cardiogenic shock; Verapamil; Propranolol

Introduction

High-dose insulin (HDI) is an accepted treatment modality in calcium channel blocker (CCB) and β -blocker (BB) toxicity.^{1,2} Insulin is a well-known cardiac inotrope, and when used in the setting of CCB- and BB-induced cardiotoxicity, this effect is potent.^{3,4} The

inotropic effects of insulin have been demonstrated in cardiac ischemia, in cardiac surgery, and in sepsis-induced myocardial dysfunction.⁵⁻⁷ Insulin also has positive effects on ventricular diastolic relaxation (lusitropy) without changing myocardial oxygen requirements. In the systemic, pulmonary, and coronary vasculature, insulin functions as a vasodilator.^{8,9} The intracellular mechanisms of inotropy and lusitropy are unknown. In a canine model of verapamil toxicity, high-stressed myocardium heavily favored glycolysis over β -oxidation of the usual fatty acid substrate to meet energy demands.⁴ Insulin promotes cellular glucose uptake by activating glucose transporters on the cell membrane. Insulin increases glucose as an energy substrate during stress to improve myocardial energy production by activating calcium and potassium channels, regenerating cytosolic ATP levels, and enhancing aerobic metabolism.¹⁰

Previous studies have shown that phospholamban (PLB) is the primary regulator of calcium uptake into the cardiac sarcoplasmic reticulum (SR), because it controls the cardiac calcium pump (SERCA2a), responsible for 70% of calcium uptake in humans.¹¹ In its unphosphorylated state, PLB inhibits SERCA2a, but phosphorylation of PLB relieves this inhibition. Increased SERCA function produces increased uptake of intracellular calcium into the SR, which promotes ventricular relaxation during diastole, and results in higher SR calcium stores.¹² The enhanced stretch of sarcomeres in diastole allows for increased preload, which may directly increase inotropy. Higher SR calcium stores allow for increased calcium release during contraction, also enhancing inotropy. PLB phosphorylation is primarily regulated via β -adrenergic stimulation of cAMP-dependent PKA and Ca^{2+} /calmodulin-dependent protein kinase.^{11,13}

The phosphatidylinositol 3-kinase (PI3K) pathway is one of the three major intracellular signaling pathways affected when insulin binds to its cell surface receptor. It has recently been shown that verapamil toxicity can deregulate the PI3K pathway and that HDI can reactivate the PI3K pathway, provided that insulin dosage far exceeds physiologic concentrations.¹⁴ Cross-talk between the cAMP/PKA pathway and the PI3K pathway has also been shown.¹⁵ It is unknown whether the inotropic/lusitropic mechanism of HDI in CCB or BB toxicity is mediated ultimately by PLB phosphorylation and subsequent restoration of the cardiac calcium pump (SERCA) activity.¹⁶ Therefore, in this study, we have used quantitative immunoblots, employing antibodies to both phosphorylated and unphosphorylated PLB (uPLB), to determine whether HDI treatment, in these states of cardiogenic shock, is mediated by phosphorylation of PLB. Phosphorylation of PLB should result in positive downstream effects, including increased SERCA activity, greater calcium stores in the SR during relaxation, greater calcium transients in the cytosol during contraction, increased diastolic lusitropy, and increased systolic inotropy. In previous studies, negligible changes in levels of PLB phosphorylation were detected in isolated pig SR membranes subject to various cardiac challenges.^{17,18} However, it is possible that the time required to isolate SR membranes in these studies allowed endogenous phosphatase activity to mask changes in PLB phosphorylation. Therefore, in this study, we analyzed flash-frozen tissue that was rapidly processed in the presence of phosphatase inhibitors and we used positive controls to validate these procedures.

Methods

Surgical protocols

Our Institutional Animal Care and Use Committee approved this research. Healthy 12-week-old Yorkshire pigs weighing 27–35 kg were sedated and anesthetized during the entire protocol. Instrumentation included placement of a tracheostomy tube for ventilation, a pulmonary artery catheter placed via an internal jugular vein, femoral arterial and venous catheters, and another catheter in the urinary bladder. This enabled continuous cardiac

output measurements, as determined by the thermodilution technique, as well as pulmonary artery and central venous pressure measurements. Arterial blood gas, pH, and mean arterial pressure (MAP) were continuously monitored, and $p\text{CO}_2$ was maintained near the baseline value. Nitrous oxide and isoflurane anesthesia were titrated by monitoring both reflexes and bispectral analysis values to minimize cardiac depression. The heart was immediately harvested at the first of two events: mortality or the end of a 4-h resuscitation protocol. Within 5 min, representative samples from the aorta, the walls of the left and right ventricles, and the intraventricular septum were flash frozen in liquid nitrogen and stored at -80°C . A total of 12 pigs were divided into six groups, consisting of two each, as follows.

Negative controls

Group 1 served as controls. These two pigs did not receive any medications, other than anesthesia for the purpose of harvesting tissue samples. Group 2 consisted of two pigs that received an insulin and a glucose infusion for 4 h. Insulin (2 units/kg/h) was infused at time zero, followed by an additional 2 units/kg/h every 10 min, until a maximum of 10 units/kg/h of insulin was reached, at 40 min. Glucose, in the form of D50, was infused and titrated to maintain this level at 60–120 mg/dL. Group 2 samples served as an additional control and allowed us to study the effects of HDI and glucose, on normal pig hearts.

Reversal of propranolol toxicity with HDI-mediated resuscitation

Groups 3 and 4 both consisted of two pigs that received propranolol, but group 3 was resuscitated with saline while Group 4 was resuscitated with HDI. An initial bolus of propranolol (0.5 mg/kg) was administered. Then, propranolol was infused at 0.25 mg/kg/min until toxicity was reached. Toxicity was defined as the time when the product of the heart rate (HR) and MAP decreased to 75% of the baseline product. At this point, the propranolol infusion rate was decreased to 0.125 mg/kg/min, to simulate continued absorption of an oral overdose. In both groups, a normal saline resuscitation bolus of 20 mL/kg of 0.9% saline was administered, over the next 10 min. This methodology has been used in previous models.³ Group 3 continued a baseline saline resuscitation for 4 h. However, HDI infusion replaced the saline in group 4, titrating from 2 to 10 units/kg/h. Glucose, in the form of D50, was infused and titrated to maintain a concentration of 60–120 mg/dL (as outlined in group 2) and continued until the first of death or 4 h.

Reversal of verapamil toxicity with HDI-mediated resuscitation

Both groups 5 and 6 consisted of two pigs that received verapamil, but group 5 was resuscitated with saline while group 6 was resuscitated with HDI. In both groups, a verapamil bolus of 0.5 mg/kg/h was increased by 0.5 mg/kg/h every 10 min until the point of toxicity, defined as in groups 3 and 4. At this time, the infusion rate was decreased by 50%. In both groups, a normal saline resuscitation bolus of 20 mL/kg of 0.9% saline was administered over the next 10 min. Group 5 continued with a baseline saline infusion until the first of death or 4 h. Group 6 was given an insulin infusion, titrating from 2 to 10 units/kg/h. Glucose, in the form of D50, was infused and titrated with the goal of maintaining this level between 60 and 120 mg/dL, as outlined in groups 2 and 4, and continued until the first of death or 4 h.

Tissue extraction

Frozen tissues were extracted at 4°C in a buffer containing 2% SDS, 1 mM PMSF, 1 mM benzamidine, 10 mM NaF, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, and 50 mM Tris-HCl (pH 7.2). Calyculin (2 nM) was added to extraction buffer just prior to use. Tissue (1 g) was cut with a knife and homogenized in 5 mL of extraction buffer with a polytronic homogenizer. The homogenates were centrifuged at $15,000 \times g$ (11,200 rpm in an SS-34

rotor) for 10 min, and the protein-containing supernatant was stored at -20°C (short-term) and at -80°C (long-term). This extract represented more than 60% of the total protein, as measured by the biuret assay, using bovine serum albumin as a standard.

SDS–polyacrylamide gel electrophoresis (PAGE) and western blot

Electrophoresis was carried out on a Criterion apparatus (Biorad Life Science, Hercules, CA, USA), on 15% polyacrylamide, containing 0.1% SDS slab gels (1.5 mm thick), using the discontinuous Laemmli buffer system.¹⁹ Samples (40 μg) were mixed in a 1:1 ratio with sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β -mercaptoethanol, and 0.01% bromophenol blue) and boiled for 2 min before loading the sample on the gel. Standards of uPLB and S16-phosphorylated PLB (pPLB) were prepared by solid-phase peptide synthesis.²⁰ Prestained, broad-range protein molecular weight SDS-PAGE standards (Bio-Rad), with molecular mass ranging from 7 to 205 kDa, were used as standards. The samples were electrophoresed at constant voltage (100 V) for 80 min.

Western blot detection of phospholamban

The proteins separated by electrophoresis were electrotransferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad), according to the method of Towbin et al.²¹ The western blot transfer was performed in the presence of Tris-glycine buffer (25 mM Tris, pH 8.3, and 192 mM glycine, containing 10% methanol) in a Transblot cell (Bio-Rad), at 280 mA constant current, for 50 min at 4°C . The membranes were blocked with 2% nonfat dry milk, for 1 h, and then washed for 10 min, three times, with PBS, containing 0.1% Tween 20. The membranes were incubated with either of two primary antibodies, 1D11Ab or 285Ab, in blocking buffer. Anti-PLB monoclonal antibody 1D11 binds both phosphorylated and uPLB. Anti-phosphoserine PLB polyclonal antibody 285, which only binds PLB, phosphorylated at serine-16. Both were produced and purified as described previously.²² 1D11Ab or 285Ab (7.2 mg/mL) was diluted between 1:2,000 and 1:3,000. After 1-h incubation, excess primary antibody was washed for 10 min, three times, with PBS, containing 0.1% Tween 20. The blots were subsequently incubated with secondary antibodies. 1D11 was incubated with 1 mg/mL stock solution of horseradish peroxidase-conjugated goat anti-mouse IgG (H+L)-HRP (Southern Biotechnology Associates, Inc., Birmingham, AL, USA), diluted between 1:1,000 and 1:2,000, in blocking buffer, without sodium azide, for 1 h at room temperature (RT). 285Ab was incubated with goat anti-rabbit IgG (H+L)-HRP (Sigma-Aldrich Corporation, St. Louis, MO, USA), diluted between 1:1,000 and 1:2,000, in blocking buffer, without sodium azide, for 1 h at RT. Excess secondary antibody was washed for 10 min, three times, with PBS, containing 0.1% Tween 20. The antigen–antibody complexes were visualized by staining for peroxidase activity with 3,3'-diaminobenzidine (DAB) tablets (Sigma), as a substrate. The color reaction was stopped by washing with deionized water. The immunoblots were scanned by a densitometer, using the reflectance mode, and the bands were quantitated using the volume (area $\times$ density) analysis method.

Results

Validation of methodology

We first performed control experiments to demonstrate that we can detect uPLB and pPLB in porcine cardiac tissue. Synthetic uPLB and pPLB were used as standards (first six lanes of Fig. 1). 285Ab only detects pPLB (Fig. 1, top), whereas 1D11Ab detects both uPLB and pPLB, with a slight preference for uPLB (Fig. 1, bottom). Both antibodies have approximately linear sensitivity in the range of 6–25 ng of PLB. Thus, 285Ab and 1D11Ab provide accurate measures of pPLB content and uPLB content, respectively. Our ability to detect both forms of PLB in porcine cardiac tissue is illustrated in the right two lanes of Fig.

1, which represent samples taken from the right ventricles of control pigs. For the pig that was given no medications (“–”), negligible pPLB, equal to or below the background, was detected (Fig. 1, top), but the total PLB was substantial (Fig. 1, bottom, 17.50 ng PLB/ μ g). Thus, less than 1% of PLB was phosphorylated for the pig receiving no medications. As a positive control, another pig was given isoproterenol, which is known to induce phosphorylation of PLB via the β -adrenergic receptor, with downstream signaling through protein kinase A (PKA).^{23,24} The pig received isoproterenol 5 (g/min for 2 h, resulting in the HR increasing from 90 to 175/min. The pig was killed, and the cardiac tissues were harvested and analyzed, as described in Methods. Isoproterenol had no significant effect on the total amount of PLB in the right ventricle (Fig. 1, bottom right, “+”), but it did produce a significant level of pPLB (Fig. 1, top right, “+”), corresponding to 1.1 ng/ μ g total protein, showing that 6.25% of the PLB was phosphorylated.

Pig survival data

Although survival was not a study aim, both pigs in group 2 (insulin and glucose alone) survived for 4 h. In group 3 (propranolol and saline), one pig died after 86 min of resuscitation and the other survived for 4 h. In group 4 (propranolol and HDI), both pigs survived for the total 4 h of resuscitation. In group 5 (verapamil and saline), both pigs died; one at 60 min and the other after 105 min of resuscitation. In group 6 (verapamil and HDI), one pig survived the 4-h resuscitation. The other died at 210 min. In summary, three of the four pigs resuscitated with HDI survived, and one of the four saline-resuscitated pigs survived.

Resuscitation from cardiogenic shock

Figure 2 demonstrates results of the average cardiac output measured for two pigs from each group during 4 h of resuscitation. Time “0” on the *x*-axis is the point of toxicity as defined in Methods, and the data from each group was standardized to 100% (baseline) for easier interpretation of the data. Group 1, given no medications, is not shown, because it was used to establish the baseline for pPLB in Fig. 3 and not to establish baseline cardiac output. The cardiac output control (Group 2; Fig. 2, black) was given only HDI and glucose, without induced toxicity. Groups 3 and 4 (dark gray) show that propranolol induces severe toxicity, which is reversed by HDI/glucose resuscitation, as reported previously.³ The curve for group 3 (dark gray, open circles) is likely high, due to inadvertent excess saline administration, during resuscitation phase, in one of these pigs. Groups 5 and 6 (light gray) show that verapamil induces severe toxicity, which is reversed by HDI/glucose resuscitation. The results show clearly that resuscitation with HDI can rescue porcine myocardia from BB- and CBB-induced cardiogenic shock, as evidenced by the return of cardiac output to a level comparable to that of controls.

Immunoblots from Groups 1–6

Immunoblots were performed on harvested cardiac tissues. Because PLB content was consistently found to be highest in the right ventricle, analysis focused on these samples. The total PLB content, measured with 1D11Ab, was essentially unchanged (4.4 ng/ μ g) by any of the treatments (Fig. 3, right). Tissues were evaluated for their pPLB content by immunoblot, with Ab285 (Fig. 3, left). Figure 3A shows that pPLB was not significantly above background in the samples corresponding to group 1, normal control (*), and group 2, insulin plus glucose (**). Figure 3B shows that there was no change in pPLB after either inducing cardiogenic shock with propranolol (*), group 3, or treating propranolol-induced shock with HDI (**), group 4. Similarly, Fig. 3C shows that there was no change in pPLB after either inducing cardiogenic shock with verapamil (*), group 5, or treating verapamil-induced shock with HDI (**), group 6. Similar results were obtained for the other four tissues studied. In summary, propranolol-induced cardiogenic shock, verapamil-induced

cardiogenic shock, and HDI-mediated reversal of cardiogenic shock all failed to affect PLB content or PLB phosphorylation.

In light of the surprising observation that pPLB was observed only in the case of isoproterenol treatment (Fig. 1), we performed control experiments to determine whether the results were affected by phosphatase activity during tissue extraction. Synthetic pPLB (25 ng) remained reactive to antibody 285 even after 1-h incubation with tissue-extract-containing phosphatase inhibitors at 37°C (data not shown). Thus, PLB phosphorylation was not masked by endogenous phosphatase activity.

Discussion

Several previous studies have sought to determine the underlying mechanisms of the inotropic properties of insulin therapy on the heart. Initial work, looking at these effects in toxicity-induced myocardial depression from CCB and BB toxicity, has focused on myocardial energy–substrate mechanisms, including glucose and lactate uptake, and pyruvate dehydrogenase activation. Under normal conditions, the heart uses primarily free fatty acids, which account for 80% of ATP production.²⁵ Under conditions of stress, glucose becomes the preferred substrate. Insulin, in high doses, dramatically increases intracellular glucose transport in cardiogenic shock and is associated with increased inotropy.²⁶ This process is activated by mitogen-activated protein kinase, a major intracellular insulin-signaling pathway.¹⁴ Translocation of GLUT 4 complexes to the cellular membrane is induced by insulin by both GTPaseTC10 activity and the PI3K pathway.²⁷ Glucose can also be transported into myocardial cells by the less abundant GLUT 1, which is not insulin sensitive, and allows for glucose uptake in the absence of insulin.²⁵ Previous work in BB-toxic pigs, demonstrating insulin in doses up to 10 units/kg/h markedly increases cardiac output to levels greater than measured in the resting, pretoxic state, led our group to hypothesize that mechanisms other than energy–substrate and metabolism may be involved in this process.³

Intracellular calcium is essential for cardiac electrical activity and is the direct activator of the myofilaments that cause myocardial contraction. Events that lead to calcium mishandling in the cell are primary causes of contractile dysfunction in the heart.¹³ The abrupt rise in free, intracellular calcium comes primarily from Ca^{2+} influx, through the L -type calcium channels, during the action potential. This triggers calcium release from the SR, through its release channels, the ryanodine receptors (RyR). Ventricular relaxation is primarily under control of PLB, which regulates the uptake of free calcium, back into the SR, by SERCA2a. The most direct and potent control of PLB is through activation of the β -adrenergic receptor, by which adenyl cyclase increases cyclic AMP (cAMP). cAMP, in turn, activates PKA.²⁸ PKA phosphorylates several proteins, including PLB, RyR, L -type calcium channels, and troponin I. Phosphorylation of PLB is the predominant mechanism of increased lusitropy mediated by accelerating SR calcium uptake.²⁹ PLB phosphorylation increases inotropy by recovering the function of SERCA2a, which pumps calcium into the SR from the cytosol, thus increasing SR calcium stores. When calcium stores are released, during muscle contraction, the calcium transient is increased, with a resultant increase in the force of cardiac contraction.

Activation of the β -adrenergic receptor, with subsequent activation of PKA and phosphorylation of PLB at serine-16, results in a relief of SERCA inhibition.²⁸ PLB can also be phosphorylated at threonine-17, in response to activation of Ca^{2+} /calmodulin-dependent kinase II, which may also be modified by β -adrenergic stimulation.^{28,30} However, serine-16 is the predominant site of PLB phosphorylation, both at baseline and after cardiac

challenge.^{18,22} 285Ab is specific for phosphorylation of PLB at serine-16. Future studies with antibodies specific for threonine-17 phosphorylation will be illuminating.

Cross-talk between the PI3K and the cAMP/PKA pathway has been established.³¹ Moreover, the effect of PI3K signaling on cardiac contractility may require activation of the cAMP/PKA pathway.¹⁵ Conversely, inhibition of PI3K signaling does not appear to have any effect on β_1 -receptor-mediated PLB phosphorylation at serine-16 by cAMP/PKA. Enhanced PI3K signaling does appear to have an anti-adrenergic effect on β_1 -induced calcium influx through L-type Ca^{2+} channels.¹⁵ The nature of this interaction in the BB- or CCB-toxicity model, as in this study, is unknown. We assumed that if HDI impacted upstream events, such as the PI3K pathway or other signaling cascades, it would be reflected in the downstream phosphorylation of PLB.

Our results clearly demonstrate that the mechanism through which HDI exerts its inotropic and lusitropic effects is not mediated by phosphorylation of PLB at serine-16 (Fig. 3). Similarly, negligible changes in levels of pPLB were detected in isolated pig SR subject to other cardiac challenges.^{17,18} There was no serine-16 PLB phosphorylation in any of the HDI-resuscitated pigs, whether made toxic by BBs or CCBs (Fig. 3). Despite these negative results in groups 1–6, cardiac tissue was obtained to compare the effect of a known activator of the β_1 -adrenergic receptor, isoproterenol, alone (Fig. 1). This result validates the methodology used. PLB phosphorylation was detected, indicating the undetectable P-serine-16 in groups 1–6 was a valid result (Fig. 1). It is remarkable that no pPLB was detected in any cardiac tissue samples (Fig. 3), except for those treated with isoproterenol (Fig. 1). However, this observation is consistent with the literature, in which quantitative measures of PLB phosphorylation are rare, and the highest reported value for the baseline level is 4% phosphorylation of PLB in ferret myocardia, which increases to 13% upon isoproterenol administration.³²

Conclusions

Neither phosphorylation of PLB (at serine-16) nor change in total PLB content is the mechanism by which HDI exerts its inotropic and lusitropic effects in CCB- and BB-induced cardiovascular toxicity. Thus, it is unlikely that these resuscitative effects are mediated by the cAMP/PKA pathway, either directly or indirectly, through cross-talk with the PI3K pathway.

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Fig. 1. Western immunoblot, isoproterenol control. Primary antibodies are 285Ab (top; specific for pPLB) and 1D11Ab (bottom). Lanes 1–6 are synthetic uPLB and pPLB standards (6, 12, and 25 ng). For tissue samples, “–” indicates no medications, while “+” indicates isoproterenol administration (see text).

Fig. 2.

HDI improves cardiac output after toxicity. Cardiac output (L/min), expressed as % of the value at time 0 (point of toxicity, as defined in text), at which resuscitation was started with either saline (open symbols) or HDI (closed symbols). Black: control (HDI and glucose only). Dark Gray: toxicity induced by propranolol. Light Gray: toxicity induced by verapamil.

Fig. 3.

Western immunoblots of samples in groups 1–6. Left: 285Ab. Right: 285Ab (as shown in Fig. 1). Lanes 1–3: synthetic pPLB standards. Right: 1D11Ab, lanes 1–3, synthetic uPLB standards. Lanes 4–6, synthetic pPLB standards. For right ventricular tissue samples, A: *normal control (group 1), **insulin and glucose (group 2). B: *propranolol only (group 3), **propranolol and insulin (group 4), C: *verapamil only (group 5), **verapamil and insulin (group 6).