BENEFICIAL EFFECTS OF HYPOTHALAMIC PROLINE-RICH PEPTIDE-1 ON THE HEART FAILURE ASSOCIATED WITH EXPERIMENTAL PANCREATIC NECROSIS AND CRUSH SYNDROME

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Hypothalamic neurosecretory cytokine, proline-rich peptide-1 (PRP-1) may protect against myocardial dysfunction and hypocalcemia induced by experimental pancreatic necrosis (PN) and/or crush syndrome (CS). 24 and 48 h after initiation of experimental PN, effective doses of PRP-1 were administered to adult Wistar male rats divided into groups corresponding to early, reparative, chronic, and chronic recurrent stages of PN. Similarly age and sex matched rats were immediately administered PRP-1 after 2 h of compression injury. The PRP-1 normalized the histopathological changes in cardiac tissues in the dynamics of both PN and CS. Study of $^{45}Ca^{++}$ binding to the membrane proteins of cardiomyocyte sarcoplasmic reticulum (SR) showed that PRP-1 could prevent an impairment in the calcium binding ability of the Ca^{2+} depot proteins caused under pathological conditions. Besides, PRP-1 suppresses a PN and/or CS-induced compensatory manifestation the affinity to calcium of the 32-kDa SR membrane protein and restores its native properties. The results highlight new prospects over the functional implications of PRP-1 and its possible therapeutic potential for the treatment of patients at high risk of cardiovascular disease associated with different pathologies.

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INTRODUCTION

It is generally accepted that the steady state of calcium flux balance is significantly necessary for myocardium.[1,2] Hypocalcemia is one of the metabolic alterations involved in the hemodynamic changes and myocardial damage observed in clinical and experimental studies in acute pancreatitis, and its severe form, pancreatic necrosis (PN).[3] It has been shown that traumatic muscle crush injury may also be a cause of cardiomyocyte specific injury.[4] Our findings suggest that muscle crush injury induced crush syndrome (CS) accompanied by total intoxication causes a cardiac muscle injury at the early stage of decompression similar to the myocardial damage occurred in experimental PN.[5,6] Moreover, we have demonstrated that both experimental acute pancreatitis and long-term compression injury are accompanied by a loss of ability to bind calcium of the membrane proteins of the cardiomyocyte sarcoplasmic reticulum (SR) that contributed to hypocalcemia which is involved in common cellular and molecular mechanisms of myocardial injury.[6,7]

Plenty of evidence suggests the interplay between cardiac endocrine system and hypothalamic neurosecretory hormones providing optimal functioning of brain and heart.[8,9] Hypothalamic neurohormones are involved in the regulation of the intracellular calcium level and exert cardioprotective properties in acute pancreatitis.[5, 10] Proline-rich peptide-1 (PRP-1), one of the neurosecretory cytokines discovered and studied at H. Buniatyan institute of Biochemistry NAS RA (acad. A.A. Galoyan) is implicated in the multiple mechanisms of neuroprotection, regulation of myelopoiesis, immune and stress response.[11] PRP-1 may also provide cardio-protective effects via regulation of phospholipids metabolism and their level in the cardiomyocyte membranes, and suppression of oxidative stress in heart tissues as it was demonstrated at cardiopulmonary insufficiency. We have shown that PRP-1 could upregulate the protein synthesis, stimulate a utilization of D-glucose, and prevent histopathological changes in tissues during CS.[12, 13] The aim of the present study was to extend our knowledge on the effects of PRP-1 on molecular mechanisms of myocardial damage particularly associated with a loss of the calcium binding ability of the cardiomyocyte proteins during experimental PN and/or CS.

EXPERIMENTAL

Materials and methods

Bovine serum albumin was from Carl Roth (GmbH, Karlsruhe). Solid-phase synthesis of proline-rich peptide-1 was performed at Moscow laboratory headed by acad. A.A. Galoyan. All other reagents were purchased from Sigma-Aldrich (USA).

Animals and study design

The experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) on care and use of animals for experimental procedures; protocols were approved by the respective Institutional Animal Care and Ethics Committee of the National Academy of Sciences the Republic of Armenia. Animals were housed six per cage at 12:12 h light/dark cycle (08.00–20.00 h) and had unrestricted access to a standard diet and tap water. Adult 6–7 month-old male Wistar rats weighing 180-220 g were randomly divided into groups (n = 12/group) and subjected to the experimental PN and/or compression injury under conditions of PRP-1 treatment. The PRP-1 preparation was dissolved in saline and filtered (0.22 μm) before use.

Experimental pancreatic necrosis was developed using previously established procedures.[5] Briefly, pancreas was removed through a surgical incision, and a tail of the pancreas, which ends abutting the spleen was cooled by chloroethyl, and the frozen part was defrosted by fingers, returned to its place and incision was sutured. Rats were sacrificed at different stages of PN (marker enzyme, serum α-amylase determined by Reagent kits (ECOlab ZAO, Russia), 1, 7, 14 and 21 d after initiation of PN corresponding to early, reparative, chronic, and chronic recurrent stages of PN respectively. 24 and 48 h after initiation of the PN, PRP-1 (10^{-6} M) was injected to rats intraperitoneally.

Experimental crush syndrome was induced by application of a standardized mechanical pressure (10 kg/100 *g* body weight) applied to the femoris muscle of rat for two hours.[13] Rats were sacrificed immediately after removal of the load, and at 2, 4, 24, and 48 h of decompression stages. Intraperitoneal administration of PRP-1 (10^{-6} M) was performed immediately at the end of compression and an hour later.

Age and sex matched intact rats serve as controls. Rats were anaesthetized with ester prior to surgical and/or compression procedures and/or decapitation.

Measurement of cardiac-specific Troponin I (cTnI) in the whole blood, a very sensitive and specific indicator of damage to the heart muscle was performed by test of i-STAT cTnI (cTnI; i-STAT, Princeton, NJ). cTnI could detect myocardial necrosis in complex clinical situations, where the usual enzymatic markers may be ineffective.[14]

Isolation of cardiomyocyte, and preparation of sarcoplasmic reticulum

After decapitation of animals under light anesthesia the heart was excised, attached to a Langendorff column, perfused with 0.15 M KCl, then crushed by a special press with micro-holes and homogenized in an ice-cold 20 mM HEPES buffer pH 7.4, containing 0.44 M sucrose and 1 mM EDTA, (1:10, w/v) using Potter homogenizer (1500 rpm for 3 min). The homogenate was centrifugated at 50 g for 3-5 min, and cardiomyocytes in the pellet were obtained, suspended in the 20 mM HEPES buffer pH 7.4, containing 300 mM sucrose, 1 mM PMSF, and 20 mM PIPES, and disrupted with a glass-glass homogenizer. The homogenates were centrifuged at 500 g for 20 min, and the supernatant was subjected to sucrose gradient centrifugation. SR was presented by the densest fraction characterized with the highest K^+/Ca^{2+}-ATPase activity, and the absence of of the Na^+/K^+-ATPase activity.[15] Purity of the SR was confirmed also morphologically.

Polyacrylamide gel electrophoresis and isoelectric focusing

Prior to initiation of PN and/or CS, radioactive $^{45}CaCI_2$ was administrated to animals. Translocation of calcium ions was assessed by measuring the distribution of $^{45}Ca^{++}$ in the cardiomyocyte cellular compartments.[16] SR membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing.[17,18] Samples were homogenized in glass-glass microhomogenizers in Tris buffer, pH 7.2 containing of 1 % SDS, 0.05 % 2-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 mcg mL^{-1} leupeptin, 50 mcg mL^{-1} antipain, and 100 mcg mL^{-1} aprotinin. The homogenates were centrifuged at 13000 g for 30 min at 4 ^oC and pellets were re-suspended in cold buffer solution. Slab gel was composed of a stacking gel of 4.75 % (W/v) acrylamide, pH 7.2, and running gel of 10 % acrylamide, pH 8.9. After electrophoresis the radioactivity of $^{45}Ca^{2+}$ was measured from the gel plates using a gas-flow meterBerthold–II (Germany).[19] Specific binding of $^{45}Ca^{2+}$ to the SR membrane proteins is expressed in counts per minute (cpm) • mg^{-1} protein. Interaction between protein and bound calcium was evaluated by Scatchard plot analysis.[20, 21]

Protein was determined by the method of Lowry et al, using crystalline bovine serum albumin as standard.[22]

Statistical analysis

Data are presented as the mean ± SEM. All statistical analysis was performed by *t*-Test for independent samples, or a one-way ANOVA followed by Holm-Sidak post hoc test (SigmaStat 3.5 for Windows). The *P*-values of 0.05 or less are considered as statistically significant differences.

RESULTS AND DISCUSSION

According to current concepts heart failure is considered as the result of changes to the heart cellular and molecular components and to mediators that drive homeostatic control.[1-4] New hypothalamic cytokine, PRP-1 (primary structure, AGAPEPAEPAQPGVY and apparent molecular mass of 1475.26 Da) represented the C-terminal 25-39 fragment of neurophysin-vasopressin-associated glycoprotein, produced in hypothalamus nuclei (n. paraventricularis and n. supraopticus) and might be involved in the regulation of homeostasis via multiple mechanisms.[11]

PRP-1 impact on the calcium binding by the cardiomyocyte membrane proteins in the dynamics of pancreatic necrosis

The sarcoplasmic reticulum (SR), the intracellular storage site of Ca^{2+} plays a major role in the contraction/relaxation machinery, and cardiac dysfunction is commonly associated with impairement in SR function, sarcolemmal calcium

influx and damage in calcium regulatory proteins.[23] Alterations in proteins involved in intracellular Ca^{2+} handling may affect cardiomyocyte contraction and contribute to heart failure.[24] Our previous findings showed that both experimental pancreatic necrosis (PN) and crush syndrome (CS) are accompanied by significant alterations in the qualitative and quantitative properties of the cardiomyocyte SR proteins, and by heart failure up to myocardial infarction.[6, 7]

The same models were applied to study the effect of PRP-1 on common molecular mechanisms of cardiac dysfunction observed at the mentioned pathological states. The effect of PRP-1 was studied at early, reparative, chronic, and chronic recurrent stages of PN (1, 7, 14 and 21 days after PN initiation respectively). Measurement of cardiac-specific Troponin I and histomorphological analysis suggested the PN-induced damage to the heart muscle.

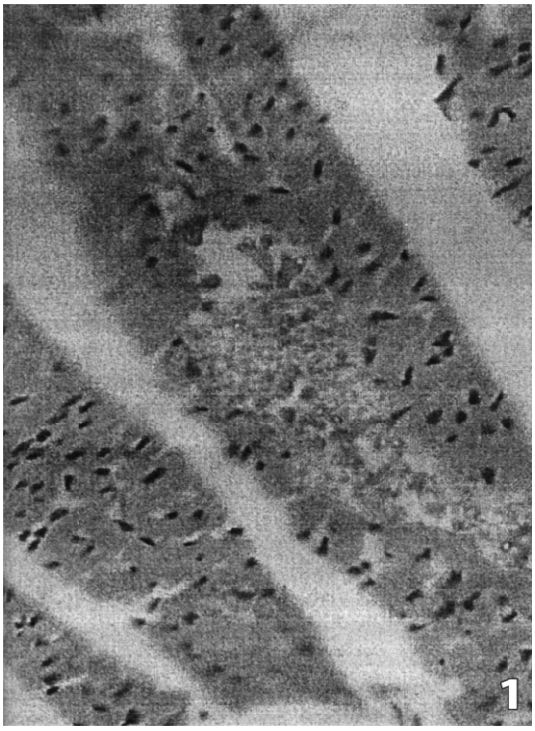

Figure 1. Focal lymphocytic infiltrate (hematoxylin, magnification 400X).

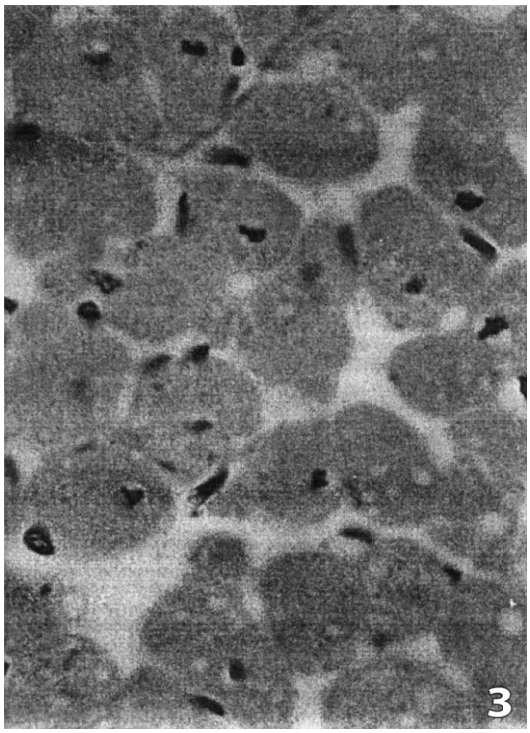

Figure 3. Myocytolysis and fuchsine stained changes in left ventricular wall (magnification 1000X).

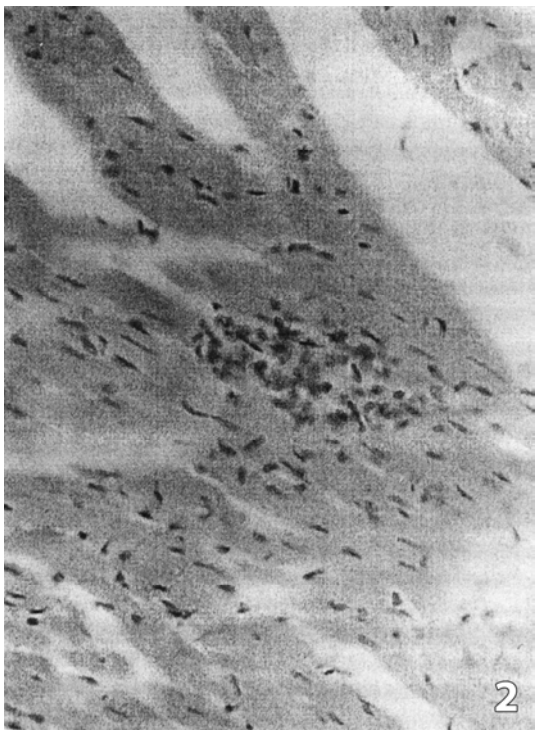

Figure 2. Vacuolation of the cardiomyocyte sarcoplasm (hematoxylin, magnification 1000X).

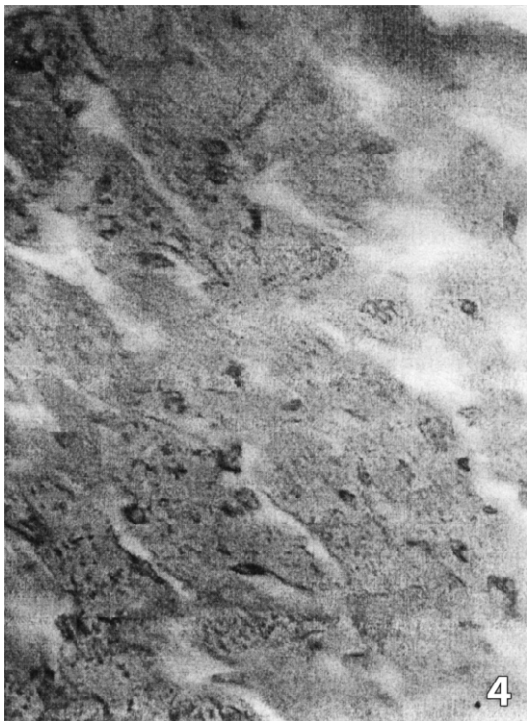

Figure 4. Hypertrophic cardiomyocytes with elongated nuclei (hematoxylin, magnification 400X).

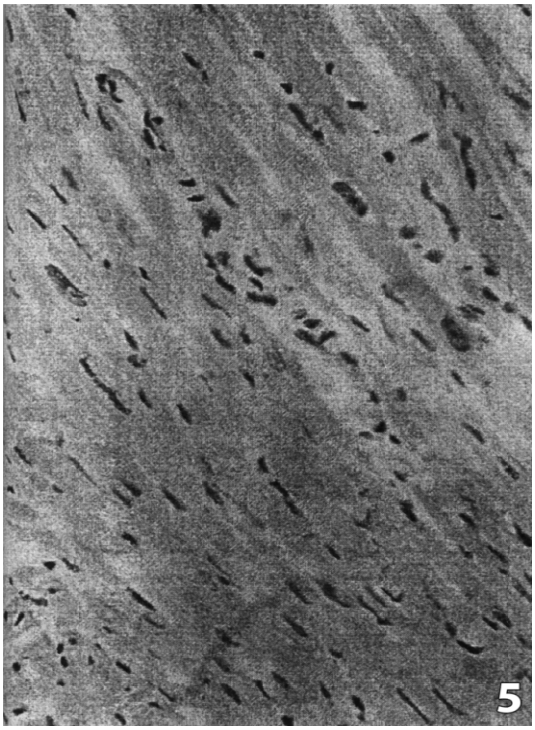

Figure 5. Contracture and degeneration of cardiomyocytes (hematoxylin, magnification 1000X).

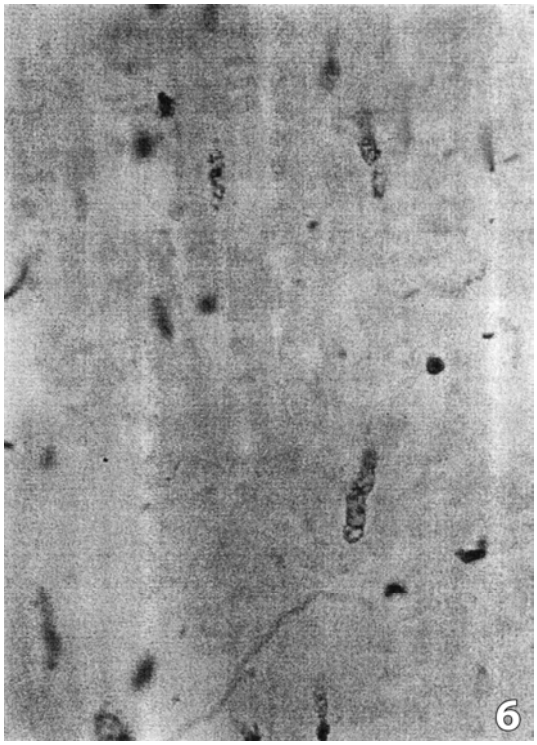

Figure 6. Perinuclear myocytolysis (staining with acid fuchsine by Selye, magnification 1000X).

Results presented in Figures 1-6 show histopathological changes in cardiac muscle corresponding to general morphologic criteria accepted for the diagnosis of degenerative myocardial lesions described for rats.[25] It may be noted that degenerating cardiomyocytes typically contained cytoplasmic vacuoles often occupied the sarcoplasm. Myocardial degeneration is associated with diffuse vacuolation of cardiomyocytes (Figure 2). In figure 5, multifocal mineralization of degenerating cardiomyocytes can be seen in hearts with more severe degeneration.

A cardiomyopathy in rats begins histologically with degeneration and necrosis of individual cardiomyocytes or small focal clusters of myofibers with eventual myofiber loss, and these foci infiltrated by inflammatory cells, and by proliferating interstitial cells.[26] Focal lymphocytic infiltrate, vacuolation of cardiomyocytes, their degeneration, and myocytolysis were observed in the heart specimens of rats with PN, as well as of those with CS.

It should be noted that certain areas of the myocardium may be more predisposed to toxic damage. So, toxins produced during PN and/or CS appeared to be contributed to the myocardial lesions found preferentially in the left ventricular wall (Figure 3), and this coincided with findings of other authors on the selective damage of the left ventricular wall by cardiotoxic compounds in Sprague-Dawley rats.[27]

Treatment with the effective doses of PRP-1 (10^{-6} M, found in the preliminary experiments) could protect from the PN-induced cardiac damage and normalize histopathological changes observed (data not shown).

At the same time, we demonstrated the beneficial effect of PRP-1 on the molecular alterations involved in the cardiomyopathy. As seen in figure 7 a, under physiological circumstances calcium binding ability is detected for five acidic proteins (3 fractions (Mr 60-80 kDa) and 2 fractions (Mr 20-30 kDa), calsequestrin (Mr 55 kDa), and Ca^{2+}-ATPase (Mr 100 kDa) (presented by two subunits after SDS-PAGE) commonly contributed to calcium accumulation in the cardiomyocyte SR. For this prior to initiation of PN $45CaCl_2$ was administered and its binding assessed after SDS-PAGE separation of the cardiomyocyte SR membrane proteins. PRP-1 was injected 24 and 48 h after the PN initiation.

24 h after the PN initiation, all the above-mentioned proteins lost their calcium binding ability, with the exception of Ca^{2+}-ATPase, which continued its affinity to calcium, presumably, protect against an instant cardiac arrest (Figure 7 b). Moreover, a concomitant manifestation of calcium binding ability of the 32-kDa cardiomyocyte SR membrane protein was observed and not only during PN, but also in the CS (vide infra), possibly, a transient attempt of heart cells to compensate hypocalcemia and protect against myocardial injury.[8] Administration of the PRP-1 cardinally prevents the loss of affinity to calcium of the Ca^{2+} depot proteins, as well as completely suppresses the calcium binding ability of the SR membrane 32-kDa protein induced under PN condition (Figure 7, c, d, e). Our previous findings show that experimental PN is accompanied by a conversion of 32-kDa alkaline protein to acidic one and its isoelectric point changes from 8.3 to 5.9, because of a 2.4-fold increase in the acidic amino-acid content (aspartic and glutamic acids) and associated manifestation of bound calcium.[28] To evaluate the PRP-1 impact on the affinity of calcium ions to the cardiomyocyte SR 32-kDa membrane protein at different stages of PN, we examined a binding curve on Scatchard coordinates that makes distinction more

Table 1. PRP-1 impact on the cardiomyocyte 32-kDa protein affinity to calcium in the dynamics of pancreatic necrosis

Calcium-binding properties Groups	B_{max}, nmol calcium·mg^{-1} protein		K_d, nmol calcium·mg^{-1} protein	
	Center of low affinity	Center of high affinity	Center of low affinity	Center of high affinity
Early stage of PN (24 h)	47.63 ± 1.1	245.43 ± 9.92	4.58 ± 0.37	13.28 ± 0.31
Reparative stage of PN (7 d)	48.17 ± 0.72$^{\#}$	245.16 ± 8.06$^{\#}$	1.81 ± 0.15***	9.07 ± 0.49**
Chronic stage of PN (14 d)	37.15 ± 0.91**	221.54±8.87$^{\#}$	1.54 ± 0.22$^{\#}$	7.51 ± 0.35**
Chronic recurrent stage of PN (21 d)	44. 35 ± 0.76*	226.68 ±7.71**	1.79 ± 0.3***	8.02 ± 0.31***
PRP-1treatment/Early stage of PN (24 h)	6.73 ± 0.5***	36.5 ± 2.98***	0.3 ± 0.02***	2.43 ± 0.02***
PRP-1 treatment/ Reparative stage of PN (7 d)	6.25 ± 0.21***	34.44 ± 2.24***	0.12 ± 0.01***	1.24 ± 0.05***
PRP-1 treatment/ Chronic stage of PN (14 d)	2.23 ± 0.29***	22.35 ± 2.98***	0.62 ± 0.02***	1.01 ± 0.02***
PRP-1 treatment/Chronic recurrent stage of PN (21 d)	5.65 ± 0.74***	29.96 ± 2.02***	0.73 ± 0.04***	1.32 ± 0.03***

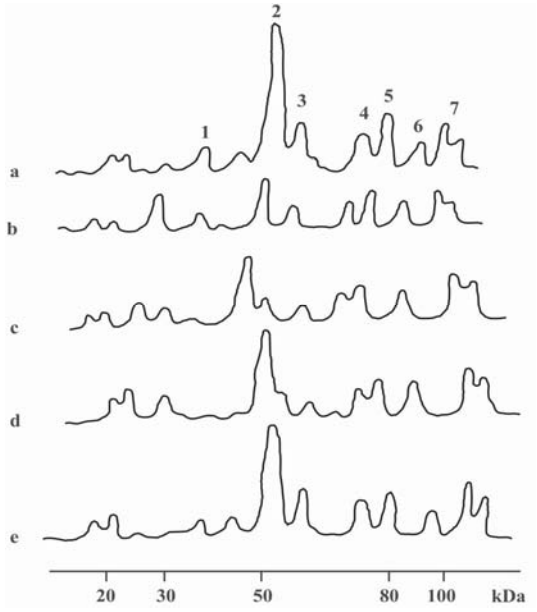

Figure 7. Effect of PRP-1 on the calcium binding to the cardiomyocyte SR membrane proteins in the dynamics of pancreatic necrosis (PN). 1, 3-6 - acidic proteins; 2 - calsequestrin; 7 - two subunits of Ca2+-ATPase (a) control: (b) early stage of PN - the 24th h after PN initiation; (c) reparative stage of PN - the 7th day of PN / PRP-1 treatment; (d) chronic stage of PN - the 14th day of PN / PRP-1 treatment; (e) chronic recurrent stage of PN - the 21st day of PN / PRP-1 treatment.

pronounced, and shows more than one population of binding sites.[20] Determined parameters are Bmax - the maximal binding capacity of the protein(s) and K_d - the steady state dissociation constant (K_d is the free concentration of calcium for which the bound concentration is equal to $B_{max}/2$; the inverse of K_d is K_a, the steady-state constant of affinity).[21]

The data obtained clearly show that PRP-1 may prevent PN-induced modification of the 32-kDa protein in cardiomyocytes that is also suggested by B_{max} and K_d values for centers of low and high affinity to calcium which are drastically decreased (Table 1). Under PN condition the number of proteins isolated from cardiomyocyte inner membrane (cytoplasmic membrane) was decreased from 28 to 5 molecules and a diminution in the energy metabolism was observed.[11]

The PRP-1 may stimulate the protein synthesis in the cardiac tissues of the rats with PN, especially at chronic recurrent stage. However, negligible changes were observed in the expression of the calcium depot proteins, as well as in that of the 32-kDa protein in the cardiomyocytes (data not shown). Therefore, it could be speculated that PRP-1 impact on the calcium binding properties of the cardiomyocyte SR membrane proteins could be realized not by their synthesis de novo, but indirectly via triggering multiple mechanisms maintaining homeostasis.

PRP-1 impact on the calcium binding by the cardiomyocyte membrane proteins in the dynamics of crush syndrome

Histomorphological changes observed in early stages of decompression (2- 48 hours) were strikingly similar to those seen in PN (data not shown), it also concerns to alterations in the calcium binding of the cardiomyocyte SR membrane proteins in CS (Figure 8, b). Moreover, the same efficient doses of PRP-1 (10^{-6} M) administered at the end of compression and an hour later modulate the calcium-binding abilities of the Ca^{2+} depot-proteins (Figure 8, c and d). PRP-1 markedly attenuates the histopathological alterations associated with cardiac injury caused by PN and/or CS. We have previously shown that PRP-1 can prevent detrimental changes in the tissues during CS.[12, 13] For this series of experiments prior to initiation of muscle crush injury $^{45}CaCl_2$ was administered, and its binding assessed after SDS-PAGE separation of the cardiomyocyte SR membrane proteins. PRP-1 was injected immediately at the end of compression and an hour later.

Table 2. PRP-1 impact on the the cardiomyocyte 32-kDa protein affinity to calcium in the dynamics of crush syndrome

Calcium-binding properties	B_{max}, nmol calcium·mg^{-1} protein		K_d, nmol calcium·mg^{-1} protein	
Groups	Center of low affinity	Center of high affinity	Center of low affinity	Center of high affinity
Crush syndrome (immediately at the end of compression)	45.62 ± 0.86	231.58 ± 9.51	1.92 ± 0.28	8.71 ± 0.26
2 h decompression	36.27 ± 0.53***	± 8.17#	3.15 ± 0.38#	11.13 ± 0.28*
4 h decompression	48.81 ± 0.92**	243.49 ± 10.01*	4.71 ± 0.42**	12.82 ± 0.29*
24 h decompression	42.39 ± 0.67**	232.68 ± 7.83#	4.68 ± 0.39**	14.73 ± 0.31**
48 h decompression	44.12 ± 0.71#	258.91 ± 8.82*	5.63 ± 1.01**	17.18 ± 0.38**
PRP-1 pretreatment crush syndrome	4.19 ± 0.08***	61.7 ± 1.34***	1.78 ± 0.26***	3.65 ± 0.13***
PRP-1 pretreatment 2 h decompression	2.53 ± 0.06***	54.22 ± 0.49***	0.92 ± 0.19***	2.45 ± 0.38***
PRP-1 pretreatment 4 h decompression	2.39 ± 0.09***	48.43 ± 0.37***	0.85 ± 0.22***	2.03 ± 0.27***
PRP-1 pretreatment 24 h decompression	1.98 ± 0.07***	31.28 ± 0.45***	0.77 ± 0.19***	1.98 ± 0.21***
PRP-1 pretreatment 48 h decompression	1.55 ± 0.08***	29.25 ± 0.33***	0.65 ± 0.21***	1.87 ± 0.23***

Scatchard plot analysis data represent the mean of 12 separate experiments ± SEM. Differences are considered significant if P=0 .05. # P> 0.05, * P< 0.05, ** P<0 .01, *** P<0.001 (vs. Crush syndrome).

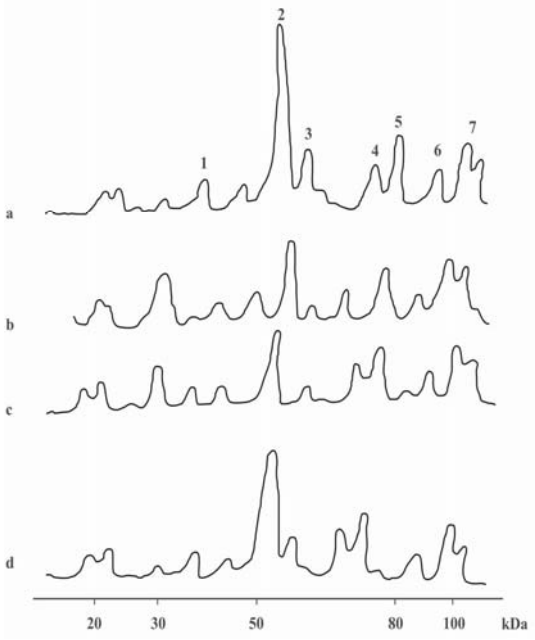

Figure 8. Effect of PRP-1 on the calcium binding to the cardiomyocyte SR membrane proteins in the dynamics of crush syndrome. 1, 3-6 - acidic proteins; 2- calsequestrin; 7 - two subunits of Ca2+-ATPase (a) control; (b) 2 h decompression; (c) 4 h decompression / PRP-1 treatment; (d) 48 h decompression / PRP-1 treatment.

Both PN and CS induce similar changes in myocardial 32-kDA protein calcium-binding properties (B_{max}) and its interaction with calcium (K_d), suggesting that there are common mechanisms involved in heart failure, regardless of pathology. As shown in Table 2, PRP-1 significantly diminishes the cardiomyocyte SR 32-kDa membrane protein affinity to calcium ions restoring its native properties which confirmed also by isoelectric focusing of 32-kDa protein (data not shown).

Notably, our previous findings on calcium-binding abilitiy of the cardiomyocyte SR membrane proteins in experimental isoproterenol-induced myocardial injury show similar changes in the qualitative and quantitative spectra of the proteins associated with a loss of their affinity to calcium, and vice versa a simultaneous posttranslational modification of the 32-kDa protein with a compensatory increase of its affinity to calcium.[10, 28]

A diffuse and granular immunoreactivity of PRP-1 was detected within the network of muscle fibers the synoatrial node of the human heart.[11] It can be only speculated that PRP-1 deficiency is one of the possible causes of the rat heart injury. It is known that PRP-1 is involved in the regulation of the cytokine expression (TNF-α, IL-1 and IL-6), activity of caspases, as well as lipid peroxidation.[11] On the other hand pathophysiological changes in heart failure include oxidative stress which is closely linked to apoptosis and inflammatory cytokines.[23] That is one of the reasons why the replenishment of PRP-1 may prevent histomorphological and molecular alterations implicated in heart injury induced under PN and/or CS conditions.

CONCLUSION

In summary, the results of the present study confirm that new neurosecretory cytokine, hypothalamic proline-rich peptide-1 preserve calcium-binding ability of the cardiomyocyte SR membrane proteins, which serve as the main calcium depot in cardiac muscle and protect against hypocalcemia implicated in common cellular and molecular mechanisms of myocardial damage during experimental acute pancreatitis and/or long-term compression injury, thus emerge from an otherwise pathological outcome. The results highlight new prospects over the functional implications of PRP-1 and its possible therapeutic potential for the treatment of patients at high risk of cardiovascular disease associated with different pathologies.

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