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Alterations in Calcium-Binding Properties of Sarcoplasmic Reticulum Membrane Proteins Following Cardiac Injury

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Author's contribution

This whole work was carried out by the author AGG.

Original Research Article

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ABSTRACT

Aims: The objective of present work is to investigate metabolic alterations associated with heart failure, particularly one of its manifestations, a sustained hypocalcemia that causes hemodynamic changes contributed to subsequent myocardial injury. Comparative study was carried out using experimental models of pancreatic necrosis (PN) and crush syndrome (CS) accompanied by cardiac damage down to myocardial infarction.
Study design: Wistar adult male rats randomly divided into groups (n=12/group). The controls are healthy intact animals. The pancreatic necrosis (PN) and crush syndrome (CS) groups were then randomly subdivided: PN group- into 3, 24 and 72 h groups concerning hemorrhage, early and late pancreatic necrosis respectively; CS group – into 2, 4, 24, and 48 h decompression stages. The rats were sacrificed to analyze spectra and calcium-binding properties of the membrane proteins isolated from the cardiomyocyte sarcoplasmic reticulum (SR). Development of pathological changes in the heart and pancreas were also monitored.
Place and Duration of Study: Department of Pathological Biochemistry and Radioisotope Methods, H. Buniatyan Institute of Biochemistry of Natl. Acad. Sci (NAS), Republic of Armenia (RA). Experiments conducted between May 2011 and October 2013.
Methodology: To study pathogenesis of hypocalcemia underlying myocardial damage a translocation of radioactive $^{45}CaCl_2$ into cardiomyocytes and its intracellular distribution was examined. Binding of $^{45}Ca^{2+}$ to the SR membrane proteins was measured after proteins separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and radioactivity from the gel plates was counted by a gas-flow meter Berthold–II.

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Isoelectric focusing of the protein isolated from the SR of cardiomyocyte was performed.
Results: Statistically significant changes in mean radio labeled calcium incorporation into a total protein fraction of the cardiomyocyte SR from control (13682±271) were determined by3h of PN (23055±168, *P*<.001), 24 h of PN (22876±240, *P*<.01), and by 72 h (3851±271,*P*<.01), *P* vs. control. Similarly, these parameters were detected following CS by 2h decompression (24179±225, *P*<.01), 4-24 hours decompression (21666±124, *P*<.001) and 48 h decompression (2941±189, *P*<.001), *P* vs. control. We demonstrate that drop in the binding calcium level observed was partially due to impaired affinity to calcium of the cardiomyocyte SR calcium-binding proteins during development of both PN and CS despite a simultaneous manifestation of affinity to calcium of the SR 32-kDa protein.
Conclusion: In the present study we have clearly shown that both experimental acute pancreatitis and long-term compression injury may cause similar changes,a loss the calcium-binding properties of the cardiomyocyte proteins, particularly those of SR serving as a main calcium depot under physiological circumstances and appear to be involved in common cellular and molecular mechanisms of myocardial injury contributing to hypocalcemia. Simultaneously, both PN and/or CS cause similar manifestations of the new calcium-binding properties of the cardiomyocyte SR 32-kDa membrane protein, and mirrored dynamic changes in its calcium affinity suggested by Scatchard plot analysis indicating a common mechanism *that* would be a transient attempt of certain heart cells to compensate hypocalcemia, and thus emerge from an otherwise pathological outcome. Thus, the above mentioned changes could be used to identify patients at high risk of cardiovascular disease in different pathologies.

Keywords: Calcium-binding proteins; crush syndrome (CS); pancreatic necrosis (PN); sarcoplasmic reticulum (SR); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

1. INTRODUCTION

There is considerable interest in elucidating mechanisms contributed to changes in the regulation of calcium level in the injured cardiac muscle. The pathogenesis of hypocalcemia is multifactorial and includes calcium-soap formation, hormonal imbalances (e.g., parathyroid hormone, calcitonin, and glucagon), binding of calcium (by proteins, free fatty acid-albumin complexes, etc.), and intracellular translocation of calcium. Hypocalcemia is one of the metabolic alterations involved in the hemodynamic changes and associated pericardium alterations and myocardial damage observed in clinical and experimental studies in acute pancreatitis [1]. Pancreatic necrosis (PN) represents a severe form of acute pancreatitis characterized by high morbidity and mortality of about 30%, and early deaths that occur one to two weeks after the onset of pancreatitis are due to multisystem organ failure [2]. Precise mechanism of myocardial injury during the course of acute pancreatitis still remains unclear, but it is of importance to identify acute pancreatitis patients at high risk of cardiovascular disease [3]. Proteolytic enzymes, lipase, kinins, and other active peptides liberated from the inflamed pancreas convert inflammation of the pancreas to a multisystem disease including myocardial depression and shock that are suspected to be secondary to vasoactive peptides and a myocardial depressant factor [4]. Moreover, PN induces acute renal failure and metabolic complications among which hypocalcemia has been long recognized as an indicator of poor prognosis [5]. Previously we developed the model of experimental pancreatitis accompanied by diffuse necrotic damage of myocardium which overgrows to a picture typical for myocardial infarction [6]. Acute pancreatitis is associated with total intoxication, influx of proteolytic enzymes into abdominal cavity, resulted in that one in 13

patients dies within 12 months of first diagnosis, with 25-35% mortality of AP caused by myocardial infarction [7,8].

Muscle crush injury commonly occurs after earthquakes, collapse of buildings etc., often induces crush syndrome (CS) if not treated promptly. Interestingly, CS and experimental CS animal model induces the total intoxication, myocardium damage, and multiple organ failure mirrored the picture occurred following NP, at that changes are observed at the early stage of decompression but not at compression - the most dangerous period of cardiomyocyte injury was at the 12th hour of decompression[9-11]. We established the experimental rat model of CS in which deep myocardial injury and acute intoxication cause a typical myocardial infarction ischemic damage and subsequent death of the animals in decompression period [12]. However, whether cardiomyocyte injury is induced after both CS and NP has not been investigated. We have performed this comparative study to observe the effects of both NP and CS on cardiomyocyte damage and its relationship to the hypocalcemia.

2. MATERIALS AND METHODS

2.1 Animals and Housing

Wistar rat strain is maintained at vivarium (H. BuniatyanInstitute of Biochemistry NAS RA) breeding facilities. 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. 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. Experiments conducted in 2011-2013.

2.2 Study Design

All experiments were carried out on 6–7 month-old white male Wistar rats weighing 180-220 grandomly divided into groups (n=12/group): one group of healthy animals served as control; The pancreatic necrosis (PN) and crush syndrome (CS) groups were then randomly subdivided: PN group - into 3, 24 and 72 h groups concerning hemorrhage, early and late pancreatic necrosis respectively; CS group – into 2, 4, 24, and 48 h decompression stages. The rats were sacrificed to analyze spectra and calcium-binding properties of the membrane proteins isolated from the cardiomyocyte sarcoplasmic reticulum (SR). Development of pathological changes in the heart and pancreas were also monitored.

All rats were anaesthetized with ester prior to decapitation or compression.

2.3 Experimental Pancreatic Necrosis

Pancreas was removed through a surgical incision, and a tail of the pancreas which ends abutting the spleen was cooled by chloroethyl, then the frozen part was defrosted by fingers, returned to its place and incision was sutured [6]. Three stages of AP were studied after initiation of PN: at3 h associated with vessel necrosis (hemorrhage), at 24 h and 72 h associated with beginning and end of the necrotic stage. As a marker enzyme of PN served α-amylase which activity increased by 8-12 times as compared with the control.

2.4 Experimental Crush Syndrome

Model of CS was induced by application of a standardized mechanical pressure (10 kg/100 g body weight) applied to the femoral muscle section of rat for two hours [12]. Thereafter rats were sacrificed at 2, 4, 24, and 48 h decompression stages.

2.5 Isolation of Rat Cardiomyocyte Sarcoplasmic Reticulum

2.5.1 Isolation of rat cardiomyocyte

After decapitation of animals under light anesthesia the heart was excised, attached to a Langendorff column, perfused with 0.15MKCl, thereafter crushed using 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). Homogenates were centrifuged at 50 *g* for 3-5 min to precipitate cardiomyocytes.

2.5.2 Preparation of SR membranes from isolated rat cardiomyocyte

To isolate SR membrane from cardiomyocytes, we used a modified procedure from Wientzek and Katz [13]. Isolated cells were suspended in a buffer containing 300 mM sucrose, 1 mM PMSF, and 20 mM PIPES, at pH 7.4, and disrupted with a glass-glass homogenizer. The homogenates were centrifuged at 500*g* for 20 minutes. The resultant supernatant was subjected to sucrose gradient centrifugation to obtain sarcoplasmic reticulum. After sucrose gradient centrifugation, the SR densest subfraction contained the highest (K+, Ca^{2+})-ATPase activity, whereas (Na2+,K+)-ATPase activity was not detected , even when its activity was unmasked. The purity of the SR was also suggested morphologically.

2.6 Polyacrylamide Gel Electrophoresis and Isoelectric Focusing

Prior to initiation of both PN and CS, radioactive $^{45}CaCl_2$ was administrated to animals and a translocation of Caions assessed by measuring the distribution of $^{45}Ca^{2+}$in the cellular compartments of cardiomyocites [14]. SR membrane proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing [15,16]. Samples were homogenized in glass-glass microhomogenizers in buffer solution1% SDS, 0.05% β-mercaptoethanol, 1mM EDTA, 50 µg/ml leupeptin, 50 µg/ml antipain, 100 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 13000g at 4ºC for 30 minand pellets were resuspended 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 activity of $^{45}Ca^{2+}$ in samples was measured from the gel plates using a gas-flow meter Berthold–II (Germany) [17].

Specific binding $^{45}Ca^{2+}$to the SR membrane proteins was measured in counts per minute (cpm) mg^{-1} protein and interaction between protein and bound calcium was evaluated using Scatchard plot analysis [18].

2.7 Protein Determination

Protein was determined by the method of Lowry et al., using crystalline bovine serum albumin as standard [19].

2.8 Statistical Analysis

Data are presented as the mean ± SEM. Relationships between biochemical parameters studied were determined calculating the Pearson linear correlation coefficient (*r*). All statistical analyses were 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-value of 0.05 is assigned to express statistically significant differences.

3. RESULTS AND DISCUSSION

Cellular and molecular alterations relevant to myocardial injury were investigated using experimental models of pancreatic necrosis (PN) and crush syndrome (CS) that accompanied by heart failure extending to myocardial infarction. Since methodological restrictions of the established acute pancreatitis models for therapy investigation, many attempts have been made to develop, modify or combine available models to more closely resemble human acute pancreatitis [20]. We have developed the most appropriate model of PN for studying a myocardial damage induced [6]. Acute pancreatitis is known varies in severity, ranging from focal edema and fat necrosis to widespread hemorrhagic parenchymal necrosis [2]. Different stages of PN were studied to assess accompanied cardiac injury and associated molecular changes. The sarcoplasmic reticulum (SR) is the intracellular storage site of Ca^{2+} and plays a major role in the contraction/relaxation machinery. Cardiac dysfunction is commonly associated with impairment in SR function, sarcolemmal calcium influx and damage in calcium regulatory proteins [21,22]. Damage of myocardium registered at hemorrhagic (3 h) and necrotic (24 and 72 hours) stages of experimental PN was accompanied by significant alterations in the qualitative and quantitative properties of the SR protein. Total protein of the inner membranes (i.e. cytoplasmic membrane) separated by SDS-PAGE exhibits 28 fractions under physiological circumstances, while PN caused a drop of the number of proteins up to 5 molecules: 3 fractions (Mr 60-80 kDa) and 2 fractions (Mr20-30 kDa) leading to a reduced cardiomyocyte energy metabolism. Moreover, analysis of dynamic changes in affinity of the SR membrane proteins to calcium following PN was shown a loss of calcium-binding ability of proteins that commonly participating in calcium accumulation in SR under physiological cirumstances, including five acidic proteins and calsequestrin (Mr 55kDa)(Fig. 1). Only exception was large subunit of Ca^{2+}-ATPase (Mr 100 kDa) (two subunits of Ca^{2+}-ATPase are separated at SDS-PAGE) that is necessary, as otherwise an instant cardiac arrest would occur.

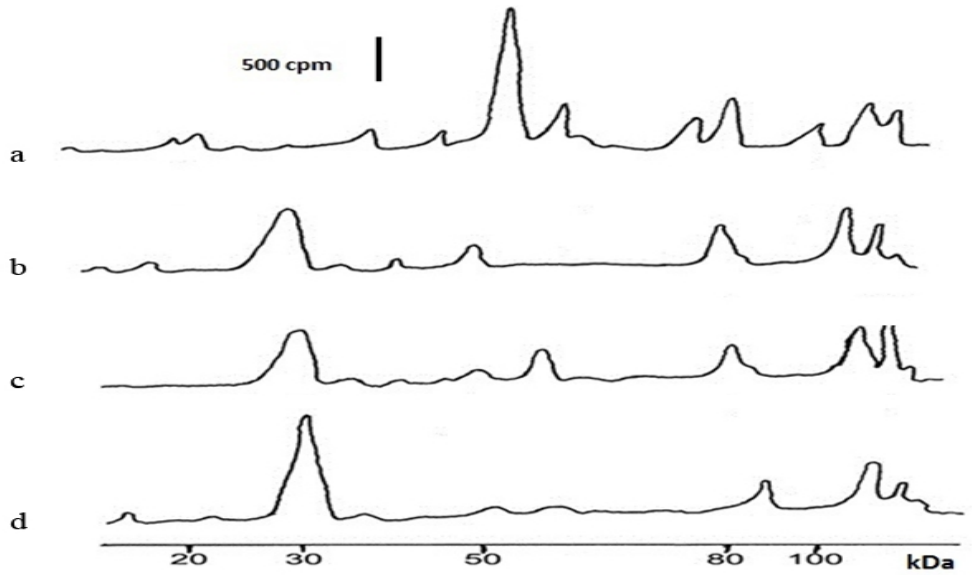

Fig. 1. Pancreatic necrosis (PN)-induced dynamic changes in the calcium-binding properties of the cardiomyocyte SR membrane proteins

Binding of the $^{45}CaCl_2$administered to rats prior to experimental PN was assessed after separation of the cardiomyocyte SR membrane proteins by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

a. control; b. 3h after initiation of PN; c. 24h after initiation of PN; d. 72h after initiation of PN.

Contrary, 32-kDa membrane protein of SR which does not exhibit affinity to calcium ions in norm, but after PN the former has acquired calcium-binding properties increasing in parallel with the development of cardiac damage. Separating the SR proteins by difference in their isoelectric points by isoelectric focusing showed that 32-kDaprotein isolated from the cardiomyocyte of control healthy rats is alkaline protein, while 32-kDaprotein isolated from cardiomyocyte of animals subjected to experimental PN is acidic protein (pHchanges were linear andit decreased from 8.3 to 5.9).We conducted a study of the 32-kDaproteinamino-acid content and showed that the acidic amino-acid (aspartic and glutamic acids)accounted for8.2% amino-acid content of the 32-kDaprotein in control animals, whereas they make up to 19.3% of the latter in rats with PN. Modeling of 32-kDa protein using special programs developed (software engineering)is shown, that in the protein tertiary structure one carboxyl group of the mentioned acidic amino-acids is embedded and the second carboxyl group remains free projecting from the protein, anda distance between the dissociated carboxyl groups allows to bind calcium ions by covalent bond there through efficiently accumulate it by modified protein [23].

Notably, it has been suggested the cardiomyocyte SR membrane proteins including changes in the 32-kDa protein affinity to calcium by 3, 24 and 72 hours of PN were almost similar to those observed in CS by 2, 4 and 24 hours that coincided with those of detected by 24 h of PN), and 48 hours decompression (Fig. 2).

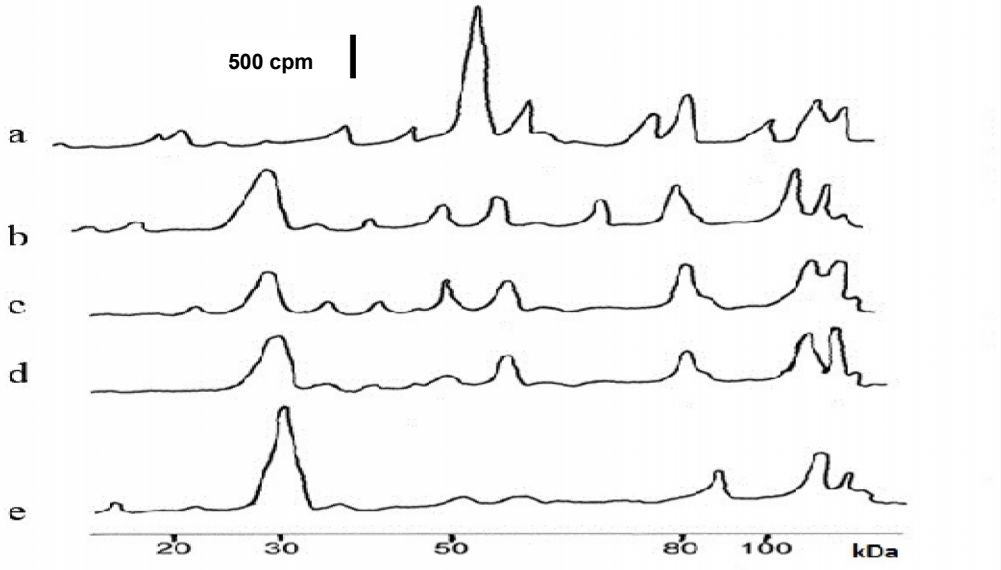

Fig. 2. Crush syndrome (CS)-induced dynamic changes in the calcium-binding properties of the cardiomyocyte SR membrane proteins

Binding of the $^{45}CaCl_2$administered to rats prior to experimental CS was assessed after separation of the cardiomyocyte SR membrane proteins by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

a. control; b. 2 h of decompression; c. 4 h of decompression; d. 24 h of decompression; e. 48 h of decompression.

Moreover, morphological analysis showed that the pattern of cardiac injury on 24 h of PN is mirrored that of detected on both 4 and 24 hours of decompression, which are not differed each from other (data not shown). Therefore, parameters studied by 2, 24 and 72 h of PN are compared with those of CS by 2, 4-24, and 48 h of decompression.

Our data were suggested by statistically significant changes in mean calcium incorporation into a total protein fraction of the cardiomyocyte SR from control (13682±271)that were determined by3h of PN (23055±168, *P*<.001), 24 h of PN (22876±240, *P*<.01), and by 72 h of PN (3851±271, *P*<.01), *P* vs. control. Similar changes of these parameters were found following CS by 2h decompression (24179±225, *P*<.01), 4-24 hours decompression (21666±124, *P*<.001) and 48 h decompression (2941±189, *P*<.001), *P* vs. control.

It is noteworthy that our previous study of calcium-binding abilities of the cardiomyocyte SR membrane proteins during an experimental isoproterenol-induced myocardial injury showed similar changes in their qualitative and quantitative spectra concerned to loss affinity to calcium, as well as a simultaneous compensatory posttranslational modification of the 32-kDa protein contributed to an increase of its affinity to calcium [14,23]. Thus, it can be speculated that these alterations might represent common cellular and molecular mechanisms contributing to hypocalcemia involved in the development of heart injury following different pathologies despite the manifestation of the new calcium-binding properties of the 32-kDA membrane protein.

To evaluate the affinity of calcium ions to the SR 32-kDa membrane proteinin norm and pathology we examined a binding curve on Scatchard coordinates that makes distinction more pronounced, and showed more than one population of binding sites [18]. Parameters determined are Bmax - the maximal binding capacity of the protein(s) and Kd - the steady state dissociation constant (Kd is the free concentration of calcium for which the bound concentration is equal to Bmax/2; the inverse of Kd is Ka, the steady-state constant of affinity). Data for centers with low and high affinity to calcium of the 32-kDa membrane protein following PN and CS are presented in Tables 1 and 2.

Table 1. Scatchard plot analysis of dynamic changes in calcium affinity to 32-kDa membrane protein from the cardiomyocyte sarcoplasmic reticulum following pancreatic necrosis

Calcium-binding properties	Bmax nmol calcium·mg^{-1} protein		Kd nmol calcium·mg^{-1} protein	
Groups	Center of low affinity	Center of high affinity	Center of low affinity	Center of high affinity
Control	45.62±0.86	231.58±9.51	1.92 ±0.28	8.71±0.26
Hemorrhage (3 h)	38.42±0.72***	201.24±8.06* *P*=.02	3.25±0.35#	11.27±0.29***
Early stage of necrosis (24 h)	47.63±1.1#	245.43±9.92#	4.58±0.37** *P*=.0017	13.28±0.31***
Late stage of necrosis (72 h)	42.63±0.75* *P*=.02	234.88±7.83#	4.99±0.39***	15.15±0.34***

Data represent the mean of 12 separate experiments± SEM. Differences are considered significant if P= .05. # P> .05, P< .05, ** P< .01,*** P<.001 (vs. control).*
Comparisons between groups, Bmax: a) Center of low affinity - F= 21.16, P<.001; b) Center of high affinity - F=4.57, P=.007; (Row 3 vs. Row 2, P= .001; Row 4 vs. Row 2, P= .01); Kd: a) Center of low affinity - F= 6.9, P<.001; b) Center of high affinity - F= 83.93, P<.001

Table 2. Scatchard plot analysis of dynamic changes in calcium affinity to 32-kDa membrane protein of the sarcoplasmic reticulum following decompression in crush syndrome

Calcium-binding properties	Bmax nmol calcium·mg^{-1} protein		Kd nmol calcium·mg^{-1} protein	
Groups	Center of low affinity	Center of high affinity	Center of low affinity	Center of high affinity
Control	45.62±0.86	231.58±9.51	1.92±0.28	8.71±0.26
2 h decompression	36.27±0.53***	211.24±8.17#	3.15±0.38#	11.13 ±0.28*
4 h decompression	48.81±0.92** *P*=.004	243.49±10.01#	4.71±0.42***	12.82±0.29**
24 h decompression	42.39±0.67** *P*=.004	232.68±7.83#	4.68±0.39***	14.73±0.31***
48 h decompression	44.12±0.71#	258.91±8.82* *P*=.034	5.63±1.01** *P*=.001	17.18±0.38***

Data represent the mean of 12 separate experiments± SEM. Differences are considered significant if P= .05. # P> .05, P< .05, ** P< .01,*** P<.001 (vs. control).*
Comparisons between groups, Bmax: a) Center of low affinity - F= 38.37, P<.001; b) Center of high affinity - F=3.86, P=.008; Row 3 vs. Row 2, P= .001; Row 4 vs. Row 2, P= .01; Kd: a) Center of low affinity - F= 4.12, P=.005; b) Center of high affinity - F=112.52, P<.001

It is of interest that both PN and CS cause similar dynamic changes in parameters characterized of myocardial 32-kDA protein calcium-binding properties (Bmax) and its

interaction with calcium (Kd). Positive correlations were detected between PN and CS in respect with Bmax values for centers of low affinity to calcium of the 32-kDa SR protein (r=0.9981, *P*=.0745), and those of high affinity (r=0.9942, *P*=.0745), as well as Kd values for centers of low affinity to calcium (r=0.9914, *P*=.0745) and those for centers of high affinity to calcium (r=0.9995, *P*=0.0745). This is once more suggested that there are common mechanisms involved in heart failure regardless of pathology.

4. CONCLUSION

In the present study we have clearly shown that both experimental acute pancreatitis and long-term compression injury may cause similar changes, particularlya loss the calcium-binding properties of the cardiomyocyte proteins, particularly those of SR serving as a main calcium depot under physiological circumstances and appear to be involved in common cellular and molecular mechanisms of myocardial injury contributing to hypocalcemia. Simultaneously, both PN and/or CS cause similar manifestations of the new calcium-binding properties of the cardiomyocyte SR 32-kDa membrane protein, and mirrored dynamic changes in its calcium affinity suggested by Scatchard plot analysis indicating a common mechanism *that* would be a transient attempt of certain heart cells to compensate hypocalcemia, and thus emerge from an otherwise pathological outcome. Thus, the above mentioned changes could be used to identify patients at high risk of cardiovascular disease in different pathologies.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "principles of laboratory animal care" (the European Communities Council Directive (86/609/EEC) on care and use of animals for experimental procedures) were followed, as well as specific national laws where applicable.

All experiments have been examined and approved by the appropriate ethics committee.”

COMPETING INTERESTS

Author has declared that no competing interests exist.

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