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Calpain Inhibition Decreases Myocardial Fibrosis in Chronically Ischemic Hypercholesterolemic Swine

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Abstract

Objectives: Calpain activation during ischemia is known to play critical roles in myocardial remodeling. We hypothesize that calpain inhibition (CI) may serve to reverse and/or prevent fibrosis in chronically ischemic myocardium.

Methods: Yorkshire swine were fed a high cholesterol diet for 4 weeks followed by placement of an ameroid constrictor on the left circumflex artery to induce myocardial ischemia. 3 weeks later animals received either: no drug, high cholesterol control group (CON; n= 8); low dose CI (0.12 mg/kg; LCI, n= 9); or high dose CI (0.25 mg/kg; HCI, n= 8). The high cholesterol diet and CI were continued for 5 weeks, after which myocardial tissue was harvested. Tissue samples were analyzed by western blot for changes in protein content.

Results: In the setting of hypercholesterolemia and chronic myocardial ischemia, CI decreased the expression of collagen in ischemic and non-ischemic myocardial tissue. This reduced collagen content was associated with a corresponding decrease in Jak/STAT/MCP-1 signaling pathway, suggesting a role for Jak 2 signaling in calpain activity. CI also decreases the expression of focal adhesion proteins (vinculin) and stabilizes the expression of cytoskeletal and structural proteins (N-cadherin, α fodrin, desmin, vimentin, filamin, Troponin-I). CI had no significant effect on metabolic and hemodynamic parameters.

Conclusions: Calpain inhibition may be a beneficial medical therapy to decrease collagen formation in patients with coronary artery disease and associated co-morbidities.

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Author contributions:

BAP and FWS contributed to study design, data collection, data analysis, manuscript writing, manuscript review. SA and IJL contributed to data collection. RTC contributed to data collection, data analysis, manuscript writing and manuscript review. MRA and AS contributed to manuscript writing and manuscript review.

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Conflict of Interest: None

Graphical Abstract

UltraMini Abstract:

In the setting of hypercholesterolemia and chronic myocardial ischemia, calpain inhibition decreases the expression of fibrosis in myocardial tissue. This decrease is associated with the Jak/STAT/MCP-1 pathway. Calpain inhibition results in stabilization of the expression of cytoskeletal and structural proteins (N-cadherin, α -fodrin, desmin, vimentin, filamin, Troponin-I).

Introduction

Calpains are calcium activated cysteine proteases, which serve to maintain cellular homeostasis by modifying functions of target proteins through truncation.¹ Following ischemic insult to the heart, myocardial remodeling is associated with changes to contractile proteins of cardiac tissue.²⁻⁴ Calpain activation during ischemia is known to play a role in myocardial remodeling by cleaving multiple myofibrillar-specific and cytoskeletal proteins. Cytoskeletal derangements during ischemia are associated with enhanced myocardial fibrosis.⁵⁻⁷ Calpain is thought to cleave and activate TGF- β . TGF- β promotes collagen formation via the TGF- β /SMAD/MMP pathway and the Jak/STAT/MCP-1 pathway.^{5,8}

We have previously shown that calpain inhibition increases blood flow, improves vessel density, improves microvessel relaxation, reduces oxidative stress and decreases cell death in the myocardium of pigs with metabolic syndrome and chronic myocardial ischemia.^{9,10} In small animal models, calpain inhibition during chronic myocardial ischemia improves capillary morphogenesis, restores cytoskeletal organization, promotes a functionally improved neovasculature that reduces underlying hypoxia, and is associated with decreased myocardial fibrosis.^{2,9-19} Therefore, calpain inhibition may serve as a potential medical therapy to reverse or prevent myocardial fibrosis and dysfunction in patients with coronary artery disease.

The aim of this study was to determine if moderate calpain inhibition would decrease fibrosis, improve pro-fibrotic signaling and stabilize cytoskeletal structure in a clinically relevant swine model of diet-induced metabolic syndrome and chronic myocardial ischemia.

Materials and Methods

Animal Model:

The animal model used in this study has been previously described.^{9,10} Twenty-five Yorkshire swine (E.M. Parsons and Sons, Hadley MA) were fed a high fat/ high cholesterol diet for four weeks. The pigs then underwent placement of an ameroid constrictor on the left circumflex artery to induce chronic myocardial ischemia. Three weeks later, animals received either: no drug, (high cholesterol control group, placebo, CON; n= 8); a low dose of the calpain inhibitor MDL28170 (CI) drug (0.12 mg/kg daily; LCI, n= 9); or a high dose of CIMDL28170 (CI) drug (0.25 mg/kg daily; HCI, n= 8). The drugs were administered by mouth. The high cholesterol diet and CI were continued for four weeks, after which the pigs were euthanized and the left ventricular myocardium was harvested and sectioned based on the location to the left circumflex and left anterior descending artery. The Rhode Island Hospital Institutional Animal Care and Use Committee approved and supervised all experiments. The Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* were used to ensure proper care of all animals. All animals survived the experiment.

Metabolic Parameters:

A glucose tolerance test was performed prior to the start of the harvest procedure. 0.5g/kg of dextrose 50 was administered at the start of the operation and blood glucose was measured at 30 and 60 minutes. The pigs were measured for length and weight prior to the start of the ameroid and harvest procedures. Blood taken at the time of the harvest procedure was sent to the Rhode Island Hospital chemistry lab for quantification of cholesterol parameters.

Hemodynamic Measurements:

A Millar catheter was inserted in the LV apex after sternotomy followed by advancement to the aorta. Hemodynamic data was recorded and analyzed with Sonosoft Software (Company Empower Technologies, Inc, Sarasota, Florida) to measure left ventricular pressure, heart rate, mean arterial pressure +dP/dt, -dP/dt, arterial pressure and pulse pressure for each of the pigs. Measurements were taken at the beginning of the harvest procedure.

Picrosirius Red Stain:

Picrosirius red stain was performed on 1cm tissue sections using the Picrosirius Red Stain Kit (Polysciences, Inc. Warrington, PA). Briefly, paraffin sections were de-waxed and hydrated. Nuclei were stained with Weigert's hematoxylin and washed with running tap water. Picrosirius stain was applied to the tissue slides for one hour and then the slides were washed in two changes of acidified water. All water was removed and the tissue samples were dehydrated using three changes of 100% ethanol. The tissue was then cleared in xylene and mounted in a resinous medium. Images were captured at 20X magnification with a Nikon E800 Eclipse microscope (Nikon, Tokyo, Japan) at the same exposure in 5 random

fields. Image J software (National Institutes of Health, Bethesda, MD) was used to measure collagen expression in a blinded fashion. Representative images have been included in the manuscript.

Immunologically Detectable Protein Content:

Tissue was lysed in radio-immuno-precipitation assay buffer (RIPA) (Boston BioProducts, Ashland, MA), 40ug were fractionated by sodium dodecyl sulfide polyacrylamide gel electrophoresis (SDS-PAGE) using 3%–8% Tris-Acetate gels (NuPage Novex Mini Gel), and the protein was transferred to polyvinylidene difluoride membranes (PVDF) (Millipore, Billerica, MA) and incubated overnight at 4 degrees C with primary antibodies against Transforming growth factor β (TGF- β), Janus family of tyrosine kinase-2 (Jak2), STAT 3, matrix metalloproteinase-9 (MMP-9), SMAD2/3, monocyte chemoattractant protein-1 (MCP-1), α -tubulin, N- Cadherin, α -fodrin, desmin, connexin 43, pan keratin, β -tubulin, Troponin I, vimentin, filamin, β -Actin and troponin T (Cell Signaling, Danvers, MA). The following day, membranes were incubated with the appropriate horseradish peroxidase (HRP)-linked secondary antibody for 1h at room temperature (Jackson ImmunoResearch, West Grove, PA). Immune complexes were visualized with enhanced chemi luminescence, images were captured with a digital camera system (G-Box, Syngene, Cambridge, England), and band densitometry was quantified as arbitrary light units using Image J Software (National Institutes of Health, Bethesda, MD). Anti-GAPDH antibody (Cell Signaling) was used on all membranes to correct for loading error. Representative images have been included in the manuscript.

Cell Culture:

H9c2 cells (ATCC) were grown to confluence in 10% fetal bovine serum (FBS) DMEM and then switched to 1% FBS DMEM for 1 week to promote increased cardiomyocyte protein expression as previously described.²⁰ Human ventricular fibroblast (VF) cells (Lonza, Walkersville, MD) were grown to confluence according to manufacturer's recommendation (Lonza, Walkersville, MD). The H9c2 and human ventricular fibroblast cells were each then split into three groups: control groups were given either DMSO (vehicle) (1:10,000) in the appropriate cell type media (H9c2 n=7, VF n=7), 10 nM of calpain inhibitor MDL28170 (LCI) (H9c2 n=7, VF n=7), or 100 nM of calpain inhibitor MDL28170 (HCI) (H9c2 n=3, VF n=4). All groups were incubated for 24 hours. The cells were then lysed in RIPA buffer with protease and phosphatase inhibitors and subjected to SDS-PAGE and immunoblotting.

Data Analysis:

Tissue and cell culture protein content data was normalized to GAPDH in Western blot experiments and is reported as fold change values compared to the high cholesterol diet (CON) group $\pm$ standard error of the mean (SEM). Kruskal-Wallis followed by Dunn's post-hoc test was used to compare the means among groups for the protein expression data and the metabolic parameter and hemodynamic data. Results are reported as fold change compared to the high cholesterol diet (CON) group $\pm$ SEM. All data was analyzed using GraphPad Prism 5.0 Software (GraphPad Software Inc, Sand Diego, Ca).

Results

Hemodynamic and Metabolic Parameters:

There was no difference in glucose tolerance, percent change in weight or cholesterol: HDL ratio between control, low-dose CI (LCI: [0.12 mg/kg daily]) and high dose CI (HCI [0.25 mg/kg]) which were all subjected to a high fat/high cholesterol diet ($p>0.05$). [Figure 1 A–C]. There were no significant differences in LV hemodynamics (LVDevP, $\pm$ dP/dt, rate pressure product) or aortic pressures (SBP, DBP, MAP, pulse pressure) between groups [Table 1].

Fibrosis Expression:

Inhibition of calpain in hypercholesterolemic pigs reduced fibrosis as assessed by picrosirius red staining in both the ischemic [Figure 2A] and non-ischemic myocardial tissue [Figure 2B].

Pro- Fibrotic Protein Expression in the Ischemic Tissue:

Calpain is thought to cleave and activate profibrotic signaling molecule TGF- β . Therefore, we investigated if classical pro-fibrotic signals were modulated by calpain inhibition in the ischemic tissue, including TGF β and downstream mediators Smad, JAK/STAT, and MCP-1. There was a decrease in TGF- β in the HCI and LCI groups compared to the control however this did not reach statistical significance ($p=0.08$). There was a significant decrease in Jak 2 in the LCI group compared to the control and a nonsignificant decrease in the HCI group compared to the control ($p<0.05$). There was a significant decrease in MCP-1 in the LCI group and a nonsignificant decrease in the HCI group compared to the control ($p=0.09$). Additionally, there was a significant decrease in the LCI group compared to the HCI group. There was a significant decrease in cardiomyocyte skeletal protein α -Actinin in the HCI group compared to the control and nonsignificant decrease in LCI compared to the control ($p<0.05$). [Figure 3A (a/b)] There were no changes in p-STAT-3 (0.46), MMP-9 ($p=0.79$), SMAD2/3 ($p=0.96$) or p-SMAD2/3 ($p=0.36$).

Pro- Fibrotic Protein Expression in the Non- Ischemic Tissue:

As fibrosis was also reduced with calpain inhibition in the non-ischemic tissue, we also evaluated profibrotic signaling in the remote territory. Similar to ischemic tissue, there was a significant decrease in Jak 2 in the LCI group compared to the control group and a nonsignificant decrease in HCI group compared to the control ($p<0.05$). TGF- β was significantly reduced in the HCI group but this decrease was non-significant in the LCI group ($p<0.05$). There was a nonsignificant increase in SMAD 2/3 in the LCI and HCI group compared to the control ($p=0.12$). There was no difference in p-SMAD 2/3 between the calpain inhibited groups and the control ($p=0.673$). [Figure 3B (a/b)]

Cytoskeletal Protein Expression in Ischemic Tissue:

Calpain has been proven to cleave several substrates which are known to play a role in fibrosis.²¹ Calpain inhibition is thought to prevent the degradation of structural proteins α -fodrin, troponin I and N-cadherin in the setting of myocardial stress.^{2,12,14,22} In the

ischemic myocardial tissue, there were significant increases in structural proteins troponin I and α -fodrin in both the LCI and HCI groups compared to the control ($p<0.05$). Desmin and vimentin, and the actin binding protein, filamin are all substrates of activated calpain. There were dose dependent increases in membrane cytoskeletal focal adhesion proteins desmin, filamin and vimentin with significant increases in the LCI group ($p<0.05$), increases in the HCI group did not reach statistical significance compared to the control ($p>0.05$). There was a significant increase in cell adhesion molecule N-cadherin in the HCI group ($p<0.05$) but not in the LCI group compared to the control [Figure 4A/B].

There were no significant changes in other membrane or cytoskeletal proteins connexin 43 ($p=0.22$), pan keratin ($p=0.94$), β -actin ($p=0.76$), Troponin T ($p=0.31$), or β -tubulin ($p=0.67$) in either the calpain inhibited group compared to the control group. [Supplemental Figure 1]

Cytoskeletal Protein Expression in Non- Ischemic Tissue:

In the non-ischemic region, calpain inhibition did not affect the expression of keratin ($p=0.27$), α -fodrin ($p=0.22$), desmin ($p=0.26$), vimentin ($p=0.24$), N cadherin ($p=0.54$), troponin T ($p=0.08$) or connexin 43 ($p=0.73$) compared to the control. [Supplemental Figure 2] However, there was a significant decrease in β tubulin in the LCI group ($p<0.05$) and a significant decrease in filamin and β actin in the HCI group ($p<0.05$) compared to the control. [Figure 5A/B]

In Vitro Calpain-Induced Changes in TGF-B Signaling:

Similar to the pig myocardial tissue, calpain inhibition *in vitro* using the cardiomyoblast h9c2 cell line decreased TGF- β ($p<0.05$) and p-SMAD 2/3 ($p<0.05$) as compared to the control. However, there was no significant difference in expression of Jak2 between the groups ($p=0.24$). [Figure 6A(a/b)] In the human ventricular fibroblast cells, calpain inhibition increased p-SMAD 2/3 ($p<0.05$) and decreased Jak2 ($p<0.05$) in the 100 nM CI groups compared to the control. There was no significant difference in expression of TGF- β between. [Figure 6B(a/b)]

Discussion

We have previously shown that calpain inhibition increases blood flow, improves vessel density, improves microvessel relaxation, reduces oxidative stress and decreases cell death in the myocardium of pigs with metabolic syndrome and chronic myocardial ischemia.^{9,10} The purpose of this study was to determine if calpain inhibition modifies fibrosis, improves pro-fibrotic signaling and stabilizes cytoskeletal structure in the myocardium of metabolic syndrome pigs.

Calpain Inhibition Leads to Decreased Fibrosis Formation

Calpain inhibition was associated with decreased fibrosis deposition in the ischemic and non-ischemic myocardial tissue. This is consistent with previous research which shows that in the setting of atherosclerosis, calpain over-activation leads to increased expression of transforming growth factor β and matrix metalloproteinases which are linked to increased collagen production, vascular calcification, myofibroblast differentiation and

fibrotic disease.^{6-8,23} Importantly, our data suggests that calpain inhibition results in decreased fibrosis formation in myocardial tissue in the setting of chronic myocardial ischemia and hypercholesterolemia.

Calpain Inhibition Attenuates The Pro-Fibrotic Jak/Stat/MCP-1 Pathway

TGF- β promotes collagen formation via the TGF- β /SMAD/ MMP pathway.^{5,8} Calpain is thought to cleave TGF- β leaving it in an active state.^{5,8} We found a decrease in TGF- β in the ischemic and nonischemic myocardial tissue in the calpain inhibited group. Interestingly, there was no change in SMAD2/3, p-SMAD 2/3, or MMP-9, which are indicators of active intracellular TGF-dependent signaling. Calpain is thought to cleave and activate TGF- β . TGF- β promotes collagen formation via several different pathways including the TGF- β /SMAD/MMP pathway and the Jak/STAT/MCP-1 pathway. These same pathways can be activated by other means as well such as BMP7 and SRC respectively. We feel that because TGF- β activation is a direct substrate of calpain, the low levels seen in our study are consistent with calpain inhibition. The recovery of the downstream signaling suggests that the activation of SMAD/MMP and Jak/Stat pathway could also be a result of cross reactivity of these different signaling pathways. Further studies are required to examine this hypothesis. Nevertheless, our data is consistent with the idea that calpain inhibition reduces pro-fibrotic signaling globally in the heart, and this reduction may be associated with modulation of TGF- β and other fibrotic signals.

Another pro-fibrotic pathway that is promoted by TGF- β is the Jak/STAT/MCP-1 pathway.²⁴ MCP-1 is produced by fibroblasts and induces monocyte chemotaxis, which further promotes myofibroblasts leading to increased fibrosis.^{6,25} MCP-1 expression is induced activated tyrosine kinase Jak which phosphorylates and activates the transcription factor Stat. Stat then moves to the nucleus and transcriptionally upregulates MCP-1 expression.²⁴⁻²⁷ We found that calpain inhibition is associated with decreased expression of Jak2 and MCP-1. This suggests a novel pathway by which calpain inhibition reduces fibrotic signaling globally in the heart via attenuation of the JAK/Stat/MCP-1 pathway

Calpain Inhibition Alters Cytoskeletal Proteins In Ischemic Myocardial Tissue

Ischemic insults are known to cause derangements and reductions in the myocardial cytoskeletal proteins. Calpain is known to cleave many of these structural proteins some of which are involved in fibrosis. Calpain inhibition results in the altered expression of several cytoskeletal proteins in the ischemic myocardial tissue. We found that in ischemic myocardial tissue, calpain inhibition coincides with preservation of structural proteins, troponin I, N Cadherin, and α -fodrin. This is consistent with previous research in rodent models which show that in the setting of myocardial stress, calpain inhibition prevents the degradation of α -fodrin, troponin I and N-cadherin.^{2,12,14,22} Our data supports the notion that in the setting of chronic myocardial ischemia and metabolic syndrome, calpain inhibition results in preservation of α -fodrin, troponin I and N-cadherin and may promote an organized left ventricular remodeling.

Interestingly, we also found that calpain inhibition increased the expression of the intermediate filament proteins, desmin and vimentin, and the actin binding protein, filamin.

All of these proteins are known to be substrates of activated calpain. Desmin is a muscle specific member of the intermediate filament family of cytoskeletal proteins. The absence of desmin in mice leads to 1) myocyte structural defects and cell death 2) calcification of the myocardium and ventricular dilatation and 3) compromised systolic function.²⁸ These data indicate that calpain inhibition may overall lead to improved cardiomyocyte integrity and function in chronically ischemic tissue as well as decreased fibrosis.

Calpain Inhibition Modulates Cytoskeletal Protein Expression In Non-Ischemic Myocardial Tissue

In the non-ischemic tissue, calpain inhibition had no effect on seven of the eleven cytoskeletal proteins tested. However, we did see a trend toward increased expression of troponin T ($p=0.08$) in the CI group compared to the control. This finding is consistent with previous research that suggests that Troponin T is a substrate of calpain cleavage. N-terminal cleavage of Troponin T by calpain is thought to alter its ability to bind to troponin C and troponin I.²⁹ Interestingly, of the 7 proteins that were found to be unaffected by calpain inhibition in the non-ischemic tissue, pan keratin, α -fodrin, desmin, vimentin, and N-cadherin had all been increased by the presence of calpain inhibition in the ischemic tissue.

One explanation for the lack of effect of CI in non-ischemic tissue is that calpain may have little basal activity. Calpain is necessary for the regular turnover of aggregated proteins, which if not cleared, will increase autophagy. Tissue hypoxia induces calpain over-activation by altering the calcium influx, thereby leading to aberrant angiogenesis in the ischemic tissue.^{17,30} Moderate inhibition of this calpain over-activation leads to improved neovessel formation while complete knock out of calpain prohibits neovessel formation.^{16,17} Therefore, some level of calpain is necessary for basic physiological functioning. This is consistent with the results of our study, which found that calpain inhibition in the ischemic territories, where calpain is likely over activated, produces a beneficial effect on cytoskeletal protein expression. However, in the non-ischemic territories, where we presumably would not have over-activation of calpain, we do not see an effect on the expression of many of the cytoskeletal proteins.

Interestingly, we also observed a decrease of filamin and β Actin in response to the high dose of calpain inhibition and a decrease of β tubulin in response to the low dose of calpain inhibition. However, this may be the result of decreased fibrosis content which was observed in both ischemic and non-ischemic tissue. In cases of greatly increased fibrosis, B-actin expression is greatly increased possibly indicative of fibroblast proliferation and activation.

Calpain Inhibition Alters Expression of Focal Adhesion Proteins In Ischemic Myocardial Tissue

Interestingly, we also found that calpain inhibition is associated with decreased expression of α -Actinin compared to the control. α -Actinin is a cell adhesion and cell migration protein. Inhibition of focal adhesion markers has been shown to attenuate cardiac fibrosis after myocardial infarct.³¹ In addition, fibroblast transition to a pro-fibrotic myofibroblast phenotype is also associated with enhanced focal adhesion formation, therefore decreased

focal adhesion protein expression with calpain inhibition may reflect decreased fibrosis signaling.

Calpain Inhibition Affects TGF- β and Jak/Stat/MCP-1 in Cardiac Cells

To confirm the effect of calpain inhibition on TGF- β and the Jak/Stat/MCP-1 pathway and to differentiate if calpain inhibition affects myocyte-like cells or fibroblasts, we examined the effects of calpain inhibition in cell cultures. Interestingly, we found that dose dependent calpain inhibition is associated with decreased expression of TGF- β and p-SMAD 2/3 in the cardiomyoblast cells and a trend toward decrease in Jak2. This suggests that calpain inhibition decreases the TGF- β and p-SMAD2/3 pathway in cardiomyoblast cells. In the ventricular fibroblast cells we found no change in TGF- β expression, an increase in p-SMAD 2/3 expression and a decrease in Jak2 expression with addition of calpain inhibitor. Taken together, this data suggests that calpain inhibition has varying effects depending on the cell type with which it is working.

Calpain Inhibition had no effect on Metabolic Parameters or Hemodynamic Analysis

We found that calpain inhibition does not modify severity of metabolic-syndrome induced impairments in glucose metabolism or total cholesterol and lipid levels. There is evidence that calpain activation is increased in the setting of metabolic syndrome and that calpain inhibition may be beneficial.^{1,13,32,33} The dosages of calpain chosen for this study were based on small animal ischemia reperfusion models. It is likely that the dose of calpain inhibition needed to have an effect on metabolic parameters is higher than those used for this study. Calpain inhibition had no significant effect on hemodynamic parameters. Our previous research studying the effects of resveratrol and metformin in the same pig model both showed lack of significant change in baseline hemodynamics, but improvement in vascular function. Both resveratrol and metformin are commonly used as effective medications for patients with coronary artery disease and metabolic syndrome.^{34–36} Therefore, we believe that calpain inhibition may be a valuable medicine in a similar subset of patients by increasing blood flow to the heart and through beneficial effects on a cellular and molecular level.

Limitations:

Calpain functions at a basal level throughout the body to maintain homeostasis. In situations of stress, calpain activity becomes overactivated which can lead to aberrant truncation of proteins and dysfunction. We believe that our inhibitor is modulating different processes at different times which contribute to complicated dose effects on different processes. The data presented in this study has not determined the optimal dose of calpain inhibition required to have a beneficial effect in the ischemic tissue and non-ischemic tissue. To obtain the precise level of calpain inhibition necessary for positive cardiovascular effects future dose-response studies will be required. Additionally, the time course of this study was just long enough to induce chronic myocardial ischemia, it would be interesting to study these pigs over a longer period of time.

Clinical Significance:

Up to one third of patients in need of revascularization for myocardial ischemia are not candidates or receive suboptimal revascularization. The prevalence of obesity and metabolic syndrome is increasing in the United States, indicating that the incidence of severe coronary artery disease not amenable to treatment is likely to increase.¹⁰ Therefore, identifying a new medical treatment for patients with chronic coronary ischemia is of significant value. In the setting of hypercholesterolemia and chronic myocardial ischemia, calpain inhibition decreases the expression of fibrosis in ischemic and non-ischemic myocardial tissue. This decrease is associated with the Jak/STAT/MCP-1 pathway. Calpain inhibition also results in stabilization of the expression of cytoskeletal and structural proteins in the ischemic myocardial tissue. [Figure 7]. Finally, calpain inhibition had varying effects on different cardiac cells. These data indicate that calpain inhibition may be a beneficial medical therapy to decrease myocardial fibrosis formation in patients with coronary artery disease and associated co-morbidities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biography

Glossary:

TGF	β -transforming growth factor beta
MMP	matrix metalloproteinase

Jak	Janus Kinase
STAT	signal transducer and activator of transcription
MCP-1	monocyte chemoattractant protein 1
CI	calpain inhibition

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Central Message:

Calpain inhibition decreases the expression of fibrosis in myocardial tissue which is associated with decreased Jak/STAT/MCP-1 signaling in a pig model of chronic ischemia and metabolic syndrome.

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Perspective Statement:

In the setting of hypercholesterolemia and chronic myocardial ischemia, CI decreases the expression of fibrosis in myocardial tissue which is associated with decreased expression of the pro-fibrotic Jak/STAT/MCP-1 pathway and stabilization of cytoskeletal and structural proteins.

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Figure 1: Calpain Inhibition Has No Effect of Metabolic Parameters.

Graphical presentation showing no difference in (A) glucose tolerance, (B) percent change in weight or (C) cholesterol: HDL ratio between the three groups as analyzed by Kruskal Wallice and Dunn's Comparisons. CON- High Cholesterol Control (n=8), LCI- Low Dose Calpain Inhibitor (n=8), HCI- High Dose Calpain Inhibitor (n=8). **BMI- Body Mass Index, BG- Blood Glucose**

b

Figure 2:

A: Calpain Inhibition Decreases Fibrosis Expression in Ischemic Myocardial Tissue. a. Graphical presentation showing decreased expression of collagen in both the low dose and the high dose calpain inhibited groups compared to the control in the ischemic myocardial tissue. Boxes represent the upper and lower quartiles. Middle horizontal lines represent the median. Upper and lower whiskers represent the maximum and minimum values of non-outliers. **b. Representative images of Picrosirius stain (used to analyze collagen), H&E stain, and Trichrome Stain.** CON- High Cholesterol Control (n=8), LCI- Low Dose Calpain Inhibitor (n=9), HCI- High Dose Calpain Inhibitor (n=8). *= p<0.05 by Kruskal Wallice and Dunn's Comparisons. **B: Calpain Inhibition Decreases Fibrosis Expression in Non-Ischemic Myocardial Tissue. a. Graphical presentation showing** decreased expression of collagen in both the low dose and the high dose calpain inhibited groups compared to the control in the non ischemic myocardial tissue. Boxes represent the upper and lower quartiles. Middle horizontal lines represent the median. Upper and lower whiskers represent the maximum and minimum values of non-outliers. **b. Representative images of Picrosirius**

stain (used to analyze collagen), H&E stain, and Trichrome Stain. CON- High Cholesterol Control (n=8), LCI- Low Dose Calpain Inhibitor (n=9), HCI- High Dose Calpain Inhibitor (n=8). *= p<0.05 by Kruskal Wallice and Dunn's Comparisons

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a

Figure 3.

A: Calpain Inhibition Decreases Expression of TGF- β , Jak/MCP-1 and α -Actinin in Ischemic Myocardial Tissue.

a: Representative Images from Western Blot with protein specific antibodies. **Duplicate loads are two different animals from each group.** Proteins in each row were run in the same gel. b. **Graphical presentation showing** significant changes in cytoskeletal structure proteins. Boxes represent the upper and lower quartiles. Middle horizontal lines represent the median. Upper and lower whiskers represent the maximum and minimum values of non-outliers. CON- High Cholesterol Control (n=8), LCI- Low Dose Calpain Inhibitor (n=9), HCI- High Dose Calpain Inhibitor (n=8). *= p<0.05 by Kruskal Wallice and Dunn's Comparisons

3B: Calpain Inhibition Decreases Expression of TGF- β and Jak2 in Non- Ischemic Myocardial Tissue.

a: Representative Images from Western Blot with protein specific antibodies. **Duplicate loads are two different animals from each group.** Proteins in each row were run in the same gel. b. **Graphical presentation showing** significant changes in cytoskeletal structure proteins. Boxes represent the upper and lower quartiles. Middle horizontal lines represent the median. Upper and lower whiskers represent the maximum and minimum values of non-outliers. CON- High Cholesterol Control (n=8), LCI- Low Dose Calpain Inhibitor (n=9), HCI- High Dose Calpain Inhibitor (n=8). *= p<0.05 by Kruskal Wallice and Dunn's Comparisons

Figure 4: Calpain Inhibition Increases Expression of Proteins Involved in Cytoskeletal Structure.

A: Representative Images from Western Blot with protein specific antibodies. **Duplicate loads are two different animals from each group.** Proteins in each row were run together in the same gel. B. **Graphical presentation showing** significant changes in cytoskeletal structure proteins. Boxes represent the upper and lower quartiles. Middle horizontal lines represent the median. Upper and lower whiskers represent the maximum and minimum values of non-outliers. CON- High Cholesterol Control (n=8), LCI- Low Dose Calpain Inhibitor (n=9), HCI- High Dose Calpain Inhibitor (n=8). *= p<0.05 by Kruskal Wallce and Dunn's Comparisons

Figure 5: Calpain Inhibition Decreases Expression of Filamin, β Tubulin, and β Actin in Non-Ischemic Myocardial Tissue.

A: Representative Images from Western Blot with protein specific antibodies. **Duplicate loads are two different animals from each group.** Proteins in each row were run together in the same gel. B. **Graphical presentation showing** significant changes in cytoskeletal structure proteins. Boxes represent the upper and lower quartiles. Middle horizontal lines represent the median. Upper and lower whiskers represent the maximum and minimum values of non-outliers. CON- High Cholesterol Control (n=8), LCI- Low Dose Calpain Inhibitor (n=9), HCI- High Dose Calpain Inhibitor (n=8). * = p < 0.05 by Kruskal Wallice and Dunn's Comparisons. **MCP-1- Monocyte Chemoattractant Protein-1**

a

a

b

Figure 6.**A: Calpain Inhibition Decreases TGF- β and SMAD Pathway in Cardiomyoblast**

cells. a. Graphical presentation showing significant changes in TGF- β and p-SMAD 2/3 expression and a dose dependent decrease in Jak2 expression in the cardiomyoblast cells. Boxes represent the upper and lower quartiles. Middle horizontal lines represent the median. Upper and lower whiskers represent the maximum and minimum values of non-outliers. b: Representative Images from Western Blots with protein specific antibodies. The number of surviving cells in the HCI groups was so low that the protein content was undetectable.

CON- DMSO (vehicle) (1:10,000), LCI- 10 nM CI, HCI-100 nM CI. *= p<0.05 by Kruskal Wallice and Dunn's Comparisons

Figure 6B: Calpain Inhibition Decreases p-SMAD2/3 and JAK2 Pathway in Ventricular Fibroblast Cells

a. Graphical presentation showing significant changes in p- SMAD 2/3 and Jak2 expression and a dose dependent decrease in TGF- β expression in the ventricular fibroblast cells. Boxes represent the upper and lower quartiles. Middle horizontal lines represent the median. Upper and lower whiskers represent the maximum and minimum values of non-outliers. b: Representative Images from Western Blots with protein specific antibodies. CON- DMSO (vehicle) (1:10,000), LCI- 10 nM CI, HCI-100 nM CI. *= p<0.05 by Kruskal Wallice and Dunn's Comparisons

Figure 7: Calpain Inhibition Decreases Fibrosis Plausibly Via Jak/STAT/MCP-1 Signaling in Ischemic Myocardium.

Schematic describing calpain effects on cellular adhesion, membrane stability, cell structure, contractile stability, focal adhesion, myofibroblast stability and Jak/STAT/MCP-1 signaling.

Table 1:

Calpain inhibition does not cause hemodynamic changes between Control, LCI, and HCI. One Way ANOVA on ranks (Kruskal-Wallis) followed by Dunn's post-hoc test was used to compare the means among groups CON- High Cholesterol Control (n=3), LCI- Low Dose Calpain Inhibitor (n=7), HCI- High Dose Calpain Inhibitor (n=7).

Hemodynamic Parameter	CON	LCI	HCI	P Value (ANOVA)
Developed Pressure	53.53+/-15.59	57.73+/-6.98	66.4+/-6.75	0.34
Heart Rate	106+/-18.43	95.6+/-4.34	94.63+/-8.35	0.69
Mean Arterial Pressure	41.17+/-9.91	57.53+/-4.37	55.5+/-2.38	0.19
Pressure Product	6358.41+/-2527.61	6115.27+/-529.42	5466.84+/-831.81	0.78
Pulse Pressure	22.43+/-6.23	30.3+/-2.86	34.03+/-2.51	0.33
+dP/dt	963.33+/-365.24	928.43+/-116.93	1036.29+/-120.98	0.36
-dP/dt	-657+/-254.80	-746.5+/-105.97	-863.00+/-74.09	0.71