Calpain inhibition modulates glycogen synthase kinase 3 β pathways in ischemic myocardium: A proteomic and mechanistic analysis

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ABSTRACT

Background: Calpain inhibition has an enhancing effect on myocardial perfusion and improves myocardial density by inhibiting glycogen synthase kinase 3 β (GSK-3 β) and up-regulating downstream signaling pathways, including the insulin/PI3K and WNT/ β -catenin pathways, in a pig model of chronic myocardial ischemia in the setting of metabolic syndrome.

Methods: Pigs were fed a high-fat diet for 4 weeks, then underwent placement of an ameroid constrictor to the left circumflex artery. Three weeks later, the animals received no drug (high-cholesterol controls [HCC]), a high-dose calpain inhibitor (HCI), a low-dose calpain inhibitor (LCI), or a GSK-3 β inhibitor (GSK-3 β I). The diets and drug regimens were continued for 5 weeks and the myocardial tissue was harvested.

Results: Calpain and GSK-3 β inhibition caused an increase in myocardial perfusion ratios at rest and during pacing compared with controls. Pigs in the LCI and HCI groups had increased vessel density in the ischemic myocardium, and pigs in the GSK-3 β I group had increased vessel density in the ischemic and nonischemic myocardium compared with the HCC group. Calpain inhibition modulates proteins involved in the insulin/PI3K and WNT/ β -catenin pathways. Quantitative proteomics revealed that calpain and GSK-3 β inhibition significantly modulated the expression of proteins enriched in cytoskeletal regulation, metabolism, respiration, and calcium-binding pathways.

Conclusions: In the setting of metabolic syndrome, calpain or GSK-3 β inhibition increases vessel density in both ischemic and nonischemic myocardial tissue. Calpain inhibition may exert these effects through the inhibition of GSK-3 β and up-regulation of downstream signaling pathways, including the insulin/PI3K and WNT/ β -catenin pathways. (J Thorac Cardiovasc Surg 2017;153:342-57)

Schematic of the proposed mechanisms by which calpain inhibition may modulate expression of WNT/GSK-3 β /β-catenin and GSK-3 β /insulin/Akt pathways.

Central Message

Calpain inhibition may improve vessel density by inhibiting GSK-3 β activity.

Perspective

Metabolic syndrome is associated with endothelial dysfunction, accelerated atherosclerosis, and a diminished angiogenic response to myocardial ischemia. Modification of major risk factors for cardiovascular disease reduce the likelihood of developing cardiovascular disease and its associated complications. Calpain may serve as a potential medical therapy for patients with chronic coronary artery disease and metabolic syndrome.

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Metabolic syndrome is associated with induction of apoptosis, increased myocardial oxidative stress, and attenuation of cell survival pathways in ischemic myocardium.¹⁻⁶ Calpains are calcium-dependent thiol-proteases. Whereas some calpains are expressed ubiquitously, others are thought to be localized to specific tissues; for example, calpain 1 and 2 to endothelial cells.⁷ In situations of stress,

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Abbreviations and Acronyms

GSK-3 β	= glycogen synthase kinase 3 β
GSK-3 β I	= glycogen synthase kinase 3 β inhibited
HCC	= high-cholesterol control
HCI	= high-dose calpain inhibited
LCI	= low-dose calpain inhibited
LEF-1	= lymphoid enhancer factor 1
MMP-7	= matrix metalloproteinase 7
PI3Kp85	= phosphoinositide 3-kinase p85
p-IRS	= phosphorylated insulin receptor substrate
p-PDK-1	= phospho-phosphoinositide-dependent protein kinase 1 (Ser241)
p-PTEN	= phosphorylated phosphatase and tensin homolog
TCF-1	= transcription factor 1
VE-cadherin	= vascular endothelial cadherin

there is an influx of extracellular calcium into cells. This influx of calcium may lead to calpain activation and has been associated with cellular apoptosis, increased leukocyte trafficking, and cytoskeletal degradation. This pathway has

been associated with renal tubule, endothelial, and myocardial cell injury in situations of stress, including ischemia and glucose intolerance.⁸⁻¹⁰ When calpains become activated, they cleave a broad spectrum of intracellular proteins involved in cellular apoptosis, inflammation, platelet function, cytoskeletal structure, cell adhesion, angiogenesis, and cell migration.^{7,11} Hyperactivation of calpain disrupts the endothelial cell cytoskeleton, causes defective capillary morphogenesis, and promotes tissue hypoxia.⁷ In metabolic syndrome, hyperactivation of calpain has been linked to myocardial and vascular inflammation and impaired collateral vessel formation.^{11,12} Modest suppression of calpain activity restores the cytoskeletal structure and promotes a functional neovasculature, resulting in reduced tissue hypoxia.^{7,11,12}

Calpain is also known to cleave the inhibitory domain of glycogen synthase kinase 3 β (GSK-3 β), keeping it in an active state.^{13,14} GSK-3 β is a serine/threonine kinase that plays a role in a broad range of activities in the cell.¹⁵ It is an essential regulator in the WNT/ β -catenin pathway, where it phosphorylates β -catenin, committing it to degradation.¹⁶ It also regulates the insulin/phosphoinositide 3-kinase p85 (PI3Kp85) pathway (Figure 1). In fact, inhibition of GSK-3 β in an animal model of diabetes has been found to reduce hyperglycemia and insulin

FIGURE 1. Schematic presentation of the proposed mechanisms by which calpain inhibition may modulate expression of WNT/GSK-3 β / β -catenin and GSK-3 β /insulin/Akt pathways. *VE-cadherin*, Vascular endothelial cadherin; *GSK-3 β* , glycogen synthase kinase 3 β ; *TCF-1*, transcription factor 1; *p-IRS*, phosphorylated insulin receptor substrate; *p-PTEN*, phosphorylated phosphatase and tensin homolog.

resistance.¹⁷ Like calpain activity, GSK-3 β activity is increased in endothelial cells in the presence of hypoxia.^{18,19} Moderate inhibition of GSK-3 β improves the architecture and function of pathological blood vessels in the setting of ischemia and has cardioprotective effects against ischemia/reperfusion and pressure overload.¹⁸⁻²⁰

We recently found that calpain activity is significantly increased in the ischemic myocardium of pigs with diet-induced metabolic syndrome, and that calpain inhibition increases coronary perfusion, improves endothelium-dependent microvessel relaxation, increases vessel density in ischemic myocardial tissue, and decreases myocardial apoptosis.^{1,2} Inhibition of GSK-3 β also increases blood flow, vessel density, and expression of proteins involved in angiogenesis and cell survival.²¹

We hypothesized that calpain inhibition has an enhancing effect on myocardial perfusion and improves myocardial density by up-regulating angiogenic and cell survival pathways, such as WNT/ β -catenin and insulin/PI3K, in a pig model of chronic myocardial ischemia in the setting of metabolic syndrome.

METHODS

Animal Model

The animal model used in this study has been described previously.^{1,21} To induce metabolic syndrome, 33 7-week-old Yorkshire swine (E.M. Parsons and Sons, Hadley, Mass) were fed a high-fat, high-cholesterol diet. To induce chronic myocardial ischemia, 4 weeks later, the pigs underwent placement of an ameroid constrictor on the left circumflex artery. Three weeks later, animals were split into 4 groups that received either: no drug, (high-cholesterol control [HCC] group; $n = 8$), a low dose (0.12 mg/kg day) of the calpain inhibitor MDL28170 (low-calpain inhibitor [LCI] group; $n = 9$), a high dose (0.25 mg/kg day) of calpain inhibitor MDL28170 (high-dose calpain inhibitor [HCI] group; $n = 8$), or 1.5 mg/kg/day of GSK-3 β inhibitor IM-12 (GSK-3 β inhibitor [GSK-3 β I] group; $n = 8$). MDL28170 is a cell-permeable selective peptidomimetic inhibitor of calpain 1 and 2 that binds to the protease enzymatic site and inhibits its action.^{22,23} IM-12 is a novel cell-permeable indolyl maleimide that inhibits GSK-3 β by competing for ATP binding.²⁴ The MDL28170 and IM-12 powders were dissolved in dimethyl sulfoxide and administered to the pigs orally. Pigs in the HCC group received a placebo of dimethyl sulfoxide with no dissolved drug. The high-fat, high-cholesterol diet and drug regimens were continued for 5 weeks. The pigs were then euthanized, and the left ventricular myocardium was harvested. Preliminary experiments using these animals were reported previously.^{1,2,21} The Institutional Animal Care and Use Committee of the Rhode Island Hospital approved all experiments.¹

Immunohistochemical staining

Immunohistochemistry staining procedures have been described previously.² Tissue slides were incubated with antibodies against p-vascular endothelial-cadherin (p-VE-cadherin) (Cell Signaling Technology, Danvers, Mass), endothelial marker CD31 (R&D Systems, Minneapolis, Minn), and smooth muscle actin (Sigma-Aldrich, St Louis, Mo). Slides were then incubated with the appropriate Alexa Fluor-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, Pa). A Nikon E800 Eclipse microscope (Nikon, Tokyo, Japan) was used to capture images at 20 $\times$ magnification in 5 random fields from each animal (4 animals per group). ImageJ was used to analyze vascular density. Capillaries were

defined as CD31-positive structures between 10 and 800 pixels, and arteries were defined as smooth muscle actin-positive structures with lumens >800 pixels.

Myocardial Blood Flow

Our technique for myocardial perfusion has been described previously.² In brief, during the ameroid placement procedure, the left circumflex artery was constricted for 2 minutes while gold microspheres (Biopal, Worcester, Mass) were simultaneously injected into the left atrium. During the harvesting procedure, europium and lutetium microspheres were injected into the left atrium (both at rest and during pacing of 150 bpm) while simultaneously withdrawing blood from a femoral arterial line. Ventricular tissue samples obtained after the final harvest procedure were divided into 10 sections and sent along with the femoral arterial line blood to BioPal, where they were exposed to a neutron beam and microspheres were measured with a gamma counter.²

Protein Expression

Protein expression was performed as described previously.¹ Tissue was lysed (Boston BioProducts, Ashland, Mass), fractionated (NuPAGE Novex Gel; Thermo Fisher Scientific, Waltham, Mass), transferred to polyvinylidene difluoride membrane (Millipore, Billerica, Mass), and incubated with primary antibodies against GSK-3 β , β -catenin, phospho- β -catenin (Ser675), eIF-2 β , phospho-eIF-2 β (Ser539), phosphorylated phosphatase and tensin homolog (p-PTEN; Ser380), CD44, Met, matrix metalloproteinase 7 (MMP-7), c-Myc, c-Jun, cyclin D1, lymphoid enhancer factor-1 (LEF-1), transcription factor 1 (TCF-1), phospho-FOXO1 (Ser256), FOXO1, phospho-insulin receptor substrate (p-IRS; Ser612), vascular endothelial cadherin (VE-cadherin), PI3Kp85, phospho-phosphoinositide-dependent protein kinase 1 Ser241 (p-PDK-1), mTOR, p-mTOR, Akt, γ -catenin, VEGF receptor 1, and VEGF receptor 2 (Cell Signaling Technology). Membranes were incubated with the appropriate secondary antibody at room temperature (Jackson ImmunoResearch, West Grove, Pa). Enhanced chemiluminescence was used to visualize images with a digital camera system (G-Box; Syngene, Cambridge, United Kingdom). ImageJ software was used to quantify band densitometry as arbitrary light units.¹

Data Analysis

Immunohistochemistry data is reported as mean $\pm$ standard error of the mean of specimens from each group. Protein density data for each pig was normalized to glyceraldehyde 3-phosphate dehydrogenase or α -tubulin for each pig. An average for each group is reported as mean $\pm$ standard error of the mean fold change values compared with the HCC group. Kruskal-Wallis one-way analysis of variance with Dunn's post hoc test was used to determine differences among 3 or more groups (Prism 5.0; GraphPad Software, San Diego, Calif). The Mann-Whitney U test was used to determine differences between 2 groups (Graph Pad Prism 5.0).

Proteomics

Ventricular tissue from the ischemic territory of each pig was homogenized in a dounce homogenizer and trypsin-digested. Spectra was then collected with a Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, Mass). Peptide and protein identification was done by matching metabolic syndrome spectra to the pig genomic sequences using Mascot software (Matrix Science, Boston, Mass). Biological replicates were aggregated using either Scaffold or ProteoIQ (Proteome, Portland, Ore). Results were analyzed using Ingenuity Pathway Analysis (Qiagen, Germantown, Md) and DDAVID Bioinformatics Database (National Institute of Allergy and Infectious Diseases, Frederick, Md). Gene sequences were compared with human sequence when pig sequences were unavailable. Gene lists were submitted to the PANTHER Classification System Database (<http://www.uniprot.org/database/DB-0069>) and sorted by protein class.

RESULTS

Arteriolar and Capillary Density

Pigs in the LCI and HCI groups had greater arteriolar and capillary densities in the chronically ischemic myocardium compared with the HCC group (Figure 2, A and C). This reanalysis confirmed previous findings of increased vessel density in chronically ischemic myocardium.² In this study, we also determined that calpain inhibition had global effects on myocardial vasculogenesis, with increased arteriolar density in the nonischemic myocardium in the LCI group compared with the control group (Figure 2, B and D). Increased capillary density in the nonischemic myocardium was seen in both the LCI and HCI groups; however, this did not reach statistical significance.

Myocardial Blood Flow

Inhibition of GSK-3 β recapitulated many of the effects of calpain inhibition. Pigs in the GSK-3 β I group (n = 8) had a significant increase in the ischemic to nonischemic myocardial perfusion ratio (left circumflex artery/left anterior descending artery), both at rest and during pacing, compared with the HCC group (Figure 3). These results are consistent with previously reported preliminary results.²¹

Similar to the calpain-inhibition groups, pigs in the GSK-3 β I group had increased arteriolar and capillary densities in the ischemic and nonischemic myocardial tissue compared with the HCC group (Figure 4, A-D).

Proangiogenic Protein Expression

VEGF receptor 1 and p-VE-cadherin expression levels were greater in the LCI and HCI groups compared with the HCC group. There was also greater expression of VEGF receptor 2, VE-cadherin, and γ -catenin in the LCI group compared with the HCC group; however, there appeared to be a biphasic dose effect, with a nonsignificant increase in the expression of these proteins in the HCI group compared with HCC group (Figure 5).

Cell Growth and Proliferation Signaling

Calpain inhibition was correlated with increased expression of the growth and proliferation proteins Akt, p-PTEN, and p-mTOR in the LCI group compared with the HCC group. There was greater expression of phosphorylated-FOXO1 in both the LCI and HCI groups compared with the HCC group, and greater expression of p-IRS and PI3-K in the HCI group compared with the HCC group (Figure 6). There were no significant differences in levels of total FOXO1 or mTOR (Table 1).

Wnt/GSK-3 β / β -Catenin Pathway

The Wnt/GSK-3 β / β -catenin pathway regulates cell development and differentiation. There was greater expression of total GSK-3 β in the HCI group compared

with the HCC group (with a nonsignificant increase in the LCI group compared with the HCC group). Consistent with GSK-3 β inhibition, the expression level of β -catenin was greater in both the LCI and HCI groups compared with the HCC group. Similarly, there was decreased phosphorylation of β -catenin in both the LCI and HCI groups compared with the HCC group; however, this did not reach statistical significance ($P = .09$). The expression levels of EIF-2B ϵ and p-EIF-2B ϵ (Ser539) were increased in the LCI and HCI groups; however, the differences were significant only in the HCI group.

Calpain inhibition was also correlated with the increased expression of downstream targets of GSK-3 β involved in cellular adhesion, growth, and angiogenesis, including CD44, TCF-1, and Met. There was greater expression of CD44 was greater in the LCI and HCI groups compared with the HCC group, and greater expression of TCF-1 and Met in the HCI group compared with the HCC group (Figure 7). Calpain inhibition did not affect the expression of downstream targets of both β -catenin and GSK-3 β involved in proliferation, including c-Myc, c-Jun, cyclin D1, and LEF-1. There was increased expression in MMP-7 in the HCI group compared with the LCI group (Table 1).

Expression levels of PI3-K and CD44 were increased in the GSK-3 β I group compared with the HCC group. There was a trend toward increased expression of P-PDK1 and eIF2B ϵ in the GSK-3 β I group compared with HCC group (Figure 8).

Proteomics

Quantitative proteomics and systems analysis revealed that calpain and GSK-3 β inhibition significantly modulated the expression of proteins enriched in cytoskeletal regulation, metabolism and respiration, and calcium-binding pathways compared with the HCC group, including increased up-regulation of β 1-catenin in the GSK-3 β I group compared with HCC group.

Expression levels of 35 proteins was increased in the GSK-3 β I group compared with the HCC group ($P < .05$), expression levels of 28 proteins were increased in the HCI group compared with the HCC group ($P < .05$), and expression levels of 25 proteins were increased in the LCI group compared with the HCC group ($P < .05$). Two proteins related to sarcomere structure and function were up-regulated in the LCI, HCI, and GSK-3 β I groups compared with the HCC group. Five proteins related to inflammation, rRNA translation, ATP breakdown, triglyceride catabolism, and ATP-dependent motor protein movement were increased in the GSK-3 β I and LCI groups compared with the HCC group. Five proteins involved in inflammation, signal transduction, ATPase activity, and cytoskeletal structure were increased in the GSK-3 β I and HCI groups compared with the HCC group. Three proteins involved in insulin resistance, redox reactions, and cellular

FIGURE 2. Calpain inhibition increases vessel density in ischemic and nonischemic myocardial tissue. **A**, Ischemic myocardium. Arteriolar cell density staining for smooth muscle actin (SMA) with SMA-specific antibody in tissue sections. Capillary cell density staining for endothelial marker CD31 with CD31-specific antibodies in tissue sections. The bar diagrams show significant increases in the low-dose calpain inhibitor (LCI) and high-dose calpain

FIGURE 3. GSK-3 β inhibition increases myocardial blood flow at rest and during pacing. *HCC*, High-cholesterol controls; *GSK-3 β I*, GSK-3 β I inhibition; *Lcx/LAD*, left circumflex artery/left anterior descending artery. * $P < .05$, Mann-Whitney U test.

homeostasis (including calpain-1 catalytic subunit) were increased in the HCI and LCI groups compared with the HCC group (Table 2).

Ten proteins were decreased in the GSK-3 β I group compared with the HCC group ($P < .05$), 18 proteins were decreased in the HCI group compared with the HCC group ($P < .05$), and 11 proteins were decreased in the LCI group compared with the HCC group ($P < .05$). Two proteins related to carbohydrate metabolism and coagulation were decreased in the GSK-3 β I and LCI groups compared with the HCC group. One protein involved in the tricarboxylic acid cycle was decreased in the GSK-3 β I and HCI groups compared with the HCC group. Six proteins involved in serine and threonine metabolism, ATP synthesis, the electron transport chain, hemoglobin binding, and cellular hemostasis were decreased in the HCI and LCI groups compared with the HCC group (Table 3).

DISCUSSION

In this study, we have demonstrated the following in a pig model of metabolic syndrome and chronic myocardial ischemia: (1) both calpain and GSK-3 β inhibition improve vessel density in ischemic and nonischemic myocardial tissue; (2) GSK-3 β inhibition improves myocardial blood flow both at rest and with pacing; (3) calpain inhibition correlates with increased expression of proteins that serve as angiogenic markers; and (4) calpain inhibition likely has beneficial effects by modulating the activity of GSK-3 β and proteins involved in the Wnt/ β -catenin and insulin/PI3K pathways (Figure 1).

We recently found that calpain activity is significantly increased in the ischemic myocardium of pigs with

metabolic syndrome, and that calpain inhibition improves myocardial blood flow, increases endothelial-dependent microvascular relaxation, increases vessel density, and decreases the expression levels of proteins involved in apoptosis.^{1,2}

Calpain is a ubiquitously expressed protein with a broad range of functions. Calpain activity is overexpressed during times of stress, and moderate inhibition of this overexpression has been found to be beneficial. GSK-3 β is a downstream target of calpain. By looking at GSK-3 β , we hoped to localize the beneficial effect of calpain and narrow the range of activity of our medications. In related preliminary studies, we found that GSK-3 β inhibition improves myocardial blood flow, increases microvascular density, and modulates proteins involved in the ischemic myocardium of pigs with metabolic syndrome.²¹ The present study validates some of our previous findings and expands on this previous work both in the extent of physiological findings, including vessel density and myocardial perfusion, and in the scope of the investigated signaling pathways and potential mechanisms.

We found that calpain inhibition increased vessel density (arteriolar and capillary) in the ischemic myocardial tissue in both the LCI and HCI groups compared with the HCC group. In addition, we found that GSK-3 β inhibition increased the ischemic to nonischemic myocardial perfusion ratio (left circumflex artery/left anterior descending artery) both at rest and during pacing compared with the HCC group. Importantly, our data are consistent with the findings of some of our preliminary studies that showed increased arteriolar and capillary density in the LCI and HCI groups compared with the HCC group, which

inhibitor (*HCI*) groups compared with high-cholesterol controls (*HCC*). B, Nonischemic myocardium. Arteriolar cell density staining for SMA with SMA-specific antibody in tissue sections. Capillary cell density staining for endothelial marker CD31 with CD31 specific antibody in tissue sections. The bar diagrams show an increase in arteriolar density in the LCI group compared with controls. C, Ischemic myocardium. Representative images in 20 $\times$ high-power fields (*HPFs*). CD31 is in red; SMA, in green. D, Nonischemic myocardium. Representative images in 20 $\times$ *HPFs*. CD31 is in red; SMA, in green. * $P < .05$, Kruskal-Wallis test with Dunn's post hoc comparison.

FIGURE 4. GSK-3 β inhibition increases vessel density in ischemic and nonischemic myocardial tissue. **A,** Ischemic myocardium. Arteriolar cell density staining for smooth muscle actin (SMA) with SMA-specific antibody in tissue sections, and capillary cell density staining for endothelial marker CD31 with CD31-specific antibody in tissue sections. The bar diagrams show significant increases arteriolar and capillary density in the GSK-3 β I inhibited groups (GSK-3 β I) group compared with high-cholesterol controls (HCC). **B,** Nonischemic myocardium. Arteriolar cell density staining for SMA with SMA-specific antibody in tissue sections, and capillary cell density staining for endothelial marker CD31 with CD31-specific antibody in tissue sections. The bar diagrams show significant increases arteriolar and capillary density in the GSK-3 β I group compared with controls. **C,** Ischemic myocardium. Representative images in 20 $\times$ high-power fields (HPFs). CD31 is in red; SMA, in green. **D,** Nonischemic myocardium. Representative images in 20 $\times$ HPFs. CD31 is in red; SMA, in green. * $P < .05$, Mann-Whitney U test.

FIGURE 5. Calpain inhibition increases expression of angiogenic proteins. A, Representative images from Western blot analysis with protein-specific antibodies as shown at the left. All images shown in for each protein are from the same Western blot membrane using different antibodies as indicated. α -tubulin shows representative images for a loading control. B, Bar diagrams showing significant changes in angiogenic proteins. HCC, High-cholesterol controls; LCI, low-dose calpain inhibitor; HCI, high-dose calpain inhibitor. * $P < .05$, ANOVA with Dunn's post hoc comparisons.

did not reach statistical significance.² In the present study, vessel density was evaluated in 5 20 $\times$ high-power fields instead of 3 20 $\times$ high-power fields, which explains the difference in significance.

In this study, we also performed vessel density analysis in the nonischemic myocardial tissue. Whereas the only significant difference from the control group was found in arteriolar density in the low dose calpain inhibited group we did observe an overall trend toward increased arteriolar and capillary densities in the LCI and HCI groups compared with the HCC group. This finding suggests that calpain inhibition not only promotes angiogenesis in ischemic

myocardium, but also has a global effect on the myocardium to induce angiogenesis.

We next evaluated the effect of GSK-3 β inhibition on vessel density in both the ischemic and nonischemic myocardial tissue, given that GSK-3 β is activated by and is a direct downstream target of calpain. We found a significant increase in capillary and arteriolar counts in both ischemic and nonischemic tissues in the GSK-3 β I group compared with the HCC group, consistent with previously reported preliminary results.²¹ Our findings demonstrate that both calpain inhibition and GSK-3 β inhibition induce new vessel growth in the ischemic

FIGURE 6. Calpain inhibition increases expression of proteins involved in cell growth and proliferation. A, Representative images from Western blot analysis with protein-specific antibodies as shown at the left. All images shown in for each protein are from the same Western blot membrane using different antibodies as indicated. GAPDH shows representative images for a loading control. B, Bar diagrams showing significant changes in prosurvival signaling proteins. *HCC*, High-cholesterol control; *LCI*, low-dose calpain inhibitor; *HCI*, high-dose calpain inhibitor. * $P < .05$, Kruskal-Wallis test with Dunn's post hoc comparisons. *p-IRS*, Phosphorylated insulin receptor substrate; *HCC*, high-cholesterol controls; *LCI*, low-dose calpain inhibitor; *HCI*, high-dose calpain inhibitor; *PI3K*, phosphoinositide 3-kinase; *p-PTEN*, phosphorylated phosphatase and tensin homolog.

myocardial tissue of pigs in the setting of metabolic syndrome. In addition, GSK-3 β inhibition may be a more potent stimulus to promote vessel growth in nonischemic myocardial tissue. Therefore, the beneficial effects of calpain inhibition and GSK-3 β inhibition likely are related to their ability to promote new vessel growth in both ischemic and nonischemic myocardial tissue.^{1,2,11,12}

Interestingly, the beneficial effect of calpain inhibition on angiogenesis seems to be more significant in ischemic

myocardium, whereas the beneficial effect of GSK-3 β is present in both ischemic and nonischemic myocardium. This is thought to occur because calpain activity is required for normal physiological functioning, whereas calpain overactivation in situations of stress, including ischemia, is detrimental. Therefore, moderate inhibition of calpain in the ischemic myocardial tissue only, where calpain activity is overactivated, would bring calpain activity back to physiological parameters. GSK-3 β is not regulated in a

TABLE 1. Proteins not affected by calpain inhibition

Protein	HCC	LCI	HCI	P value
FOXO1	1.00 ± 0.28	1.24 ± 0.18	1.59 ± 0.23	.31
mTOR	1.00 ± 0.14	0.56 ± 0.10	0.63 ± 0.10	.50
c-Myc	1.00 ± 0.08	1.0 ± 0.07	1.04 ± 0.08	.84
c-Jun	1.00 ± 0.1	1.19 ± 0.05	0.24 ± 0.10	.26
Cyclin D1	1.00 ± 0.19	0.78 ± 0.10	1.05 ± 0.10	.29
Lymphoid enhancer factor-1	1.00 ± 0.18	0.72 ± 0.07	1.07 ± 0.12	.10
Matrix metalloproteinase 7	1.00 ± 0.14	0.85 ± 0.10	1.39 ± 0.18	.04*

Analyzed by the Kruskal–Wallis test with Dunn's post hoc comparison. HCC, High-cholesterol control; LCI, low-dose calpain inhibitor; HCI, high-dose calpain inhibitor.

*Indicates significant differences between the LCI and HCI groups only.

similar fashion, and thus we do not see a difference of effect in the ischemic and nonischemic myocardium.

We then evaluated the signaling pathways that calpain and GSK-3 β inhibition may be affecting to exert the changes in vessel density.² VEGF receptors activate several intracellular signaling processes that promote angiogenesis. In this study, we have verified that calpain inhibition up-regulates VEGF receptors 1 and 2, similar to our previous work that demonstrated up-regulation of VEGF and its associated receptors.² We then extended our analysis of potential mechanistic signaling pathways further and examined the Wnt/GSK-3 β / β -catenin signaling pathway. β -catenin is an integral VE-cadherin cell–cell adhesion adaptor protein. Up-regulation of VE-cadherin in the cell membrane of vascular cells serves as an indicator of increased angiogenesis. VE-cadherin binds to γ -catenin and β -catenin on the cell membrane. Interestingly, we identified up-regulation of VE-cadherin, phospho-VE-cadherin, γ -catenin, and β -catenin in the LCI and HCI groups compared with the HCC group. We also previously showed that GSK-3 β inhibition up-regulates the expression of the similar proteins, including VEGF-R1 and -R2, VE-cadherin, γ -catenin, and β -catenin, in the same pig model.²¹ These findings corroborate the increased vessel density seen in the LCI, HCI, and GSK-3 β I groups compared with the HCC group. Cytosolic β -catenin is known to function as a transcriptional coregulator that promotes cell growth and differentiation.²⁵ Therefore, these findings also suggest that calpain inhibition may have an effect via up-regulation of cytosolic β -catenin and its downstream signaling pathways.

The Wnt/ β -catenin pathway is essential for cell fate and angiogenesis. One of the most well-described cellular functions of GSK-3 β is its regulation of Wnt/ β -catenin signaling.¹⁶ In this study, we found that calpain inhibition increases the expression of total GSK-3 β , suggesting that in the LCI and HCI groups, there is less cleavage of GSK-3 β by calpain, possibly leading to less GSK-3 β activity. To test this hypothesis, we investigated proteins

downstream of GSK-3 β . GSK-3 β works to phosphorylate β -catenin, which marks it for degradation. Stabilization of β -catenin allows it to translocate to the nucleus, where it interacts with TCF-1, displaces corepressors, and recruits additional coactivators of Wnt target genes (such as CD44 and Met).²⁶ CD44 is a transmembrane glycoprotein that mediates cell–cell and cell–matrix interactions. Met is a tyrosine kinase receptor involved in cell growth. We found that calpain inhibition is associated with increased expression levels not only of β -catenin, but also of its coactivator TCF-1 and its downstream targets CD44 and Met. We also found that GSK-3 β inhibition is associated with increased expression levels of CD44. GSK-3 β also works to directly phosphorylate eIF2B ϵ at Ser535 (after it is primed by another kinase via phosphorylation at Ser539) which inhibits its ability to initiate mRNA translation.¹⁵ We found up-regulation of both eIF2B ϵ and p-eIF2B ϵ (Ser539) in the LCI and HCI groups, and a trend toward increased eIF2B ϵ expression in the GSK-3 β I group compared with the HCC group. The next step will be to evaluate the expression of the inhibited form, p-eIF2B ϵ (Ser535), which will be a more direct way to measure GSK-3 β function. These data suggest that calpain inhibition modulates proteins involved in the Wnt/ β -catenin pathway via GSK-3 β . Future work will need to evaluate GSK-3 β activity in the calpain-inhibited and GSK-3 β -inhibited groups.

The insulin/PI3K pathway is known to promote cell survival. Tyrosine phosphorylation of IRS allows its association with PI3K. PI3K activation leads to phosphorylation of PDK-1, which then leads to activation of Akt, a serine kinase, which phosphorylates and modulates numerous effectors proteins (eg, mTOR, FOXO1). mTOR is an intracellular protein involved in protein synthesis. FOXO1 is a member of the forkhead box transcription factor family and is important in cellular atrophy. FOXO1 is directly phosphorylated by Akt, which inactivates it and excludes it from the nucleus.^{27,28} PTEN is a tumor-suppressive protein that functions to dephosphorylate and turn off Akt activity. Phosphorylation of PTEN suppresses its ability to turn off Akt.²⁹ We identified increases in these proteins (Akt, p-PTEN, PI3K, p-IRS, p-mTOR) in the ischemic myocardium of pigs with calpain inhibition compared with the control pigs. These data suggest that calpain inhibition may function through up-regulation of the insulin/PI3K pathway and modulation of its downstream pathways. Importantly, GSK-3 β is known to degrade IRS-1, which is an upstream protein in the insulin/PI3K pathway. Interestingly, we found increased expression of activated PI3K (p85) and a trend toward increased expression of activated p-PDK1 (Ser241) in the GSK-3 β I group compared with the HCC group. We previously found that GSK-3 β inhibition also increases the expression of many of these proteins in ischemic myocardium in

FIGURE 7. Calpain inhibition increases expression of proteins in the WNT/ β -catenin pathway. A, Representative images from Western blot analysis with protein-specific antibodies as shown at the left. All images shown for each protein are from the same Western blot membrane using different antibodies as indicated. GAPDH shows representative images for loading control. B, Bar diagrams showing significant changes in prosurvival signaling proteins. HCC, High-cholesterol controls; LCI, low-dose calpain inhibitor; HCI, high-dose calpain inhibitor; GSK-3 β , glycogen synthase kinase 3 β ; TCF-1, Transcription factor-1. * $P < .05$, Kruskal-Wallis test with Dunn's post hoc comparison.

FIGURE 8. GSK-3β inhibition increases expression of proteins involved cell growth and the WNT/β-catenin pathway. A, Representative Western blot images with protein specific-antibodies as shown at the left. All images shown in for each protein are from the same Western blot membrane using different antibodies, as indicated. GAPDH shows representative images for loading control. B, Bar diagrams showing significant changes in prosurvival signaling proteins. PI3Kp85, phosphoinositide 3-kinase; p-PDK-1, phospho-phosphoinositide-dependent protein kinase 1 (Ser241); *HCC*, High-cholesterol controls; *GSK-3βI*, GSK-3β inhibited group. *P < .05, Mann-Whitney *U* test.

TABLE 2. Proteomic analysis: Proteins with increased expression in the GSK-3 β I, HCI, and LCI groups compared with the HCC group

GSK-3 β protein expression>HCC	LCI protein expression>HCC	HCI protein expression>HCC
60S ribosomal protein L32*	26S proteasome non-ATPase regulatory subunit 5	α -soluble NDK attachment protein
AHNAK nucleoprotein	60S ribosomal protein L32*	Annexin A6†
α -actinin-1	Apolipoprotein A-1*	Armadillo repeat-containing protein 10
α -actinin-4	Calpain-1 catalytic subunit‡	β 2-syntrophin
α -soluble NSF attachment protein	Glucosamine-6-phosphate isomerase‡	Calpain 1 catalytic subunit‡
Annexin A6†	Homer protein homolog 1 (Homer-1)	Calreticulin†
Apolipoprotein E	Integrin-linked protein kinase	Coiled-coil domain-containing protein 141
β -catenin	MHC class II antigen	Fatty-acid amide hydrolase 1
Calreticulin†	Mitochondrial import inner membrane translocase subunit TIM50	Fumarate hydrolase, mitochondrial
Cat eye syndrome critical region protein	MYL2	Glucosamine-6-phosphate isomerase‡
Chaperonin containing, TCP	Myomesin-3 (myomesin family member 3)§	GTP-AMP phosphotransferase AK3
Complexin-1	Myosin-9	GTP-binding nuclear protein Ran
Cysteine-rich motor neuron 1 protein	Myotilin§	Homer IF (Homer protein homolog 1)
Cytoplasmic aconitate hydratase	NADH-ubiquinone oxidoreductase chain 4‡	Myomesin-3§
Domain-containing protein 4	Polypyrimidine tract binding protein 1	Myotilin§
Integrin α -V*	Probably C->U editing enzyme APOBEC-2 (EC3.4.5)	NADH-ubiquinone oxidoreductase chain 4
LIM domain-only protein 7	Protein CDV3 homolog	NAP1L1, NRP
Myomesin-3§	Pyridoxal kinase (EC 2.7.1.35) pyridoxine kinase	Phosphoacetylglucosamine mutase
Myotilin§	Ribosomal protein	Proteasome subunit α type 6
NAD(P) transhydrogenase, mitochondrial	Ryanodine receptor 2	Protein NipSnap homolog 3A
NADH dehydrogenase (ubiquinone)1	Tropomodulin-1 (erythrocyte tropomodulin)	RuvB-like 1†
Obscurin	Voltage-dependent anion-selective channel protein 2	Sorting nexin 18
Peptidyl-prolylcis-trans-isomerase		Spectrin α chain, nonerythrocytic 1
Protein CDV3 homolog*		Synemin (Desmuslin)†
Pyridoxal kinase*		Talin-1†
RuvB like 1†		Uncharacterized protein TMEM70
Synemin†		
Talin-1†		
Tubulin β -chain		
Uncharacterized protein (ILK)		
Uncharacterized protein (FN1)		
Unconventional myosin-X		
Vitamin D binding protein		

GSK-3 β I, Glycogen synthase kinase inhibitor; HCC, high-cholesterol control; LCI, low-dose calpain inhibitor; HCI, high-dose calpain inhibitor. *Proteins were increased in the GSK-3 β I and LCI groups compared with the HCC group. †Proteins were increased in the GSK-3 β I and HCI groups compared with the HCC group. ‡Proteins were increased in the HCI and LCI groups compared with the HCC group. §Proteins were increased in all 3 experimental groups compared with the HCC group. All proteins listed showed statistically significant differences in expression between each experimental group (GSK-3 β I, HCI, and LCI) and the HCC group by Scaffold or ProteoIQ analysis.

calpain-inhibited animals, including akt, FOXO1, and p-FOXO1.²¹ Therefore it is possible that calpain inhibition is modulating the insulin/PI3K pathways via GSK-3 β . Future work should examine this mechanism in more detail.

Finally, we used proteomic analysis to evaluate differences in protein expression among the study groups. We found that calpain and GSK-3 β inhibition significantly modulated the expression of proteins enriched in cytoskeletal regulation, metabolism and respiration and calcium-binding pathways compared with the HCC group. These pathways are critical for tissue survival under stress. These data support the overall idea that calpain and GSK-3 β inhibition have a protective effect on the heart. Importantly, quantitative mass spectrometry studies confirmed Western blot

analysis results demonstrating a significant increase in β -catenin in the GSK-3 β I group compared with the HCC group.²¹ However, not all proteins investigated using Western blots were found in our unbiased proteomic analysis. Certain limitations of both methods likely play a role, specifically that the proteomic methods that we used are most suitable for detecting changes in proteins that are very highly expressed in the myocardium.

Calpain overexpression is induced by the increased calcium released by cells in situations of stress. Therefore, we would expect to see similar changes in the ischemic tissue of pigs on a normal diet. Several early large animal studies looking at chronic myocardial ischemia and angiogenesis showed promising results; however, most of those studies failed to translate to successful phase I and II clinical trials. One likely explanation for these failures in

TABLE 3. Proteomic analysis: Proteins with decreased expression in the GSK-3 β I, LCI, and HCI groups compared with the HCC group

GSK-3 β protein expression<HCC	LCI protein expression<HCC	HCI protein expression<HCC
α -1,4, glucan phosphorylase*	Alanine aminotransferase 1 (ALT1)‡	14-3-3 protein θ
Fibrinogen β chain*	Alpha-1,4 glucan phosphorylase*	40S ribosomal protein SA
Ribonuclease inhibitor	ATP synthase F(0) complex subunit B1, mitochondrial	Alanine-glyoxylate aminotransferase‡
Serum deprivation-response protein	ATP synthase protein 8‡	Antithrombin protein
Succinyl-CoA ligase subunit β †	Carbonic anhydrase 1	ATP synthase protein 8
Uncharacterized protein (CHCHD3)	Cytochrome c oxidase subunit 5A‡	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial
Uncharacterized protein (COX6B)	Fibrinogen beta chain*	Cytochrome c oxidase subunit 5A‡
Uncharacterized protein (PURA)	Hemoglobin subunit α (α -globin) (hemoglobin α chain)‡	Glyceraldehyde-3 phosphate dehydrogenase
Uncharacterized protein (COX5A)	LETM1 and EF-hand domain-containing protein 1, mitochondrial‡	Glycogen phosphorylase
	Voltage-dependent anion-selective channel protein 1 (VDAC 1)‡	Hemoglobin subunit alpha‡
	V type proton ATPase catalytic subunit A	LETM1 and EF-hand domain containing protein 1, mitochondrial‡
		Malate dehydrogenase, mitochondrial
		NADH ubiquinone oxidoreductase chain 4
		Peptidyl-prolyl <i>cis-trans</i> isomerase
		Succinate dehydrogenase
		Succinyl-CoA ligase subunit β †
		Voltage dependent anion-selective channel protein 1 (VDAC-1)‡

GSK-3 β I, Glycogen synthase kinase inhibitor; HCC, high-cholesterol control; LCI, low-dose calpain inhibitor; HCI, high-dose calpain inhibitor. *Proteins were decreased in the GSK-3 β I and LCI groups compared with the HCC group. †Proteins were decreased in the GSK-3 β I and HCI groups compared with the HCC group. ‡Proteins were decreased in the HCI and LCI groups compared with the HCC group. All proteins listed showed statistically significant differences in expression between each experimental group (GSK-3 β I, HCI, and LCI) and the HCC group by Scaffold or ProteoIQ analysis.

human subjects is that patients with end-stage coronary artery disease have multiple comorbid conditions, including hypertension, hyperlipidemia, diabetes, and endothelial dysfunction, making them vastly different from healthy animals with induced coronary ischemia.³⁰ Our animal model has established a clinically relevant metabolic syndrome pig model to approximate the comorbidities seen in patients with chronic myocardial ischemia; therefore, we chose to begin our study by looking at pigs with metabolic syndromes. However, in the future it would be interesting to compare our results with those of normal-diet controls.

Limitations

The data presented in this study did not determine the optimal dose of calpain inhibition required to have a beneficial effect. The optimal dose of calpain inhibition may plausibly lie between the doses used in our LCI and HCI protocols. Future dose–response studies are needed to identify the precise optimal level of calpain inhibition required for positive cardiovascular effects. In addition, the optimal treatment time will require further investigation. Our Western blot data identify differences in protein expression in the pig myocardial tissue compared with loading controls. These differences could be related to differences in protein stability or differences in the relative proportions of cell populations expressing

different quantities of proteins. Our animal model is designed to emulate patients with coronary artery disease (amerooid constrictor model) and its associated comorbidities (diet-induced metabolic syndrome). However, it is important to note that humans develop these complications owing to a variety of complex factors over many years. Whereas our animal model is very instructive for elucidating early changes associated with chronic ischemia, numerous differences may exist, and extrapolation of our findings to pathophysiological processes in human coronary artery disease and ischemia should be done very cautiously.

CONCLUSIONS

In summary, we found that in the setting of metabolic syndrome, calpain and GSK-3 β inhibition increase vessel density in both ischemic and nonischemic myocardial tissue. Importantly, we also found that calpain inhibition may have exerted these effects by inhibition of GSK-3 β and up-regulation of its downstream effectors, including the GSK-3 β /insulin/PI3K and WNT/GSK-3 β /catenin pathways. This study confirmed that GSK-3 β inhibition increases blood flow to ischemic myocardial tissue both at rest and during pacing. These findings have important implications for new vessel formation in the setting of myocardial ischemia under conditions known to be associated with reduced angiogenesis and collateral vessel formation.

Webcast

You can watch a Webcast of this AATS meeting presentation by going to: http://webcast.aats.org/2016/Video/Monday/05-16-16_Room_340_1812_Pötz-800.mpg4.

Conflict of Interest Statement

Dr Sellke reports fees from Stryker and Boehringer Ingelheim. All other authors have nothing to disclose with regard to commercial support.

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Key Words: calpain inhibition, chronic myocardial ischemia, metabolic syndrome, angiogenesis

Discussion

Dr J. Crestanello (Columbus, Ohio). I would like to congratulate Dr Pötz and colleagues for a well-designed set of experiments and for an excellent presentation. This study addresses an important topic given the increased incidence of morbid obesity, diabetes, hypercholesterolemia, and metabolic syndrome in patients with chronic ischemic heart disease. The authors examined the effects of calpain inhibition and of a downstream intermediary, GSK-3β, in a pig model of chronic myocardial ischemia in the setting of metabolic syndrome. They found that calpain inhibition and GSK-3β inhibition increases angiogenesis, capillary and arteriolar density is ischemic and nonischemic myocardium and myocardial perfusion. This effect appears to be mediated

by the increased expression of angiogenic proteins, cytoskeletal regulation, increasing the proteins in both the regulation of metabolism and respiration and in calcium-binding pathways.

I have the following questions: What evidence do you have that calpain is overexpressed in your metabolic syndrome pig model? And if you do, is there a relationship between the severity of the metabolic syndrome and the level of expression of calpain?

Dr Potz (*Providence, RI*). Thank you for that review and for the question. The evidence that we have for increased activity of calpain actually comes from research of a collaborator of ours, Dr Donald R. Senger. Using a small animal model, he has shown that ischemia increases calpain activity and that moderate calpain inhibition improves cytoskeletal structure and reduces underlying hypoxia. In our study, we used the same calpain inhibitor at similar dosages. We have not yet looked at calpain activity officially in our pig model but instead we look at the expression levels of specific calpain substrates and inferred decreased activity in the calpain inhibited groups in that way.

Dr Crestanello. So the question is why did the effect of calpain inhibition on angiogenesis seem to be limited to or more significant in the ischemic myocardium while the effect of GSK-3 β is present in both the ischemic and nonischemic myocardium?

Dr Potz. Thank you. That's a really good question. I think the reason we see a greater effect of calpain inhibition in the ischemic tissue is that calpain is activated by stress and the release of calcium (among other things). So in the ischemic tissue there is release of calcium and subsequent overactivation of calpain. When this overactivation gets inhibited, the calpain activity is brought to an appropriate level for cellular function. We do not see this same ischemic versus nonischemic effect with GSK-3B because, as far as we know, GSK-3B has a broader spectrum of activation and activity.

Dr Crestanello. And my final question is probably the most important. Did this improvement in myocardial angiogenesis and flow result in any improvement in ventricular function or any decrease in the size of the myocardial infarction?

Dr Potz. That is a very good question. We were mostly looking at blood flow and angiogenesis. We did insert catheters into the left ventricle and the ascending aorta to measure pressure. We haven't analyzed that data, but we plan on doing that.

And in terms of determining the ischemic area of the heart, we injected gold microspheres into the heart after occluding the left circumflex artery. We later isolated those gold microspheres in the tissue and read their levels (BioPal, MA). This helped us to determine what area of the myocardium was ischemic versus non-ischemic. We did not measure the size of infarct in our pigs. However, to determine the effect of calpain inhibition on tissue ischemia, we measured vessel density, blood flow and oxidative stress in the ischemic myocardial tissue from the control and experimental pigs and found that calpain inhibition was beneficial. Thank you.

Dr M. Ruel (*Ottawa, Ontario, Canada*). It looks like this is one of perhaps many master switches that occur in metabolic syndrome. Do we know how it interrelates with other ones such as methylglyoxal and eNOS uncoupling? Have you been able to kind of figure out the story together?

And what does it do to insulin resistance and the overall effects of the metabolic syndrome?

Dr Potz. That's a very good question. Our calpain inhibitor inhibits calpain 1 and 2, which is localized both in the heart and in the endothelium. We have thus far really only looked at the effect of calpain on the blood flow and have focused on changes in vessel density and the endothelium. However, we agree that calpain inhibition may be important in regulating metabolic syndrome, and we're now looking to explore that further.