Glycogen Synthase Kinase 3β Inhibition Improves Myocardial Angiogenesis and Perfusion in a Swine Model of Metabolic Syndrome

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Background—Inhibition of glycogen synthase kinase 3β (GSK-3β) has been reported to be cardioprotective during stressful conditions.

Methods and Results—Pigs were fed a high-fat diet for 4 weeks to develop metabolic syndrome, then underwent placement of an ameroid constrictor to their left circumflex artery to induce chronic myocardial ischemia. Two weeks later, animals received either: no drug (high cholesterol control group [HCC]) or a GSK-3β inhibitor (GSK-3β inhibited group [GSK-3βI]), which were continued for 5 weeks, followed by myocardial tissue harvest. Coronary blood flow and vessel density were significantly increased in the GSK-3βI group compared to the HCC group. Expression levels of the following proteins were greater in the GSK-3βI group compared to the HCC group: vascular endothelial growth factor receptor 1, vascular endothelial cadherin, γ-catenin, β-catenin, protein kinase B, phosphorylated forhead box O1, and superoxide dismutase 2.

Conclusions—In the setting of metabolic syndrome, inhibition of GSK-3β increases blood flow and vessel density in chronically ischemic myocardium. We identified several angiogenic, cell survival, and differentiation pathways that include β-catenin signaling and AKT/FOXO1, through which GSK-3β appears to improve vessel density and blood flow. These results may provide a potential mechanism for medical therapy of patients suffering from coronary artery disease and metabolic syndrome. (J Am Heart Assoc. 2016;5:e003694 doi: 10.1161/JAHA.116.003694)

Key Words: angiogenesis • cardiovascular diseases • diabetes mellitus • hypercholesterolemia • obesity

Metabolic syndrome (hypertension, obesity, and glucose intolerance leading to type 2 diabetes mellitus and hyperlipidemia) is a significant cause of cardiovascular disease. The endothelial dysfunction associated with metabolic syndrome leads to a diminished angiogenic response to chronic coronary ischemia. This vascular dysfunction includes formation of aberrant collateral vessels.

Glycogen synthase kinase 3β (GSK-3β) is a serine/threonine kinase that regulates a diverse range of cellular pathways, including those involved in growth and development, endocrine function, immunity, and neuronal function. GSK-3β signaling is regulated by growth factors, hedgehog, Wnts, G-protein-coupled ligands, and cytokines. GSK-3β is ubiquitously expressed, and nearly 100 proteins have been identified as substrates, which are involved in numerous cellular processes. Abnormal GSK-3β activity is associated with metabolic and cardiovascular disease.

One important pathway through which GSK-3β acts is the Wnt signaling cascade. The Wnt/β-catenin pathway promotes β-catenin stability, which can help correct vascular defects, reduce permeability, and increase stability of new blood vessel growth. GSK-3β acts as an inhibitor of this pathway by phosphorylating β-catenin and marking it for degradation. Some of the pathways involved in GSK-3β regulation and some of the established substrates for GSK-3β are demonstrated in Figure 1A. Importantly, inhibition of GSK-3β is cardioprotective. GSK-3β inhibition promotes functional neovessel formation, suppresses apoptosis of cardiomyocytes, and improves ischemia/reperfusion injury in myocardial tissue. Together, these findings suggest that GSK-3β may serve as a potential therapeutic target for patients with ischemic heart disease and metabolic syndrome.

Patients with metabolic syndrome, as defined by the National Institutes of Health (NIH) National Heart, Lung, and Blood Institute, must have at least 3 of the 5 identified...
metabolic risk factors for heart disease. These risk factors include the following: obesity, high triglyceride level, low high-density lipoprotein (HDL) cholesterol, high blood pressure, or high fasting glucose (which is an early sign of diabetes mellitus). We have developed a pig model that adequately represents the molecular, microcirculatory, and metabolic abnormalities associated with metabolic syndrome observed in adult patients with coronary artery disease and metabolic syndrome. In our model, the pigs fed a high-cholesterol diet develop 4 of the 5 risk factors associated with metabolic syndrome when compared to the control diet pigs. These risk factors include (1) greater weight gain, (2) higher total cholesterol and low-density lipoprotein/HDL ratios, (3) increased mean arterial pressure, and (4) elevated blood glucose levels in response to a glucose tolerance test.10 Using this model, we found that moderate inhibition of calpain decreases myocardial apoptosis, improves collateral dependent perfusion, and increases expression of signaling proteins that promote angiogenesis.11,12 Interestingly, GSK-3β is a substrate for calpain. Calpain activation can promote GSK-3β activation by cleaving off its inhibitory domain (Figure 1B). Importantly, inhibition of calpain has been shown to inhibit GSK-3B function.13

The objective of this study is to examine the effects of inhibition of GSK-3β on myocardial blood flow and vessel density in the setting of chronic myocardial ischemia and metabolic syndrome conditions. We hypothesized that GSK-3β inhibition would have a protective effect on absolute blood flow and angiogenic signaling in chronically ischemic myocardial tissue in a clinically relevant swine model of metabolic syndrome.

Methods

Animal Model

Twelve Yorkshire swine (E.M. Parsons and Sons, Hadley MA) were fed a 500 g/day high-fat/high-cholesterol diet (4% cholesterol, 2.3% corn oil, 17.2% coconut oil, 1.5% sodium cholate, and 75% regular chow) for 4 weeks (Sinclair Research, Columbia, MO).10 Pigs then underwent general anesthesia, and a minithoracotomy was performed. The left circumflex artery was identified and manually occluded for 2 minutes while gold microspheres were injected into the left atrium. To induce chronic myocardial ischemia, a titanium ameroid constrictor was then placed on the left circumflex. At the completion of the

Figure 1. A, Glycogen synthase kinase 3β (GSK-3β) regulates a diverse range of cellular pathways. Inhibition of GSK-3β, by phosphorylation of its N terminal, has been reported to be cardioprotective. B, Calpain regulates GSK-3β. Calpain is thought to inactivate GSK-3β by truncation of its N terminal. AKT indicates protein kinase B; MCL-1, myeloid cell leukemia 1; Mdm2, E3 ubiquitin-protein ligase; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI3K, phosphatidylinositol 3-kinase; p-IRS, phosphorylated insulin receptor substrate; VE, vascular endothelial; VEGFR, vascular endothelial growth factor receptor.
experiment the amount of gold microspheres in each tissue section allowed us identify ischemic versus nonischemic tissue. Two weeks later, animals were split into 2 groups: pigs received either a placebo (high-cholesterol control group, 1.5 mg/kg per day of DMSO, termed as HCC group; n=8); or a GSK-3β inhibitor (IM-12, 1.5 mg/kg per day dissolved in DMSO, termed as GSK-3β inhibited [GSK-3βI] group; n=4). The high-cholesterol diet and GSK-3β inhibitor were continued for 5 weeks. Seven weeks after ameroid placement surgery, pigs were anesthetized and a terminal procedure was performed. The heart was exposed through a sternotomy, coronary angiography was performed, and microspheres were injected at rest and with pacing (150 beats per minute [bpm]) in the left atrium. Euthanasia was performed by exsanguination under deep anesthesia. Endocardial and peripheral organ tissue was collected for molecular analysis. Endocardial tissue was collected from the chronically ischemic myocardium and the nonischemic myocardium. Ischemic tissue was identified based on the proximity to the left circumflex artery (distal to the ameroid constrictor), and nonischemic tissue was identified based on the proximity to the left anterior descending artery. Tissue was both rapidly frozen in liquid nitrogen and placed in formalin. One pig died at the end of the ameroid placement procedure. This pig was excluded from the study. 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. The surgical procedures used in this study have been previously described.11 The metabolic data for these pigs is shown in Table 1. A glucose tolerance test was performed before the start of the harvest procedure: 0.5 g/kg of dextrose 50 was administered at the start of the operation and glucose was measured at 30 and 60 minutes. Pigs were measured for length and weight before 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.

Table 1. Metabolic Data for Pigs

| Metabolic Parameter | HCC (n=8) | GSK-3βI (n=4) | P Value |
|---------------------|-----------|--------------|---------|
| Percent change in weight | 27.38±2.11 | 22.17±4.12% | 0.17 |
| Cholesterol/HDL ratio | 5.41±0.78 | 6.48±0.76 | 0.53 |
| Initial BG | 88.9±13.0 | 98±4.32 | 0.22 |
| 30-minute BG fold change from initial value | 2.50±0.19 | 2.22±0.24 | 0.23* |
| 60-minute BG fold change from initial value | 1.57±0.15 | 1.20±0.42 | 0.76* |

There were no differences in metabolic parameters between the 2 groups. Initial blood glucose values are listed (before dose of dextrose 50 [0.5 g/kg]). Thirty- and 60-minute blood glucose values are expressed as fold change from initial blood glucose value. Weights are expressed as percent change of body weight at the harvest procedure from the weight of the pigs at the ameroid placement operation. There was no difference in percent change in weight, cholesterol/HDL ratio or initial glucose values between the 2 groups as determined by unpaired Mann–Whitney U test between the GSK-3βI and HCC groups. BG indicates blood glucose; GSK-3βI (n=4), GSK-3β inhibited group; HCC (n=8), high cholesterol control group; HDL, high-density lipoprotein.

There was no effect of GSK-3β on blood glucose as determined by 2-way repeated-measures ANOVA (P=0.473) or at 30 (P=0.23) or 60 minutes (P=0.756), as determined by a Student-Newman-Keuls post-hoc test (*).

Myocardial Perfusion

The methods used to determine perfusion to the myocardium (both during rest and with demand pacing) have been previously described.14 During the first surgical procedure, the left circumflex artery was occluded and gold microspheres (BioPhysics Assay Laboratory Inc., Worcester, MA) were injected into the left atrium. This procedure helps to identify the ischemic (fed by the left circumflex artery) and nonischemic myocardial territory (fed by the left anterior descending artery). During the final surgical procedure, lutetium microspheres were injected at rest and europium microspheres were injected during demand pacing while simultaneously withdrawing blood from the femoral artery. After cardiac harvest, myocardial tissue samples were divided into 10 sections (1 cm width) and sent to BioPhysics Assay Laboratory for analysis of blood flow in the ischemic and nonischemic regions of the left ventricle.11

Immunohistochemical Staining

Immunohistochemical staining was performed as previously described.12 Slides were incubated against endothelial marker CD-31 antibody (R&D System Metabolic, Minneapolis, MN) and smooth-muscle actin (SMA) antibody (Sigma-Aldrich, St Louis, MO) overnight followed by appropriate secondary antibody. Secondary was applied on day 2. A Nikon E800 Eclipse microscope (Nikon, Tokyo, Japan) was used to take images (×20 magnification at 5 random fields). Structures with lumens between 10 and 800 pixels were defined as capillaries and those between 800-infinity pixels were defined as arteries. Vessel densities from the 5 randomly selected myocardial tissue sections were averaged.

Protein Expression

Western blot analysis was performed as previously described.12 PVDF membranes (Millipore, Billerica, MA) were primed with primary antibodies against VEGFR1 (vascular endothelial growth factor receptor 1), VEGFR2 (vascular endothelial growth factor receptor 1), ERK1/2 (extracellular signaling kinase 1 and 2), eNOS (endothelial nitric oxide synthase), p-FOXO1 (phosphorylated forkhead box O1), AMPK-α (AMP-activated protein kinase-α), FOXO1 (forkhead...
box O1), BAD (B-cell lymphoma 2 [Bcl-2]-associated death promotor), SOD2 (superoxide dismutase 2), p-IRS-1 Ser332 (phosphorylated insulin receptor substrate 1 serine 332), p-Bcl-2 ser70 (phosphorylated Bcl-2 serine 70) and p-mcl-1 ser159 (phosphorylated myeloid leukemia cell differentiation protein 1 serine 159; all from Cell Signaling Technology, Danvers, MA). ImageJ software (NIH, Bethesda, MD) was used to quantify band densitometry as arbitrary light units. Loading error was controlled for by probing membranes with an antibody against GAPDH.

Statistical Analysis

Results are reported as a mean±SEM. GraphPad software (version 5.0; GraphPad Software Inc., San Diego, CA) was used to perform an unpaired Mann–Whitney U test between the GSK-3β and HCC groups. SigmaPlot (Systat Software, Inc., San Jose, CA) was used to perform 2-way repeated-measures ANOVA with a Student-Newman-Keuls post-hoc test on the glucose tolerance data. Protein expression was normalized to GAPDH in all pigs and is reported as fold change compared to HCC group. Data for GSK-3β substrates were pooled before analysis.

Results

Metabolic Parameters

There was no difference in percent change in weight, cholesterol/HDL ratio, or initial glucose values between the two groups (Table 1). There was no effect of GSK-3β on blood glucose as determined by 2-way repeated-measures ANOVA (P=0.473) or at 30 (P=0.23) or 60 minutes (P=0.756) as determined by a Student-Newman-Keuls post-hoc test.

Blood Flow

Myocardial perfusion ratios (left circumflex [ischemic]/left anterior descending [nonischemic]) were significantly increased in the GSK-3β inhibited group compared to the control group, both in ischemic and nonischemic myocardial tissue (Figure 2).

Arteriolar and Capillary Counts

Pigs in the GSK-3β inhibited group had increased capillary (P=0.01) and arteriolar (P<0.01) densities in the chronically ischemic myocardium compared to the control group (Figure 3A). Pigs in the GSK-3β inhibited group had increased capillary (P=0.04) and arteriolar (P<0.01) densities in the nonischemic myocardium compared to the control group (Figure 3B).

Pro-Angiogenic Signaling Pathway

There were greater expression levels of the following proangiogenic protein markers in the GSK-3β inhibited group compared to the control group including: VEGFR1 (P=0.046), γ-catenin (P=0.02), and VE-cadherin (P=0.01) and β-catenin that failed to reach significance (P=0.07). There was no significant difference in VEGFR2. There were also increases in expression in ERK1/2 (P=0.17) and eNOS (P=0.12) in the GSK-3β inhibited group compared to the HCC control; however, these did not reach statistical significance (Table 2).

Apoptotic Pathway

Expression levels of antiapoptotic signaling proteins Akt (P=0.03) and p-FOXO1 (P=0.03) were greater in the GSK-3β inhibited group compared to the control group. There was a trend toward greater expression in AMPK-α (P=0.08) and FOXO1 (P=0.05) in the GSK-3β inhibited group compared to the control group. Expression level of proapoptotic protein BAD was less in the GSK-3β group compared to the control group (P=0.059), although this failed to reach significance. There was no significant difference in p-AKT (P=0.20; Table 2).

Antioxidant Proteins

The expression level of antioxidant proteins SOD2 (P=0.02) was greater in the GSK-3β inhibited group compared to the control group. However, there was no significant change observed in catalase (P=0.24; Table 2).

GSK-3β Activity

The GSK-3β inhibited myocardial tissue had decreased expression of GSK-3β substrates p-Bcl-2 (ser70), p-mcl-1 (ser159), and p-IRS-1 (Ser332) compared to the control (P=0.01).

Discussion

In this study, we examined the role of GSK-3β inhibition on myocardial blood flow and vessel density in the setting of chronic ischemia and metabolic syndrome. We hypothesized that GSK-3β inhibition, like calpain inhibition, would beneficially effect myocardial blood flow and angiogenic signaling in chronic ischemic myocardium.11 We found that in the setting of metabolic syndrome and chronic myocardial ischemia, inhibition of GSK-3β increases blood flow and vessel density in ischemic and nonischemic tissue. We identified several angiogenic, cell survival, and cell differentiation pathways through which GSK-3β inhibition could be having an effect, including the VEGF receptor/β-catenin and Akt/FOXO1.
pathways. Pathways found to be affected by GSK-3β inhibition were similar to the pathways previously found to be affected by calpain inhibition (B.A. Potz, et al, unpublished data). This suggests that the beneficial effect observed by calpain inhibition on myocardial blood flow and vessel density may be through its inhibition of GSK-3β. Future work will need to evaluate the downstream signaling pathways of GSK-3β in myocardial tissue of our animal model in the presence of both GSK-3β inhibition and calpain inhibition.

Figure 2. Glycogen synthase kinase 3β (GSK-3β) inhibition increases myocardial perfusion. Myocardial perfusion ratios (Ischemic/Nonischemic tissue) were increased in the GSK-3β inhibited group compared to the control group at rest and with demand pacing. GSK-3βI indicates GSK-3β inhibited group (n=4); HCC, high cholesterol control group (n=8). *P<0.05 by Mann–Whitney U test.

Both capillary and arteriolar vessel densities were increased in nonischemic and ischemic myocardial tissue in the GSK-3β inhibited group compared to the control. Importantly, this increase in vessel density led to an increase blood flow to ischemic and nonischemic tissue both at rest and with demand pacing.

To determine whether GSK-3β inhibition decreases GSK-3β activity in myocardial tissue, we examined specific downstream targets of GSK-3β in ischemic left ventricular tissue.
GSK-3β is known to phosphorylate and inhibit antiapoptotic proteins p-Bcl-2 (ser70) and p-mcl-1 (ser159).\textsuperscript{15,16} Mcl-1 localizes to the mitochondria, where it interacts with and antagonizes proapoptotic Bcl-2 family members and inhibits apoptosis. Bcl-2 is an antiapoptotic protein. Therefore, inhibition of GSK-3β would increase the antiapoptotic activity of both Bcl-2 and mcl-1.\textsuperscript{17,18} GSK-3β also phosphorylates IRS-1 (Ser332), which inhibits its ability to activate the Akt pathway. We found decreased expression of the phosphorylated form of these proteins in GSK-3β inhibited tissue.

Figure 3. A, Glycogen synthase kinase 3β (GSK-3β) inhibition increases vessel density in ischemic tissue. GSK3β inhibition caused an increase in both capillary and arteriolar density in the ischemic myocardium compared to the HCC group. B, GSK-3β inhibition increased vessel density in nonischemic tissue. GSK-3β inhibition caused increased arteriolar and capillary density in the non ischemic myocardium compared to the HCC group. Representative images of myocardial tissue at $\times$20 high-power field. DAPI is blue, CD31 is red, SMA is green. DAPI indicates 4',6-diamidino-2-phenylindole; GSK-3β, GSK-3β inhibited group (n=4); HCC, high cholesterol control group (n=8); SMA, smooth-muscle actin. *$P<0.05$ by Mann–Whitney $U$ test.
Table 2. Protein Expression in Ischemic Myocardial Tissue

| Targets                | HCC (n=8) | GSK-3β (n=4) | P Value |
|------------------------|-----------|--------------|---------|
| Angiogenic proteins    |           |              |         |
| VEGF-R1                | 1.0±0.25  | 3.32±0.9     | 0.046   |
| γ-Catenin              | 1.0±0.36  | 2.47±0.29    | 0.02    |
| VE-cadherin            | 1.0±0.19  | 1.96±0.19    | 0.01    |
| β-catenin              | 1.0±0.26  | 2.71±0.70    | 0.06    |
| VEGFR2                 | 1.0±0.18  | 0.83±0.24    | 0.60    |
| ERK1/2                 | 1.0±0.25  | 6.57±3.57    | 0.17    |
| eNOS                   | 1.0±0.29  | 1.85±0.38    | 0.12    |
| Apoptotic proteins     |           |              |         |
| Akt                    | 1.0±0.26  | 2.01±0.26    | 0.03    |
| p-FOXO1                | 1.0±0.21  | 2.70±0.58    | 0.03    |
| AMPK-α                 | 1.0±0.19  | 4.54±1.63    | 0.08    |
| FOXO1                  | 1.0±0.36  | 2.54±0.53    | 0.05    |
| BAD                    | 1.0±0.39  | 0.10±0.03    | 0.05    |
| p-AKT                  | 1.0±0.10  | 0.66±0.21    | 0.20    |
| Antioxidant proteins   |           |              |         |
| SOD2                   | 1.18±0.22 | 2.70±0.32    | 0.02    |
| Catalase               | 1.0±0.29  | 1.42±0.15    | 0.24    |

Proteins expression is listed as fold change±SEM as compared to the control group (HCC). GraphPad software (version 5.0; GraphPad Software Inc., San Diego, CA) was used to perform an unpaired Mann–Whitney U test between the GSK-3β and HCC groups. Akt indicates protein kinase B; AMPK-α, AMP-activated protein kinase-α; BAD, B-cell lymphoma 2–associated death promotor; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signaling kinase 1 and 2; GSK-3β (n=4), GSK-3β inhibited group; HCC (n=8), high cholesterol control group; p-FOXO1, phosphorylated forkhead box 01; SOD2, superoxide dismutase 2; VE-cadherin, vascular endothelial cadherin; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2.

compared to control. Decreases in downstream substrates of GSK-3β suggest that the inhibitor is working by inhibiting the GSK-3β signaling pathway. Future studies will be carried out to address these pathways further.

To further explore the molecular mechanism responsible for GSK-3β inhibitor–induced increases in blood flow and vessel density, we examined expression of several angiogenic markers and apoptotic pathways. We found greater expression levels of important markers of angiogenesis, including VEGF-R1, VE-cadherin, β-catenin, and γ-catenin, in the GSK-3β inhibited group compared to control. β-catenin works by acting as an integral adaptor protein for VE-cadherin cell-cell adhesion. Interestingly, cytosolic β-catenin promotes cellular differentiation and growth by functioning as a transcriptional coregulator. β-catenin is also a major protein involved in the Wnt pathway, where it functions to promote vascular stability. GSK-3β is known to phosphorylate β-catenin, which marks it for proteasomal degradation. Therefore, it is possible that GSK-3β inhibition is having a beneficial effect on myocardial blood flow and vessel density by promoting stabilization of β-catenin. Future work will be needed to further identify the downstream consequences of β-catenin signaling.

We also found that expression levels of Akt and p-FOXO1 were upregulated in the GSK-3β inhibited group compared to control. FOXO1 is a member of the “forkhead box” family of transcription factor proteins. It plays an important role in regulation of gluconeogenesis, glycolysis, and cell survival. FOXO1 is phosphorylated by Akt, which inactivates it and prohibits it from entering the nucleus and causing cellular atrophy. Akt is also known to inhibit apoptosis by inhibiting BAD, a protein directly involved in programmed cellular death. We found less expression of BAD in the GSK-3β inhibited group compared to control. Interestingly, GSK-3β is known to phosphorylate and degrade IRS-1, which is upstream of the Akt/FOXO1 pathway. We found decreased expression of this form of phosphorylated IRS-1 in the GSK-3β inhibited tissue. The IRS/PI3K/Akt pathway is crucial for regulation of endothelial cell proliferation and survival. It is possible that GSK-3β inhibition stabilizes (IRS-1), which allows upregulation of the Akt/FOXO1 pathway. Together, the above findings suggest that GSK-3β inhibition works through the IRS-1/Akt pathway to prevent apoptosis and promote cellular survival in the ischemic myocardium. GSK-3β is known to modulate other proteins involved in cellular apoptosis as well, including hypoxia-inducible transcription factor, Bcl-3, and others. Future work will look into these pathways more directly to identify exactly how GSK-3β inhibition is having an effect.

We found that there was greater expression of antioxidant protein SOD2 and a trend toward greater expression of catalase in the GSK-3β inhibited group compared to control. It is unclear how GSK-3β inhibition modulates antioxidant protein levels in ischemic myocardium. However, GSK-3β has been found to influence redox conditions in Caenorhabditis elegans by inhibiting transcription of the SKN-1 protein, which is known to orchestrate the response to oxidative stress. It is currently unclear whether GSK-3β inhibitor-mediated effects on proteins involved in mitochondrial respiration and metabolism could also impact oxidant status in the cell. Future studies will be needed to carefully evaluate the levels and localization of antioxidant proteins and reactive oxygen species (ROS) to determine whether GSK-3β modulates ROS.

Limitations

Using a fixed dose of the inhibitor, we have shown that GSK-3β inhibition is beneficial. However, this study did not determine the optimal dose of GSK-3β inhibition needed for improved angiogenesis. Additionally, the following 2
parameters could have varying effects on the observed outcomes: time from the induction of ischemia to GSK-3β inhibitor administration and treatment time. The amioder constrictor fully occludes in 10 to 11 days. Therefore, in order to simulate chronic cardiac ischemia in the setting of metabolic syndrome before the start of therapy, pigs were started on the GSK-3β inhibitor on postamioder placement day 14. The GSK-3β inhibitor was given over 5 weeks, and tissue was analyzed at the end of the study, at only 1 time point. In the future, the drug should be given at different doses and intervals to develop a dose-response curve and determine optimal time and duration of drug treatment. It is also possible that a longer duration of ischemia may alter the beneficial effects we observed with GSK-3β inhibition. Future studies will need to evaluate whether GSK-3β inhibition is beneficial after alterations and remodeling associated with chronic ischemia over a prolonged period (weeks to months). Additionally, it is also possible that GSK-3β inhibition may have side effects; however, none were observed during our study. Finally, we have attempted to mimic many of the alterations observed in humans with chronic myocardial ischemia and impaired angiogenic by performing this study in a large animal model of metabolic syndrome. However, there are shortcomings of any animal model, including the potential for species specific responses and the age of the animals. At this time, we do not know the effect of GSK-3β inhibition on normal diet control pigs with and without chronic myocardial ischemia. We do have preliminary data, using reverse-transcriptase quantitative polymerase reaction, showing that in the left ventricle of hyperglycemic swine, there is upregulation of GSK-3β mRNA compared to normoglycemic control animals. However, future work will need to answer this important question.

**Conclusion**

In the setting of metabolic syndrome and chronic myocardial ischemia, inhibition of GSK-3β increases blood flow and vessel density in ischemic and nonischemic myocardial tissue. We identified several angiogenic, cell survival, and cell differentiation pathways through which GSK-3β inhibition may have an effect, including the β-catenin and AKT/FOXO1 pathways. Signaling alterations affected by GSK-3β inhibition in this study were similar to effects of calpain inhibition we have previously reported (B.A. Potz, et al, unpublished data). Therefore, inhibition of calpain/GSK3β activity may promote a functional increase in coronary angiogenesis after chronic ischemia in the setting of metabolic syndrome. The results of this article may provide a potential mechanism for medical therapy of patients suffering from coronary artery disease and metabolic syndrome.
19. Elmadhun NY, Lassaletta AD, Chu LMSF. Metformin alters the insulin signaling pathway in ischemic cardiac tissue in a swine model of metabolic syndrome. J Thorac Cardiovasc Surg. 2013;145:258–266.

20. Badorff C, Holzmann Y, Zeiher AM, Dimmeler S. Glycogen synthase kinase-3 couples AKT-dependent signaling to the regulation of p21Cip1 degradation. J Biol Chem. 2002;277:9684–9689.

21. Flügel D, Görlich A, Kietzmann T. GSK-3B regulates cell growth, migration, and angiogenesis via Fbw7 and USP28-dependent degradation of HIF-1. Blood. 2012;119:1292–1302.

22. An JH, Vranas K, Lucke M, Inoue H, Hisamoto N, Matsumoto K, Blackwell TK. Regulation of the Caenorhabditis elegans oxidative stress defense protein SKN-1 by glycogen synthase kinase-3. Proc Natl Acad Sci USA. 2005;102:16275–16280.