Metabolic syndrome abolishes glucagon-like peptide-1 receptor agonist stimulation of SERCA in coronary smooth muscle

Running title: Metabolic syndrome and GLP-1 in CSM

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Abstract

Metabolic syndrome (MetS) doubles the risk of adverse cardiovascular events. Glucagon-like peptide-1 (GLP-1) receptor agonists induce weight loss, increase insulin secretion, and improve glucose tolerance. Studies in healthy animals suggest cardioprotective properties of GLP-1 receptor agonists, perhaps partially mediated by improved sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) activity. We examined the acute effect of GLP-1 receptor agonists on coronary smooth muscle cells (CSM) enzymatically isolated from lean, healthy Ossabaw miniature swine. Intracellular Ca\(^{2+}\) handling was interrogated with fura-2. The GLP-1 receptor agonist, exenatide, activated SERCA, but did not alter other Ca\(^{2+}\) transporters. Further, we tested the hypothesis that chronic, in vivo treatment with GLP-1 receptor agonist, AC3174, would attenuate coronary artery disease (CAD) in swine with MetS. MetS was induced in 20 swine by six months feeding of a hypercaloric, atherogenic diet. Swine were then randomized (N=10/group) into placebo or AC3174 treatment groups and continued diet for an additional six months. AC3174 treatment attenuated weight gain, increased insulin secretion, and improved glucose tolerance. Intravascular ultrasound and histology showed no effect of AC3174 on CAD. MetS abolished SERCA activation by GLP-1 receptor agonists. We conclude that MetS confers vascular resistance to GLP-1 receptor agonists, partially through impaired cellular signaling steps involving SERCA.
Metabolic syndrome (MetS) is defined as the presence of three or more of the following five risk factors: obesity, hypertension, glucose intolerance, insulin resistance, and dyslipidemia (1). Obesity, MetS, and type 2 diabetes are all independent risk factors for cardiovascular disease. The presence of MetS doubles risk of experiencing an adverse cardiovascular event (2). Thus, it is important to understand the effect of diabetes and MetS treatment modalities on cardiovascular health.

Insulin-sensitizing drugs and those which enhance insulin secretion from pancreatic beta cells, have been employed in the treatment of type 2 diabetes and MetS. Others, such as dipeptidyl peptidase-4 inhibitors, and glucagon-like peptide-1 receptor agonists (exenatide, liraglutide, AC3174, etc.) increase both insulin sensitivity and insulin secretion (3;4). Recently, insulin-sensitizing thiazolidinediones have been implicated in increased risk of fluid retention, LDL and triglyceride accumulation, heart failure, and myocardial infarction (5-7). Hence, there is great attention on cardiovascular outcomes of anti-diabetic agents and the Food and Drug Administration requires cardiovascular safety profiles for all new agents (8).

Glucagon-like peptide-1 (GLP-1) receptor agonists are attractive treatment options for MetS, because GLP-1 is an endogenous hormone which functions in normal physiology to increase insulin sensitivity, biosynthesis, and secretion (3). Additionally, GLP-1 receptor agonists reduce myocardial infarct size in ischemia/reperfusion injury (9), improve cardiac function in chronic heart failure (10), and attenuate neointimal formation following vascular injury (11). At the cellular level, GLP-1 receptor agonists improve endothelial calcium homeostasis following simulated ischemia/reperfusion (12). Further, GLP-1 receptor agonists improve sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) activity in multiple cell types (13;14). These studies provide rationale for a protective role of GLP-1 in the coronary vasculature.
A recent study revealed impaired GLP-1 stimulation of myocardial glucose uptake in human patients with type 2 diabetes and in MetS swine (15). This finding raises the question of potential resistance to GLP-1 in MetS and type 2 diabetes and highlights the need for rigorous examination of GLP-1 action on coronary artery disease (CAD), specifically in MetS. Given the stimulation of SERCA in macrophages and ventricular myocytes (13;14) and altered SERCA in coronary smooth muscle (CSM) in MetS and diabetes (16;17), potentially vaso-protective actions of GLP-1 could be mediated through cellular Ca^{2+} signaling. This study was investigated the effect of GLP-1 receptor agonists on SERCA stimulation in CSM from lean swine and on coronary artery disease (CAD) and CSM SERCA stimulation in MetS.

**Methods:**

**Animals.**

All protocols involving animals were approved by the institutional animal care and use committee at Indiana University School of Medicine, and fully complied with animal use standards (18;19). Ossabaw miniature swine were separated into two treatment groups: placebo (N=10) and GLP-1 receptor agonist AC3174-treated (N=10; 0.25µg/kg body weight subcutaneously, twice daily; Amylin Pharmaceuticals, San Diego, CA), which displays nearly identical pharmacokinetic and pharmacodynamics profiles as the marketed GLP-1 receptor agonist, exenatide (4). CAD and MetS were induced in both treatment groups as described in the online supplement. One animal in the placebo group died prior to collection of endpoint data.

**Isolation of coronary smooth muscle cells.**

Epicardial coronary arteries were cleaned of adherent tissue and CSM were isolated with a collagenase solution as described (16;17).

**Measurement of intracellular Ca^{2+}.**
CSM were loaded with fura-2/AM and whole cell intracellular Ca\(^{2+}\) levels were measured as described in the online supplement.

**Acute in vitro exenatide treatment.**

CSM from lean, healthy Ossabaw swine or a separate group of swine with MetS and CAD were treated with 100 nM exenatide during assessment of intracellular Ca\(^{2+}\). The selective SERCA inhibitor, cyclopiazonic acid (CPA, 10 µM) was utilized as a negative control for SERCA function.

**Intravenous glucose tolerance testing.**

Intravenous glucose tolerance testing (IVGTT) was performed as described (16;20) and in the online supplement.

**Plasma Lipid, Electrolyte, and Enzyme Assays**

Blood samples were obtained at time of IVGTT prior to intravenous injection of glucose. Lipid electrolyte, and enzyme content were measured offsite (Antech Diagnostics, West Lafayette, IN).

**Intravascular ultrasound.**

After 12 months on diet, intravascular ultrasound pullbacks were performed as described in the online supplement.

**Histology**

Coronary arterial rings were placed in phosphate-buffered formalin at time of euthanasia. Hematoxylin & Eosin (H&E), Verhoeff-van Gieson (VVG), and Trichrome (TC) staining were performed on sections of these rings. Plaque burden and collagen content were determined using commercially available software (ImageJ 1.48v, National Institutes of Health, USA) as described (16).

**Results:**

**Acute in vitro exenatide treatment in CSM from lean, healthy swine**
We investigated the acute effect of the GLP-1 receptor agonist, exenatide, on intracellular Ca\(^{2+}\) handling in CSM isolated from lean, healthy Ossabaw swine. SR Ca\(^{2+}\) stores were released with caffeine in the absence of extracellular Ca\(^{2+}\) in the presence and absence of 100 nM exenatide (Fig 1A), and the subsequent undershoot of cytosolic Ca during recovery was assessed. Exenatide treatment did not alter resting cytosolic Ca\(^{2+}\) (Fig 1B) or Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels (Fig 1C). Exenatide elicited improved recovery of cytosolic Ca\(^{2+}\) below baseline levels and this effect was completely ablated in the presence of the SERCA inhibitor CPA (Fig 1D). The GLP-1 receptor agonist liraglutide also demonstrated improved recovery of cytosolic Ca\(^{2+}\), which was prevented by the GLP-1 receptor antagonist, exendin (9-39) (Fig S1). In the presence of caffeine, SERCA buffering of cytosolic Ca\(^{2+}\) is functionally inhibited, because of the much more rapid release of Ca\(^{2+}\) through ryanodine receptors on the SR membrane. Therefore, we assessed the rate of recovery to 50% recovery to baseline (T\(_{50}\)), in the presence of caffeine as a measure of Ca\(^{2+}\) extrusion and/or Ca\(^{2+}\) uptake into caffeine-insensitive intracellular stores and to 100% recovery to baseline (T\(_{100}\)) in the absence of caffeine as another measure of SERCA activation. Exenatide significantly decreased time to 100%, but not 50% recovery to baseline (Fig 1E).

**Clinical measurement of MetS in Ossabaw swine**

We then investigated the chronic effect of GLP-1 receptor antagonism *in vivo*. To examine the effects of diet and GLP-1 receptor treatment on glucose metabolism, kidney function, and plasma electrolytes, blood profiles of these parameters were obtained (Table S1) and IVGTTs were performed. Overall, feeding of an excess calorie, atherogenic diet increased body weight, which was significantly attenuated by AC3174 treatment (Fig 2A). Blood pressure was not altered by diet or AC3174 treatment. While fasting plasma glucose was not altered by diet or AC3174 treatment (Table S1), IVGTT assessment of glucoregulation revealed improved glucose handling in AC3174 treated swine, compared
to placebo (Fig 2B-D). This was corroborated by augmented plasma insulin levels following intravenous glucose challenge in AC3174 treated animals, compared to placebo (Fig 2E-G).

**Intravascular ultrasound and histology measurement of CAD**

Intravascular ultrasound (IVUS) was employed to examine severity of CAD in vivo and was confirmed by histology. Both wall coverage and percent plaque burden were assessed as previously described (16) in placebo and AC3174-treated pigs. AC3174 treatment did not alter CAD severity as indicated by either wall coverage or percent plaque burden (Fig 3C-D). In vitro, histological examination of coronary arterial rings revealed no effect of AC3174 treatment on plaque burden (Fig 3E-F; I-J; H), collagen deposition (Fig 3G; K-L).

**Effect of chronic, in vivo GLP-1 receptor agonist treatment on CSM Ca^{2+} handling in Met-S induced CAD.**

We have previously demonstrated alterations in coronary smooth muscle (CSM) Ca^{2+} handling in MetS-induced CAD (16;17;21). We therefore investigated whether chronic, in vivo AC3174 treatment resulted in improved intracellular Ca^{2+} handling in CSM. Fig. 4A shows the Ca^{2+} signaling protocol to assess baseline Ca^{2+} levels, sarcoplasmic reticulum (SR) Ca^{2+} store release assessed by the caffeine-induced Ca^{2+} peak in the absence of extracellular Ca^{2+}, and SERCA activity assessed by subsequent recovery of cytosolic Ca^{2+} levels and undershoot of cytosolic [Ca^{2+}] below baseline levels. Chronic AC3174 treatment did not alter intracellular Ca^{2+} handling in CSM from swine with MetS-induced CAD (Fig 4B-D).

**Effect of acute, in vitro exenatide treatment on CSM in MetS and CAD**

Further, we assessed the acute, direct effect of GLP-1 receptor agonists on Ca^{2+} handling in CSM isolated from swine with MetS and CAD. Cells were exposed to the GLP-1 receptor agonist, exenatide (100 nM), which has an identical pharmacological profile as AC3174 (4) (Fig. 4A) for three minutes
prior to treatment with high K+. We again assessed caffeine-induced SR Ca\textsuperscript{2+} store release by peak Ca\textsuperscript{2+} response to caffeine and SERCA activity by the subsequent undershoot of cytosolic Ca\textsuperscript{2+} during recovery. Exenatide did not alter either SR Ca\textsuperscript{2+} store release or the undershoot of cytosolic Ca\textsuperscript{2+} below baseline (Fig 4E-F).

**Discussion**

The principle finding of this study is that MetS confers *vascular* resistance to the effects of GLP-1 receptor agonists. AC3174 did improve several cardiovascular risk factors, such as the metabolic factors, glucose tolerance and insulin secretion, indicating that longer term treatment may have indirect benefits on cardiovascular health. Treatment of MetS and type 2 diabetes is confounded by potential secondary and detrimental cardiovascular effects. Previous studies in lean, healthy animals indicate that GLP-1 receptor agonists provide a potential cardioprotective treatment for type 2 diabetes and MetS (9-11). In the current study, we examined possible Ca\textsuperscript{2+} regulatory mechanisms for GLP-1 receptor agonist action in CSM from lean, healthy Ossabaw swine. Our finding that GLP-1 receptor agonists exert a positive effect on SERCA activity in coronary smooth muscle from lean Ossabaw swine is in agreement with findings that GLP-1 receptor agonists enhance SERCA activity in other cell types, including endothelial cells (12), macrophages (13), and cardiomyocytes (14), and is the first study examining the effect of GLP-1 receptor agonists in CSM. The kinetics of the Ca\textsuperscript{2+} transient shown in Fig 1-E demonstrate a lack of exenatide effect on Ca\textsuperscript{2+} extrusion. We have previously shown these kinetic measurements to be an appropriate assay of Ca\textsuperscript{2+} extrusion (17). Reduced SERCA activity has been implicated in CSM proliferation and neointimal formation in the progression of CAD (22;23). One possible mechanism explaining this phenomenon is induction of endoplasmic reticulum (ER) stress. As the single means by which Ca\textsuperscript{2+} may enter the ER, SERCA is a crucial regulator of ER Ca\textsuperscript{2+} homeostasis, and SERCA inhibition elicits an ER stress response through depletion of ER Ca\textsuperscript{2+} (24). ER
stress is associated with development of atherosclerosis (13). Inhibition of ER stress through heightened activation of SERCA could provide a novel means by which to treat CAD.

We also examined the effect of GLP-1 receptor agonists on CAD progression in MetS. This study was needed because of recent evidence of MetS-induced resistance to the cardioprotective effects of GLP-1 (15). The improvement of systemic glucoregulation with AC3174 treatment provides essential positive evidence for GLP-1 receptor agonist action in the Ossabaw swine model of MetS and CAD. Further, we demonstrated that GLP-1 receptor agonist, AC3174 has no effect on MetS-induced CAD. This is in contrast with the recent finding that GLP-1 prevents myocardial ischemia-reperfusion injury (9) and injury-induced neointimal hyperplasia (11), providing evidence that the MetS phenotype itself confers cardiovascular resistance to the beneficial effects of GLP-1. It is important to note that, while AC3174 treatment did not attenuate CAD progression, the absence of adverse effects of AC3174 provide a cardiovascular safety profile for GLP-1 receptor agonists.

Additionally, we examined the acute, direct effect of GLP-1 receptor agonists on intracellular Ca$^{2+}$ regulation in CSM from Ossabaw swine with MetS and CAD who had not received any treatment with GLP-1 receptor agonists. Here, we found that exenatide had no effect on Ca$^{2+}$ regulation in CSM from swine with MetS and CAD. This finding is in contrast with other studies in which GLP-1 receptor agonism resulted in increased SERCA activity (12-14). A study in humans with type 2 diabetes revealed that exenatide treatment enhanced endothelial-dependent vasodilation (25), although it is important to note that blood cholesterol levels were controlled in these patients. The current study, revealing a lack of effect of GLP-1 receptor agonism on SERCA activity in the setting of MetS corroborates the earlier finding that MetS ablated the effect of GLP-1 receptor agonists in myocardium and highlights the need for additional studies to investigate specific aspects of MetS (obesity, hyperinsulinemia, glucose
intolerance, hypertension, and dyslipidemia) that may underlie resistance of the coronary vasculature to GLP-1 receptor agonists.

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S.L.D. researched and evaluated data and wrote the manuscript. M.L.M. researched data and reviewed and edited the manuscript. L.N.B. and A.F. researched and organized data and reviewed the manuscript. K.A.S. researched data and reviewed the manuscript. M.A., N.C., and M.S. conceptualized the chronic, in vivo study, researched data, and reviewed and edited the manuscript. M.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Figure Legends**

**Figure 1:** Effect of acute exenatide treatment on CSM Ca$^{2+}$ handling in CSM isolated from lean, healthy Ossabaw swine. A) Representative tracing of data from Ca$^{2+}$ imaging protocol. Horizontal dashed line indicates baseline. (Control n = 114 CSM; Exen n = 82 CSM; CPA n = 68 CSM) B) Resting cytosolic Ca$^{2+}$. C) Area under the curve (AUC) for Ca$^{2+}$ influx during high K$^+$, corresponding to the shaded region in panel A. D) Undershoot of Ca$^{2+}$ below resting levels, corresponding to the black vertical arrow in panel A. E) Time to half ($t_{50}$) and total ($t_{100}$) recovery from caffeine-induced rise in cytosolic Ca$^{2+}$, corresponding to the horizontal black arrows in panel A.

**Figure 2:** AC3174 attenuates weight gain and improves glucose handling in Ossabaw miniature swine. Closed circles = placebo treatment group. Open circles = AC3174 treatment group. A) Weight gain in Ossabaw miniature swine. Inset timeline indicates time on placebo or AC3174 treatment, which corresponds with times in B-D and Table S1. B-D) Time course of plasma glucose responses during IVGTT at B) beginning, C) 12 weeks, and D) 24 weeks of AC3174 treatment. E-G) Time course of plasma insulin responses during IVGTT at E) beginning, F) 12 weeks, and G) 24 weeks of AC3174 treatment.

**Figure 3:** AC3174 treatment does not attenuate CAD progression as measured by intravascular ultrasound (IVUS) and histology. A) IVUS image of artery with CAD. Lumen traced in white dashed line. L = Lumen, C = Catheter. Distance between dots is 1 mm. Lumen traced in white dotted line. Arterial wall traced in white dashed line. Atherosclerotic plaque is indicated between dotted and dashed lines. B) The heart and major epicardial coronary arteries. RC = right coronary artery, LAD = left anterior descending, CFX = circumflex. The RC and CFX on the anterior aspect of the heart are shown.
by the solid lines and the arteries traversing to the posterior aspect of the heart are shown by the dashed lines. The IVUS catheter is shown in the RC positioned for a pullback. C,D) Quantification of IVUS pullbacks performed in the RC in both placebo (n = 6) and AC3174-treated (n = 3) swine. AC3174 did not attenuate CAD as measured by percent plaque burden (panel C) or percent wall coverage (panel D).

**E-G; I-K:** Coronary arterial rings from placebo (n = 4; panels E-G) and AC3174-treated (n = 5; panels I-K) swine. In panels F and J: A = Adventitia; I = Intima; L = Lumen; M = Media. A,E) Coronary artery rings stained with hematoxylin & eosin (H&E). F,J) Coronary artery rings stained with Verhoff-van Gieson (VVG) stain for elastin. An overt atherosclerotic plaque is traced in panel H. G, K) Coronary artery rings stained with Masson’s trichrome (TC) for collagen. H) Graphical representation of plaque burden, (p = 0.35) L) Graphical representation of total collagen area, (p = 0.79).

**Figure 4: Effect of GLP-1R agonists on CSM Ca^{2+} handling in MetS Ossabaw swine.** A) Representative tracing of data from Ca^{2+} imaging protocol. Dashed line indicates baseline. B-D) Effect of chronic *in vivo* AC3174 treatment on CSM Ca^{2+} handling. (Placebo n = 4 swine; AC3174 n = 6 swine) B) Resting cytosolic Ca^{2+} levels. C) Caffeine-induced SR Ca^{2+} store release. Corresponds to brackets in panel A. D) Undershoot of cytosolic Ca^{2+} levels below baseline. Corresponds to black arrow in panel A. E-F) Effect of acute *ex vivo* exenatide treatment on Ca^{2+} handling in CSM from MetS Ossabaw swine. (0Ca n = 38 CSM; Exen n = 24 CSM) E) Caffeine-induced SR Ca^{2+} store release. Corresponds to brackets in panel A. F) Undershoot of cytosolic Ca^{2+} levels below baseline. Corresponds to black arrow in panel.
Data Supplement

Animals.

Coronary artery disease (CAD) and metabolic syndrome (MetS) were induced in both treatment groups by feeding, once daily for 6 months, 1 kg of atherogenic diet, containing 16% kcal from protein, 41% kcal from complex carbohydrates, 19% kcal from fructose, and 43% kcal from fat, and supplemented with 2.0% cholesterol and 0.7% sodium cholate by weight (KT324, Purina Test Diet, Richmond, IN), as previously described (1-3). Following 6 months on this diet, swine were placed on placebo or AC3174 treatment twice daily and feeding was altered to 0.5 kg twice daily for an additional six months. Water was provided ad libitum. Six lean, age-matched Ossabaw miniature swine fed a standard chow diet (5L80, Purina Lab Diet, Richmond, IN) were a control group to verify presence of MetS. Coronary smooth muscle (CSM) cells were isolated from additional lean and MetS swine for acute, in vitro assessment of GLP-1 receptor agonist action on intracellular Ca^{2+} handling (see acute in vitro exenatide treatment methods).

Measurement of intracellular Ca^{2+} levels.

CSM were loaded with the fluorescent intracellular Ca^{2+} indicator, fura-2/AM. Fura-2 loaded cells were placed on a coverslip contained in a constant-flow superfusion chamber mounted on an inverted epifluorescence microscope (model TMS-F, Nikon, Melville, NY), with flow maintained at 1-2 ml/min. Whole cell intracellular Ca^{2+} levels were assessed as the 360 nm/380 nm excitation ratio of the fura-2 emission at 510 nm at room temperature (22-25 °C), using the InCa++ Ca^{2+} Imaging System (Intracellular Imaging, Cincinnati, OH) as previously described (1;2;4-10). Basal Ca^{2+} levels were measured in physiologic salt solution (PSS) composed of the following (in mM): 2 CaCl_{2}, 138 NaCl, 1 MgCl_{2}, 5 KCl, 10 HEPES, 10 glucose; pH 7.4). Voltage-gated calcium channels were activated by depolarization with high (80 mM) K^{+} solution (2 CaCl_{2}, 63 NaCl, 1 MgCl, 80 KCl, 10 HEPES, 10 glucose; pH 7.4). Sarcoplasmic reticulum (SR) Ca^{2+} stores were released with 5 mM caffeine in Ca^{2+}-free solution (138 NaCl, 1 MgCl_{2}, 5 KCl,
Intravenous glucose tolerance testing.

Briefly, swine were pre-acclimated to sling-restraint. Following an overnight fast, swine were restrained in the low stress sling and baseline blood samples and tail-cuff blood pressures were obtained. Next, glucose (1 g/kg body weight) was administered intravenously as a bolus and timed blood samples were collected. Blood glucose was measured immediately (YSI 2300 STAT Plus Glucose analyzer, YSI Life Sciences, Yellow Springs, OH). Plasma insulin assays were performed offsite (Millipore, Inc., St Charles, MO).

Intravascular ultrasound.

After 12 months on diet, following an overnight fast, swine received 2.2 mg/kg xylazine and 5.5 mg/kg telazol, similar to previous reports (1-3;5;11;12). Swine were intubated and anesthesia was maintained at 2-4% isoflurane in 100% O₂ as a carrier gas. A 7 F introducer sheath was inserted into the right femoral artery and heparin (200 U/kg) was administered. A 7 F guiding catheter (Amplatz L, sizes 0.75-2.0; Corndis, Bridgewater, NJ) was advanced to engage either the right or left coronary ostium. A 3.2 F, 40 MHz IVUS catheter (Boston Scientific, Natick, MA) was advanced over a guide wire and positioned in the coronary artery. Automated IVUS pullbacks were performed at 0.5 mm/sec. Angiography was performed throughout the procedure to assist in catheter placement.
Figure S1: Liraglutide increases SERCA activity in CSM from lean, healthy Ossabaw Swine, and this effect is prevented in the presence of GLP-1 receptor antagonist, Exendin (9-39).
Table S1: Phenotypic Characteristics of Ossabaw Swine Groups.

| Parameter                          | Lean  | Placebo | AC3174 | Significance, * (p < 0.05) |
|------------------------------------|-------|---------|--------|----------------------------|
| **Week on Treatment**              | 0     | 12      | 24     | 0                          | 12                          | 24                          |
| **Body Weight**                    | 61 ± 6| 75 ± 3  | 100 ± 2*| 73 ± 4                     | 87 ± 5                      | 100 ± 6*                    |
| **Blood Pressure**                 |       |         |        |                            |                             |                             |
| **Systolic (mmHg)**                | 143 ± 8| 149 ± 8| 151 ± 4| 170 ± 7                    | 132 ± 5                     | 149 ± 6                     | 152 ± 6                     | None                       |
| **Diastolic (mmHg)**               | 76 ± 5| 79 ± 5  | 84 ± 3 | 89 ± 5                     | 74 ± 4                      | 82 ± 5                      | 85 ± 4                      | None                       |
| **Mean Arterial Pressure (mmHg)**  | 98 ± 5| 102 ± 5 | 106 ± 2| 116 ± 5                    | 93 ± 4                      | 104 ± 5                     | 107 ± 4                     | None                       |
| **Carbohydrate Metabolism**        |       |         |        |                            |                             |                             |                             |                           |
| **Fasting plasma glucose (mg/dL)** | 77 ± 5| 74 ± 2  | 78 ± 2 | 81 ± 2                     | 72 ± 3                      | 73 ± 3                      | 68 ± 2                      | None                       |
| **Peak plasma insulin (µU/dL)**    | 88 ± 33| 47 ± 17 | 47 ± 6 | 67 ± 12                    | 46 ± 10                     | 139 ± 29*                   | 122 ± 25*                   | Placebo < AC3174           |
| **Lipids**                         |       |         |        |                            |                             |                             |                             |                           |
| **Total cholesterol (mg/dL)**      | 66 ± 10| 430 ± 95| 420 ± 68| 240 ± 20                  | 460 ± 78                    | 369 ± 65                    | 354 ± 61                    | Lean < Placebo, AC3174     |
| **Triglycerides (mg/dL)**          | 29 ± 5| 67 ± 16 | 46 ± 6 | 41 ± 4*                    | 60 ± 6                      | 64 ± 13                     | 74 ± 7*                     | Lean < Placebo < AC3174    |
| **Kidney Function**                |       |         |        |                            |                             |                             |                             |                           |
| **BUN (mg/dL)**                    | 13 ± 1.0| 15 ± 1.4| 13 ± 0.9| 13 ± 1.2                  | 16 ± 1.2                    | 14 ± 1.4                    | 15 ± 1.2                    | None                       |
| **Creatinine (mg/dL)**             | 1.2 ± 0.09| 1 ± 0.04| 0.9 ± 0.05| 1 ± 0.04                  | 1.1 ± 0.09                  | 1 ± 0.07                    | 1 ± 0.04                    | None                       |
| **Plasma Proteins**                |       |         |        |                            |                             |                             |                             |                           |
| Protein (g/dL)           | 7.1 ± 0.2 | 6.2 ± 0.4 | 6.7 ± 0.2 | 6.8 ± 0.2 | 6.4 ± 0.4 | 6.8 ± 0.7 | 6.6 ± 0.2 | None |
|-------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Albumin (g/dL)          | 4 ± 0.2   | 3.6 ± 0.1 | 3.6 ± 0.1 | 3.7 ± 0.2 | 3.7 ± 0.1 | 3.9 ± 0.1 | 3.8 ± 0.1 | None |
| Globulin (g/dL)         | 3.3 ± 0.2 | 2.9 ± 0.1 | 3.1 ± 0.2 | 3.1 ± 0.1 | 2.8 ± 0.2 | 2.9 ± 0.1 | 2.8 ± 0.1 | None |

**Electrolytes**

| Phosphorus (mg/dL)     | 5.8 ± 0.2 | 7 ± 0.1*  | 6.7 ± 0.2 | 6.2 ± 0.2 | 6.7 ± 0.2* | 6.1 ± 0.2* | 6 ± 0.2  | Lean < Placebo > AC3174 |
|------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Calcium (mg/dL)        | 10.3 ± 0.1| 9.7 ± 0.2 | 9.4 ± 0.2 | 9.5 ± 0.3 | 9.7 ± 0.3 | 9.8 ± 0.3 | 9.5 ± 0.2 | None |
| Magnesium (mEq/L)      | 1.6 ± 0.1 | 1.8 ± 0.1 | 1.7 ± 0.1 | 1.6 ± 0.1 | 1.7 ± 0.1 | 1.7 ± 0.1 | 1.6 ± 0.1 | None |
| Sodium (mEq/L)         | 140 ± 1   | 140 ± 1   | 137 ± 2   | 139 ± 1   | 141 ± 1   | 137 ± 2   | 138 ± 2   | None |
| Potassium (mEq/L)      | 4.3 ± 0.3 | 4.2 ± 0.1 | 4.3 ± 0.1 | 4 ± 0.1   | 4.3 ± 0.1 | 4.2 ± 0.1 | 3.9 ± 0.1 | None |
| Chloride (mEq/L)       | 102 ± 1   | 99 ± 1    | 97 ± 1    | 106 ± 8   | 100 ± 1   | 97 ± 1    | 98 ± 1    | None |

**Other**

| Creatine Phosphokinase | 537 ± 225 | 422 ± 40 | 502 ± 56 | 448 ± 54 | 364 ± 54 | 410 ± 72 | 645 ± 321 | None |

Lean control; Placebo = MetS + Placebo treatment; AC3174 = MetS + AC3174 treatment.
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