Metformin and Metabolic Syndrome Attenuate Autophagy in Myocardium

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Abstract

Objective: Autophagy is a highly conserved cellular process by which damaged organelles and proteins are degraded, and is critical for cell survival under conditions of stress such as starvation or ischemia. Given the possible role of autophagy in cardio protection, we developed a swine model of metabolic syndrome and chronic myocardial ischemia to investigate the effects of metformin on myocardial autophagy.

Methods: Ossabaw pigs were fed a regular diet (OC, n=8), or a hypercaloric, diet (OHC, n=16) to induce metabolic syndrome. After nine weeks, all animals underwent placement of an ameroid constritor to induce chronic myocardial ischemia and collateral myocardial circulation. OHC animals were split into hypercaloric diet alone (OHC, n=8) or hypercaloric diet supplemented with metformin (OHCM, n=8). After seven weeks, the myocardium was harvested.

Results: In the collateral-dependent myocardium, there was down-regulation of markers of autophagy including pMTOR, LC3a, LC3b and LAMP1, and up-regulation of autophagy flux marker p62 in the OHCM group compared to OC. In the normally perfused myocardium, there was down-regulation of markers pP70S6K, pULK1, LC3b, and LAMP1 and up-regulation of p62 in both OHC and OHCM compared to OC. There was further down-regulation of autophagy markers MTOR, Beclin1, ATG5, LC3a and LAMP1 in the OHCM group compared to OHC and OC.

Conclusions: Animals with metabolic syndrome had significant autophagy down-regulation in both the collateral dependent and normally perfused myocardium, suggesting that metabolic syndrome inhibition of autophagy is not reversed with metformin.

ABBREVIATIONS

OC: Ossabaw Control; OHC: Ossabaw Hyper Cholesterolemic Diet; OHCM: Ossabaw Hyper Cholesterolemic Diet + Metformin; pMTOR: Phosphorylated Mammalian Target of Rapamycin; MTOR: Mammalian Target of Rapamycin; PAMPK: Phosphorylated AMP-Activated Protein Kinase; AMPK: AMP-Activated Protein Kinase; ATG5: Autophagy Related Protein 5; LC3a: Microtubule-Associated Protein 1 Light Chain 3b; LC3b: Microtubule-Associated Protein 1 Light Chain 3b; LAMP1: Lysosomal-Associated Membrane Protein 1; LAMP2: Lysosomal-Associated Membrane Protein 2; pP70S6K: Phosphorylated S6 Ribosomal Protein Kinase; p70S6K: S6 Ribosomal Protein Kinase; p62: Ubiquitin Binding Protein p62; pULK 1/2: Phosphorylated Unc-51 Like Autophagy Activating Kinase½; ULK 1/2: Unc-51 Like Autophagy Activating Kinase½

INTRODUCTION

Autophagy is an evolutionarily conserved mechanism to degrade long-lived or damaged proteins and organelles, and is critical for cellular homeostasis. Autophagy occurs constitutively at low levels for routine turnover, and can be up-regulated under conditions of cellular stress, such as hypoxia or starvation, or down-regulated in a well fed state [1,2]. Both up-regulation and down-regulation of autophagy is in part regulated by the insulin signaling pathway via AMPK and MTOR, which suggests that perhaps excessive caloric intake, causes maladaptive autophagy suppression, and contributes to the deleterious effects of key components of metabolic syndrome including obesity and...
Autophagy can promote cell survival by generating amino acids from proteins and organelle degradation, or by sequestering damaged mitochondria to prevent stress induced mPTP pore opening and subsequent cell death [4,5]. In the heart, up-regulation of autophagy has been shown to be cardio protective, whereas inhibition of autophagy results in loss of cardio protection, suggesting that autophagy is an integral component to cardiac recovery after an ischemic insult [3]. This observation in animal studies has been confirmed in humans: in patients undergoing coronary artery bypass or valve surgery requiring cardiopulmonary bypass, autophagic flux correlated with cross-clamp time and was inversely related to morbidity and mortality risk scores [6].

The possibility of pharmacologically optimizing adaptive autophagy is an increasingly appealing strategy to protect against myocardial ischemia-reperfusion injury. Metformin is an orally administered biguanide frequently prescribed for the treatment of type 2 diabetes mellitus. Metformin reduces blood glucose by increasing glucose uptake and reducing hepatic glucose production. In addition to its glucose lowering effects, studies have shown that metformin also has direct cardio protective properties and that patients treated with metformin had decreased all-cause mortality [7,8]. In animal models of myocardial infarction, metformin has been shown to preserve myocardial function, reduce infarct size, and reduce the development of heart failure after myocardial infarction [9]. In our lab, we demonstrated that metformin supplementation in a swine model of chronic myocardial ischemia and metabolic syndrome resulted in reversal of diet-induced hypertension and glucose intolerance [10]. In the same animal model, we found that metformin decreased cell death and mitigated apoptosis signaling in the collateral-dependent myocardium [11]. We also demonstrated that metformin supplementation up-regulated the insulin signaling pathway in collateral-dependent myocardium, which is at the crossroads of known metabolic and survival benefits of metformin [12].

Given metformin’s cardio protective properties, we sought to investigate the hypothesis that metformin would increase autophagy in collateral-dependent myocardium in the setting of metabolic syndrome.

MATERIALS AND METHODS

Animal model

Twenty-four intact male Ossabaw miniswine (Purdue Ossabaw Facility, Indiana University, and Indianapolis, IN) were split into three groups according to diet at 6 weeks of age. The control group was fed 500g/day of regular chow (OC, n=8). The high-cholesterol animals were fed 500g/day of high-cholesterol chow consisting of 4% cholesterol, 17.2% coconut oil, 2.3% corn oil, 1.5% sodium cholate, and 75% regular chow (Sinclair Research, Columbia, MO) (OHC, n=8). High cholesterol metformin animals were also fed high-cholesterol chow (OHC M, n=8). After 9 weeks of diet initiation, all animals underwent surgical placement of an ameroid constrictor to induce chronic myocardial ischemia (see surgical interventions). Postoperatively, the OHC M group was supplemented with 500mg metformin orally twice daily and all animals continued on their respective diets. The dose in this study was selected based on metformin dosing in adult males. Typically, a 70kg male would be prescribed 1g metformin twice daily. Since the animals in this study were approximately 35kg at the time of the harvest, we selected half the dose that would prescribed to a 70kg male, 500mg metformin twice daily.

Seven weeks after ameroid constrictor placement, all animals underwent euthanasia and cardiac tissue harvest. All animals were observed to ensure complete consumption of food and supplement, had unlimited access to water, and were housed in a warm non-stressful environment for the duration of the experiment.

Surgical interventions

Anesthesia was induced with an intramuscular injection of telazol (4.4 mg/kg). Animals were endotracheally intubated, mechanically ventilated at 12-20 breaths per minute, and general anesthesia was maintained with a gas mixture of oxygen at 1.5-2 liters/min and isoflurane at 0.75-3.0% concentration.

Ameroid constrictor placement

Animals were given a single dose of antibiotic prophylaxis, intravenous enrofloxacin 5mg/kg, and general anesthesia was induced and maintained. Animals were prepped and draped in the usual sterile fashion. The heart was exposed through a left mini-thoracotomy. The left atrial appendage was retracted and the proximal left circumflex artery was dissected at the take off of the left main coronary artery. The ameroid constrictor was placed around the left circumflex artery (Research Instruments NW, Escondido, CA). The pericardium was loosely re-approximated followed by a layered closure of the surgical incision. Post-operative pain was controlled with a single dose of intramuscular buprenorphine (0.03 mg/kg) and 72-hour fentanyl patch (4µg/kg). All animals received 325mg of aspirin daily for thromboembolic prophylaxis starting 1 day pre-operatively and continuing for a total of 5 days. All animals continued perioperative antibiotics: enrofloxacin 68mg orally daily for 5 days.

Cardiac harvest

Under general anesthesia, the heart was exposed via a median sternotomy and animals were euthanized by exsanguination. Of note, prior to euthanasia and harvest, myocardial perfusion was measured by injecting isotope-labeled microspheres at rest and with demand pacing at 150 beats per minute.

Cardiac tissue from the collateral-dependent myocardium and normally perfused myocardium was collected for further analysis. The hearts were sectioned in a systematic manner to ensure that the same part of the heart was sampled in every animal. The normally perfused myocardium was consistently sampled from the left ventricle in the left anterior descending coronary artery territory, and the collateral-dependent myocardium was sampled from the left circumflex coronary artery territory distal to the ameroid constrictor. After myocardial tissue sectioning, the samples were immediately frozen in liquid nitrogen.

The Institutional Animal Care and Use Committee of the Rhode Island Hospital approved all experiments. Animals were cared for in compliance with the “Principles of Laboratory Animal
Protein expression

Forty micrograms of the Radio-Immuno precipitation Assay (Boston Bio Products, Ashland, MA) soluble fraction of myocardial lysates from each animal were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis 3-8% Tris-acetate gel (NuPage Novex Mini Gel, Invitrogen, Carlsbad, CA) for molecular weight targets >100 kilodaltons and 4-12% Bis-Tris gels for molecular weight targets <100 kilodaltons (NuPage Novex Mini Gel, Invitrogen). The protein was then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) and incubated overnight at 4°C with primary antibodies at dilutions recommended by the manufacturer against phosphorylated mammalian target of rapamycin (pMTOR Ser2481), MTOR, phosphorylated AMP-activated protein kinase (pAMPKThr 172), AMPK, LAMP1, phosphorylated P70S6K (pP70S6K Ser371), ATG5, Beclin1, phosphorylated ULK1 (pULK1 Ser623), LC3a, LC3b, p62 (all from Cell Signaling, Danvers, MA) and LAMP2 (Invitrogen, Carmillo, CA). Membranes were incubated with the appropriate horseradish peroxidase-linked secondary antibody for one hour at room temperature (Jackson Immuno Research, West Grove, PA). Immune complexes were visualized with enhanced chemiluminescense and images were captured with a digital camera system (G-Box, Syngene, and Cambridge, England). Band densitometry was quantified as arbitrary light units using Image-J software (National Institutes of Health, Bethesda, MD). All membranes were probed with GAPDH (Cell Signaling) to correct for loading error. Two representative bands from each of the three animal groups were selected using Photoshop (Adobe, San Jose, CA) and displayed in tabular form.

Data analysis

All results are reported as mean ± standard error of the mean. A one-way ANOVA was used to compare the means among groups followed by a post-hoc Bonferroni test to compare the means between groups using Graph Pad Prism 5.0 Software (Graph Pad Software Inc., San Diego, CA). Protein expression is reported as fold change compared to OC.

RESULTS

Animal model

All animals included in the analysis survived the entire experiment. One animal in the OC group and 1 in the OHC group died postoperatively. The first animal died 8 days postoperatively and the second 2 days postoperatively. Although necropsy did not reveal a clear cause of death, it was assumed that the animals died after an acute arrhythmia leading to sudden cardiac death. One animal in the OHCM group had a prolonged myocardial infarction at the time of ameroid placement and was subsequently euthanized. The animals that did not survive to completion of the experiment were excluded from analysis and replaced with new animals. We previously reported that there was no difference in myocardial perfusion, contractility, capillary density, or arteriolar density in the normally perfused or collateral dependent myocardium [10]. We also reported that animals fed a high fat diet in the OHC group developed obesity, hypertension, dyslipidemia and glucose intolerance, which are all components of metabolic syndrome [10]. Animals that were supplemented with metformin had reversal of the diet induced hypertension and glucose intolerance.

Autophagy protein expression in the normally perfused myocardium

In the normally perfused myocardium, there was a significant down-regulation of autophagy markers MTOR, Beclin1, ATG5, LC3a, and LAMP1 in the OHCM animals compared to OHC and OC. There was up-regulation of AMPK in the OHCM group compared to OC. There was also a significant up-regulation of autophagy markers LAMP2, pP70S6K, and autophagy flux marker p62 expression in the high fat fed OHCM and OHC animals compared to OC. In the OHC animals, there was a significant up-regulation of autophagy markers LC3b and down-regulation of pULK1 compared to OC (Table 1).

Autophagy protein expression in collateral-dependent myocardium

In the collateral dependent myocardium, there was a significant down-regulation of autophagy markers pMTOR, LAMP1, and LC3a in the OHCM group compared to OC. Also, there was a significant down-regulation of autophagy marker LC3b expression in the high fat fed OHCM and OHC animals compared to OC. There was up-regulation of autophagy flux marker p62 in the OHCM group compared to OC and OHC. There was up-regulation of pAMPK in the OHCM group compared to OHC (Table 2).

DISCUSSION

Autophagy is a mechanism to degrade oxidized or aggregated proteins and organelles to preserve optimal cell function. This process is of utmost importance in long-lived, terminally differentiated cells, such as neurons or cardiomyocytes. This is the first study in a large animal model to investigate the effects of metformin and metabolic syndrome on autophagy in the heart. In this clinically relevant animal model of chronic myocardial ischemia and metabolic syndrome, we previously reported that although metformin supplementation did not affect myocardial perfusion or function [10], there was a significant decrease in cardiomyocyte apoptosis and attenuation of apoptosis signaling [11]. Given the changes in apoptosis signaling and cell death in the myocardium, we sought to investigate the effects of metformin on autophagy. We report that metformin supplementation significantly decreased markers of autophagy in both the ischemic myocardium and normally perfused, non-ischemic myocardium.

Autophagy activation begins with the formation of the phagophore, which is a double-membrane structure that is believed to originate from the endoplasmic reticulum and other membrane organelles. The phagophore elongates and gradually surrounds and sequesters cytoplasmic material including proteins and organelles targeted for degradation. The phagophore fuses at the edges forming a double-membrane vesicle (autophagosome). The autophagosome goes through a process of maturation whereby it fuses with the lysosome to create a single membrane autophagolysosome. The inner membrane of the autophagosome...
and its contents are degraded into amino-acids, lipids and sugars (Figure 1) [13].

Under nutrient-rich conditions, the insulin signaling pathway is activated, which activates MTOR, a potent inhibitor of autophagy. MTOR stimulates protein synthesis and suppresses autophagy by inhibiting ULK 1 and 2, which control early autophagosome formation. However, under conditions of cell stress, such as ischemia or starvation, or with metformin treatment, the ratio of AMP to ATP is increased, which activates AMP-activated protein kinase (AMPK) [14,15]. AMPK acts as a cellular energy sensor, and when activated it phosphorylates and activates ULK 1 and 2 and inactivates MTOR [16]. In this study, we found that there was significant

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Data represented as fold change with respect to OC. (*) Indicates significant difference between OC and OHCM groups. (¶) Indicates significant difference between OC and OHC groups. (§) indicates significant difference between OHC and OHCM groups.

**Abbreviations:** OC: Ossabaw Control; OHC: Ossabaw High Cholesterol Diet; OHCM: Ossabaw High Cholesterol diet supplemented with metformin (OHCM), MTOR: Mammalian target of Rapamycin, pMTOR: Phosphorylated Mammalian Target of Rapamycin; AMPK: AMP-Activated Protein Kinase, pAMPK: Phosphorylated AMP-Activated Protein Kinase, pP70S6K: Phosphorylated S6 Ribosomal Protein Kinase, pULK1: Phosphorylated Unc-51 Like Autophagy Activating Kinase 1/2, ATG5: Autophagy Related Protein 5, LC3a/b: Microtubule-Associated Protein 1 Light Chain 3 a/b, LAMP1/2: Lysosomal-Associated Membrane Protein 1/2, p62: Ubiquitin Binding Protein p62.

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up-regulation of AMPK and pAMPK and down-regulation of MTOR and pMTOR in the non-ischemic and ischemic myocardium respectively. This result was expected given metformin’s known mechanism of action. It is interesting that metformin increased AMPK activation (pAMPK) in Ossabaw miniature swine, which have impaired AMPK function due to a gene mutation [17]. Furthermore, we found that there was up-regulation of pP70S6K in the non-ischemic myocardium, which is also expected since both AMPK and pP70S6K inhibit MTOR activity. We found that despite the up-regulation of two inhibitors of MTOR activity, AMPK and pP70S6K, and down-regulation of MTOR expression in the myocardium, there was still decreased expression of pULK1.

This suggests that in the setting of metabolic syndrome, there is an alternate pathway to inhibit autophagy that is independent of MTOR activity (Figure 2).

Once autophagy is activated, Beclin1 triggers downstream formation of the ATG5/ATG12 complex on the phagophore and mediate phagophore elongation. When the ATG 5/ATG12 complex is formed, LC3 is cleaved and modified to LC3-II, which facilitates linkage between the targeted cargo and autophagy machinery. The phagophore closes forming the autophagosome. Finally, LAMP1 and LAMP2 are recruited to facilitate the fusion between the autophagosome and lysosome to form the autophagolysosome [18]. In this study, we found that there was a marked decrease in autophagy markers LC3a, LC3b and LAMP1 in the ischemic myocardium and decrease in autophagy markers Beclin1, ATG5, LC3a, and LAMP1 in the non-ischemic myocardium in the OHCM group compared to OC. Although there was an increase in expression of LAMP2 and LC3b in the OHC and OHCM group, the overall trend of the rest of the markers of autophagy and autophagy flux indicates that there is a decrease in autophagy in the OHC and OHCM groups.

The results in this study suggest that metformin inhibits autophagy in metabolic syndrome. In fact, metformin further attenuated the metabolic syndrome induced autophagy inhibition. We demonstrated that in both the ischemic and non-ischemic myocardium, there was an increase in p62 in the OHCM group compared to OC and OHC. p62 is a key autophagy protein that links ubiquitin-rich mitochondria and protein aggregates to the forming autophagosome. When autophagy is inhibited, autophagosomes build up and there is an increase in total p62 levels. When autophagy is activated, p62 and its associated damaged mitochondria and protein aggregates are degraded and p62 levels are decreased [19-21]. Therefore p62 is a useful marker to assess autophagic flux.

Limitations

Though this work represents the first study in a large animal model to investigate the effects of metformin and metabolic syndrome on autophagy in the heart, there are several limitations. Autophagy is a dynamic cellular process and we only investigated one dose of metformin at one time point. It would be useful to conduct a study with multiple doses of metformin at multiple time points to establish a dose-response curve and...
determine if there is a difference in autophagy regulation with chronic myocardial ischemia. Furthermore, we did not directly measure autophagy in this model. In this study, we utilized western blot to measure specific autophagy markers that are key to the formation of autophagolysosomes. Transmission electron microscopy is considered the gold standard for measuring autophagy in tissues; however it is highly subjective as it can be very difficult to distinguish between autophagosomes, autolysosomes and other cellular vesicles. Electron microscopy only gives a snapshot of autophagy at one time and does not measure autophagy flux. If there is an increase in autophagosomes, it may represent an increase in autophagy or a decrease in degradation and reduction in autophagy [22]. Therefore, we did not use electron microscopy or immunohistochemistry, which is subject to the same limitations to measure autophagy in this study. Unfortunately, many of the sophisticated molecular tools for objectively quantifying autophagy are only available in cell culture or select murine models [23-25].

CONCLUSION

Under conditions of stress, autophagy up-regulation is one mechanism to increase the availability of substrates used for energy production and cell remodeling in cardiomyocytes. Removal of damaged organelles and misfolded proteins through autophagy is important for efficient ATP production and cardiomyocyte contractility [26-27]. Stress-induced autophagy up-regulation is known as adaptive autophagy. However, in the setting of metabolic syndrome, there is sustained nutrient availability and autophagy is suppressed, which attenuates the heart’s ability to compensate and following an ischemic insult [28-29]. Consistent with this, our results demonstrate that animals in the high fat groups, OHC and OHCM, had a reduction in autophagy markers, and increase in autophagy flux marker p62. Surprisingly, there was a further reduction in autophagy in the metformin treated animals despite the metformin-mediated decrease in MTOR activation, and increase in AMPK and P70S6K expression that should increase autophagy. Thus metformin treatment did not reverse the maladaptive autophagy inhibition caused by metabolic syndrome. The paradoxical down-regulation of autophagy suggests that there may be an alternate mechanism for maladaptive autophagy suppression that is independent of MTOR [30]. Further investigation into the effects of metabolic syndrome and metformin on autophagy in the heart is warranted to elucidate the mechanism of maladaptive autophagy inhibition.

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