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Adiponectin partially rescues high glucose/high fat-induced impairment of mitochondrial biogenesis and function in a PGC-1α dependent manner

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Abstract. – OBJECTIVE: Plasma adiponectin (APN) levels are decreased in diabetic patients. Dysfunctional mitochondrial biogenesis is involved in type 2 diabetes (T2DM) pathogenesis, by unclear mechanisms. The present study determined (1) whether myocardial mitochondrial biogenesis was impaired in cardiomyocytes exposed to a high glucose/high fat (HGHF) medium (a T2DM in vitro model), (2) the effects of APN administration upon mitochondrial biogenesis in cardiomyocytes affected by HGHF incubation, and 3) the involved underlying mechanisms.

MATERIALS AND METHODS: Neonatal rat ventricular myocytes (NRVMs) were isolated and incubated in HGHF medium. Mitochondrial function was assessed by ATP content, and fluorescent microscopic analysis of myocardial apoptosis was determined by TUNEL staining and caspase-3 activity.

RESULTS: HGHF treatment reduced mitochondrial biogenesis, altered mitochondrial structure, and induced mitochondrial dysfunction in NRVMs. Administration of APN partially rescued these effects. However, siRNA-mediated knock-down of peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1α) significantly blocked the beneficial effects of APN in mitochondria and cardiomyocytes subjected to hypoxia/reoxygenation injury.

CONCLUSIONS: In the current study, we have provided the direct in vitro evidence that APN partially rescues HGHF-induced impairment of mitochondrial biogenesis and function via PGC-1α-mediated signaling.

Key Words:
Adiponectin, High glucose/high fat, Peroxisome proliferator-activated receptor-gamma coactivator-1alpha, Type 2 diabetes, Mitochondrial biogenesis.

Introduction

It is estimated approximately 29 million people in the United States have diabetes mellitus¹. About 90% of this population has type 2 diabetes (T2DM), characterized by insulin resistance. In 2010, diabetes was the seventh leading cause of death in the United States. A major complication associated with T2DM is ischemia heart disease (IHD), leading to significant morbidity and mortality in patients with obesity and T2DM². Despite marked technological advances in percutaneous coronary intervention (PCI) techniques, the cardiac recovery and prognosis of diabetic patients post myocardial infarction (MI) remains significantly worse compared to non-diabetic patients. Complete understanding of the underlying mechanisms responsible for the increased vulnerability of diabetic myocardial cells in response to ischemia/reperfusion injury remains largely elusive.

An adipocytokine secreted from adipose tissue, adiponectin (APN) is abundant in human circulating plasma. Several clinical trials demonstrate elevated circulating APN levels tend to be protective against T2DM and AMI³⁻⁵. A study of > 18,000 male patients demonstrated a strong negative correlation between plasma APN level and MI morbidity⁶. Moreover, data from Shibata et al. and our group demonstrated that myocardial infarction/reperfusion(MI/R) injury is markedly exacerbated in the APN-KO mouse, ameliorated by APN administration⁷⁻⁸. Together, these results suggest APN is cardioprotective, and reduced plasma APN concentration may contribute to T2DM development. The mechanisms respon-
sible for hypoadiponectinemia-mediated T2DM development are likely complex and have not been fully investigated.

The mitochondria are intracellular centers regulating cellular energy metabolism and apoptosis. Mitochondrial dysfunction has been demonstrated in T2DM patients. Self-renewal via mitochondrial biogenesis helps maintain mitochondrial integrity. Interruptions in the self-renewal process have been implicated in multiple diseases, including aging, neurodegeneration and T2DM. Dysfunctional mitochondrial biogenesis impairs enzyme activity and increases intra-myocellular lipid (IMCL) levels, stimulating insulin resistance leading to and exacerbating type 2 diabetes. Recent studies demonstrate the involvement of APN signaling in mitochondrial biogenesis regulation in skeletal muscle. However, definitive evidence linking impaired myocardial mitochondrial biogenesis with enhanced MI/R injury in T2DM remains lacking. Therefore, the aims of the present study were 1) to determine whether T2DM may impair myocardial mitochondrial biogenesis in vitro, and if so, 2) to determine whether APN can exert cardioprotection by attenuating impaired mitochondrial biogenesis in type 2 diabetic heart, and 3) to determine the involved underlying mechanisms.

Materials and Methods

Isolation and Culture of Cardiomyocytes

Primary neonatal rat ventricular myocytes (NRVMs) were obtained from 1-3 day old neonatal Sprague-Dawley rats, and cultured as previously described. NRVMs were plated at density 6.6×10⁴ cells/cm² in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) with 0.1 mM 5-bromo-2-deoxyuridine, 2 mM L-glutamine, and penicillin-streptomycin. The purity of these cultures was > 98% cardiac myocytes, as demonstrated by positive staining for smooth muscle α-actin.

Real-time PCR

Total RNA was extracted from NRVM using TRizol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 1 μg RNA via reverse transcription reagent kit (DRR047A, TaKaRa, Otsu, Shiga, Japan). Expression analysis of the reported genes was performed by real-time PCR via commercial kit (DRR081A, TaKaRa, Otsu, Shiga, Japan) and the ABI 7500 Sequence Detection System (Foster City, CA, USA) using SYBR GREEN as a double-stranded DNA-specific dye. GAPDH served as endogenous control. The primers for mRNA expression analysis by real-time PCR are listed in Supplemental Table I.

Quantitative Real-time PCR for Mitochondrial DNA Content

To quantify mitochondrial DNA copy number per nuclear genome, ND-5 and GADPH served as respective markers for mitochondrial and nuclear DNA. DNA was extracted from NRVM by a QIAamp DNA mini kit (QIAGEN, Valencia, CA, USA). Rat ND-5 and GAPDH (sequences for probe and primers are listed in Supplemental Table I) were utilized as previously described. Primers were ordered from TaKaRa (Otsu, Shiga, Japan). The amplification and quantitation of mitochondrial DNA were accomplished by PCR detection kit (DRR039A, TaKaRa, Otsu, Shiga, Japan) and ABI 7500 Sequence Detection System.

Table I. Primers sequences for Real-time PCR analysis.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ppargc1a (mouse)</td>
<td>5’T – CACTGACAGATGGAGGCCGTGA – 3’</td>
</tr>
<tr>
<td>5’T – TGTTGGGCTGGTCCAGGTAAGAG – 3’</td>
<td></td>
</tr>
<tr>
<td>Nrf1 (mouse)</td>
<td>5’T – CATGCTTCAGAACTGCAACCA – 3’</td>
</tr>
<tr>
<td>5’T – GGTATCTTTACCACCCCTTGAAC – 3’</td>
<td></td>
</tr>
<tr>
<td>Tfam (mouse)</td>
<td>5’T – TCAAGGACGACACGACTA3’</td>
</tr>
<tr>
<td>5’T – CGAGTCTCCGAGTCTTGAACAC – 3’</td>
<td></td>
</tr>
<tr>
<td>Ppargc1a (Rat)</td>
<td>5’T – TCAAGAACAACCCCTGCAATTGA – 3’</td>
</tr>
<tr>
<td>5’T – AAGTCATTGTTTGGTCCAGATA – 3’</td>
<td></td>
</tr>
<tr>
<td>Tfam (Rat)</td>
<td>5’T – TGAAGCTTGAATACGGCTTGAAC – 3’</td>
</tr>
<tr>
<td>5’T – GAGATGTTCCTGCCCACACTTAC – 3’</td>
<td></td>
</tr>
<tr>
<td>GAPDH (Rat)</td>
<td>5’T – GCCACAGTCAAGGCTGAAATG – 3’</td>
</tr>
<tr>
<td>5’T – ATGTTGGTAAAGCCAGCAGTA – 3’</td>
<td></td>
</tr>
<tr>
<td>ND-5 (for mtDNA)</td>
<td>5’T – CCTATCACCTTGGCATTGTA3’</td>
</tr>
<tr>
<td>5’T – GAGGCTGTTGCTTGTGAC – 3’</td>
<td></td>
</tr>
</tbody>
</table>
Small Interfering RNA (siRNA) Sequences

siRNA against PGC-1α mRNA (21 nucleotide length) were designed and purchased from GenePharma (ShangHai, China). The sequences of siRNA are as follows: Rat PGC-1α, sense: 5’-GCUCUGAGAAUGGAUAUATT-3’, anti-sense: 5’-UAUACUCAUUCCAGACGTT-3’. Scrambled PGC-1α siRNA, sense: 5’-UUCUCCGAACGUGACACGUTT-3’, anti-sense: 5’-ACGUGACACGUUCGAGAATT-3’. NRVMs were transfected with siRNA by Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) per manufacturer’s instructions. The efficiency of gene knockdown was determined by real-time PCR 48 hours after siRNA transfection.

Assessment of Mitochondrial Function

Mitochondrial function was assessed by ATP content (via commercial assay kit S0026, Beyotime) and fluorescent microscopic analysis (via JC-1 Assay Kit C2005, Beyotime) per manufacturer’s instructions.

Determination of Myocardial Apoptosis

Myocardial apoptosis was determined by TUNEL staining and caspase-3 activity as previously described7.

Statistical Analysis

Data are presented as mean ± SEM. The 2-tailed Student’s t-test was employed for comparisons between 2 groups, and ANOVA (followed by Bonferoni correction for post-hoc t-test) was used when 3 or more groups were compared. p-values less than 0.05 were considered statistically significant.

Results

High glucose/High fat Treatment Decreased Mitochondrial Biogenesis in NRVMs.

Previous studies6,17 have demonstrated that mitochondrial biogenesis and function are significantly diminished in the fat and muscle tissue of obese animals. The mitochondria in ob/ob mice exhibit a global defect in oxidative phosphorylation, with reduced expression of complexes I, III, and V of the electron transport chain. Together, past data demonstrates myocardial mitochondrial impairment and dysfunction is present in ob/ob hearts. To exclude complex in vivo factors, we designed a series of in vitro experiments to determine whether T2DM may impair mitochondrial biogenesis. Neonatal rat ventricular myocytes (NRVMs) were isolated and cultured in medium containing excess glucose (concentration 25 mmol/L) and palmitate (16:0, a common saturated fatty acid, concentration 500 μmol/L). Treatment with this high glucose and high fat (HGHF) medium reduced mRNA levels of PPARGC1A and Nrf-1, important mediators of mitochondrial biogenesis and function, in a time-dependent manner in NRVMs (Figure 1). After

![Figure 1](image-url)

Figure 1. HGHF (high glucose/high fat, 25 mM glucose and 500M Sodium palmitate) treatment decreases mitochondrial biogenesis in neonatal rat ventricular myocytes (NRVMs). Real-time PCR analysis of Nrf-1 [A] and Pgargc-1a [B] mRNA levels in NRVMs. All values are presented as mean ± SEM. n=3-4. *, p < 0.05, **, p < 0.01. vs. Control group.
18 hours of HGHF exposure, mRNA levels of both PPARGC1A and Nrf-1 were significantly reduced. Based upon this time point result, all subsequent experiments concluded after 18 hours of HGHF incubation.

Mitochondrial Structure is Altered in NRVMs Treated with HGHF
To obtain direct evidence supporting the deleterious influence of HGHF upon NRVM mitochondrial biogenesis, transmission electron microscopy (TEM) was performed upon NRVMs subjected to 18 hours of incubation with HGHF. TEM revealed morphological mitochondrial defects, such as edema, as well as ambiguous and destroyed cristae (Figure 2B). Taken together, our quantitative and qualitative data suggest impaired mitochondrial biogenesis occurs in cardiomyocytes subjected to HGHF exposure.

Mitochondrial Dysfunction Occurs in NRVMs Subjected to HGHF Treatment
Having demonstrated that impaired mitochondrial biogenesis occurs in cardiomyocytes subjected to HGHF exposure, we next determined whether the mitochondrial function is affected by impaired mitochondrial biogenesis. We assessed mitochondrial membrane potential (MMP) via commercial JC-1 test kit. Neonatal rat cardiomyocytes incubated with HGHF for 18 hours exhibited decreased red fluorescence and increased green fluorescence in comparison to control ($p < 0.05$), indicating diminished mitochondria membrane potential and mitochondrial dysfunction (Figure 4).

Adiponectin Partially Rescues Impaired Mitochondrial Biogenesis Induced by HGHF Treatment in Cultured Cardiomyocytes
Our previous study demonstrated that plasma APN levels were markedly reduced in the ob/ob mice. To determine whether a causal relationship exists between reduced plasma APN and mitochondrial biogenesis impairment in cultured cardiomyocytes subjected to HGHF, we determined the effect of administering the globular domain of APN (gAd, 3 μg/mL) in NRVMs treated with HGHF. As summarized in Figure 3A-C, 18 hours of HGHF incubation significantly reduced the mRNA levels of Nrf-1, PPARGC1A, and Tfam (another key regulator of mitochondrial biogenesis) in NRVMs compared to control. Mitochondrial and nuclear DNA was extracted from NRVMs. Real-time PCR revealed that gAd administration increased the mitochondrial DNA copy number (mtDNA, Figure 3D). Together, these results suggest that gAd supplementation can rescue mitochondrial biogenesis impairment in cultured cardiomyocytes treated with HGHF.
Adiponectin Promotes Mitochondrial Biogenesis and Function via PGC-1α Signaling

The data obtained in the above experiments demonstrate gAD may partially reverse the deleterious effects of HGHF treatment upon mitochondrial biogenesis and function in NRVMs. We next determine the underlying protective mechanism by which APN acts upon mitochondrial biogenesis and function. It is well-known PGC-1α controls mitochondrial biogenesis and function in the postnatal heart\textsuperscript{19,20}. The absence of PGC-1α reduces available ATP supply, leading to significant contractile defects\textsuperscript{21}. Massive PGC-1α overexpression in normal hearts leads to cardiomyopathy\textsuperscript{20,21}. It is a plausible expression of active PGC-1α at physiological levels may be beneficial. The activity of PGC-1α may be modulated, via phosphorylation by AMPK and deacetylation by AMPK or Sirtuin 1 (SIRT1)\textsuperscript{14,22}. We pre-treated NRVMs with PGC-1αsiRNA, we determined the mtDNA and ATP content. As summarized in Figure 5, knockdown of PGC-1α by specific siRNA blocked the beneficial effect of gAd upon mitochondrial biogenesis and function in NRVMs subjected to HGHF, suggesting APN-mediated mitochondrial effects are PGC-1α-dependent.

Figure 3. Mitochondrial dysfunction occurs in NRVMs subjected to HGHF treatment. JC-1 staining of NRVMs with or without HGHF indicates reduced mitochondrial membrane potential in NRVMs with HGHF. Green fluorescence reveals the JC-1 stained mitochondrial plasma with a low JC-1 concentration (monomer) that mitochondrial membrane potential depolarized (left panel). While red fluorescence represents JC-1 aggregates accumulated in mitochondria due to high mitochondrial membrane potential (middle panel). The right panel is representative of merge pictures. Original magnification, ×100 (Row 1, 3), ×400 (Row 2, 4).
Anti-apoptotic Effects of APN in NRVMs Subjected to HGHF Treatment is PGC-1α-Dependent

Multiple previous studies have demonstrated gAd exerts significant protection against ischemic myocardial injury.4, 5 NRVMs were cultured in HGHF medium for 18 hours, and subjected to 12 hours hypoxia followed by 6 hours reoxygenation. Assessment of apoptosis by TUNEL (Figure 6A) and caspase-3 activity (Figure 6B) revealed incubation of NRVMs with HGHF resulted in increased cellular apoptosis. gAd administration (concentration 3 μg/ml, given in concert with HGHF treatment) protected cardiomyocytes against hypoxia/reoxygenation-induced apoptosis after HGHF medium incubation, as evidenced by TUNEL staining (Figure 6A) and caspase-3 activity (Figure 6B). However, when NRVMs were first subjected to PGC-1α knockdown by siRNA and then hypoxia/reoxygenation, supplemental gAd had no effect upon NRVM apoptosis compared to control. Taken together, the results of this and our previous experimental results suggest gAd supplementation partially rescues both mitochondrial biogenesis impairment and apoptosis in NRVMs subjected to HGHF incubation, in a PGC-1α-dependent manner.
This *in vitro* study excluded complex *in vivo* factors in an attempt to understand the effects of diabetes upon mitochondrial biogenesis and the resultant implications. Neonatal ventricular cardiac myocytes were subjected to 18 hours of incubation with HGHF medium, simulating the diabetic condition. The present work provides direct evidence that mitochondrial biogenesis is impaired in the HGHF environment.

Early researches reported impaired lipid oxidation and reduced mitochondrial content in the skeletal muscle of obese and type 2 diabetic patients\(^2\),\(^3\),\(^4\). Furthermore, others have confirmed the role of mitochondria in type 2 diabetes by examining healthy individuals with or without insulin resistance\(^5\). Insulin-resistant individuals harbor increased IMCL levels. IMCL are stored as droplets in skeletal muscle cells, and serve as a local energy supply for endurance workloads. Insulin-resistant individuals also have 40% reduction in ATP levels and oxidative capacity compared to the healthy population. Diabetics exhibit reduced mitochondrial oxidative capacity\(^6\). It has been demonstrated that the A3243G mutation in the tRNA\(^{Leu}\) gene of mitochondrial DNA may result in maternally inherited diabetes and deafness (MIDD); this mutation markedly increases the risk of T2DM development\(^7\). However, very few studies have provided direct evidence of mitochondrial disorder in T2DM\(^8\).

In the current study, we performed transmission electron microscopy, and presented direct evidence of structural mitochondrial abnormalities in NRVMs subjected to HGHF treatment. These data suggest that mitochondrial disorder may be an early event in T2DM development.

An adipocytokine of adipose origin, adiponectin has well documented beneficial bio-functions, including increasing insulin sensitivity, decreasing inflammation, and anti-ischemia/reperfusion injury effects. Hypoadiponectinemia in T2DM (indicative of down-regulated APN) contributes to the development of insulin resistance and T2DM\(^9\),\(^10\),\(^11\). In the current study, gAd administration partially restored the HGHF-induced reduction of mitochondrial biogenesis-associated gene mRNA levels, giving evidence adiponectin may attenuate impaired mitochondrial biogenesis and mitochondrial function in the diabetic heart. To dissect the underlying mechanisms responsible for gAd-mediated mitochondrial effects during
Hypoadiponectinemia-induced mitochondrial biogenesis dysfunction in heart

In HGHF conditions, we utilized siRNA-mediated knockdown of PGC-1α expression in NRVMs. Suppressed PGC-1α expression resulted in marked reduction of gAd-amelioration of HGHF-impaired mitochondrial biogenesis and mitochondrial function. PGC-1α, a member of the PGC-1 family, regulates a series of nuclear transcription factors (e.g. NRF-1, ERRα, and Tfam). Although PGC-1α critically mediates mitochondrial biogenesis and mitochondrial function both in vivo and in vitro, whether PGC-1α-mediated mitochondrial biogenesis and mitochondrial function play a positive or negative role in T2DM remains debated. To directly investigate this question, we subjected NRVMs to 12 hours of hypoxia followed by 6h reoxygenation (H/R). HGHF incubation increased NRVM apoptosis. gAd administration

![Figure 6](image_url)

Figure 6. Anti-apoptotic effects of APN in NRVMs subjected to HGHF treatment is PGC-1α dependent. NRVMs were first treated with PGC-1α siRNA or scramble RNA for 6h, then cultured in HGHF medium for 18 hours, and subjected to 12 hours hypoxia followed by 6 hours reoxygenation. gAd (3 μg/mL) was treated as the same time as HG/HF culture. TUNEL staining assessment of apoptotic NRVMs (A). Top row is representative of DAPI for nuclear; Bottom row is representative of TUNEL for apoptosis. Caspase-3 activity analysis for apoptosis (B). gAd: globular adiponectin domain. All values are presented as mean±SEM. n=3-4. *, p < 0.05,**, p < 0.01. vs. Control group; ^, p <0.05, ^^, p < 0.01. vs. HG/HF+sI/R group.
reduced apoptosis in NRVMs subjected to H/R, but this effect was abrogated when PGC-1α expression was knocked down by siRNA. Taken together, these results suggest PGC-1α is requisite in APN-induced cardioprotection in the diabetic heart.

Conclusions

Several significant observations were made in the present study. First, we have provided a direct in vitro evidence that a high glucose and high-fat environment, the major contributors to T2DM, can reduce mitochondrial biogenesis and induce mitochondrial dysfunction. Second, we have demonstrated that reduced adiponectin and PGC-1α signaling impairment directly contribute to deleterious altered mitochondrial biogenesis and mitochondrial dysfunction in cultured cardiomyocytes subjected to HGHF treatment. Finally, we have provided a direct in vitro evidence that APN-induced cardioprotection in the diabetic heart is PGC-1α dependent.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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