Thyroid Hormone Metabolite 3-Iodothyronamine (T1AM) Alleviates Hypoxia/Reoxygenation-Induced Cardiac Myocyte Apoptosis via Akt/FoxO1 Pathway

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Background: The thyroid hormone metabolite 3-iodothyronamine (T1AM) is rapidly emerging as promising compound of decreasing heart rate and lowering cardiac output. The aim of our study was to fully understand the molecular mechanism of T1AM on cardiomyocytes and its potential targets in cardiovascular diseases.

Material/Methods: We developed an in vitro myocardial ischemia-reperfusion injury model of AC-16 cells by hypoxia-reoxygenation injury. Cell viability of AC-16 cells was detected using CCK-8 assay and apoptosis was detected by flow cytometry. RNA-seq was used to characterize the gene expression in H/R-induced AC-16 cells after T1AM treatment. The mRNA levels of FoxO1, PPARα, Akt, and GCK and the protein levels of PPARα, GCK, and components of the Akt/FoxO1 pathway were detected by qRT-PCR and Western blotting, respectively.

Results: Exogenous T1AM increased the H/R-induced AC-16 cell viability in a relatively low concentration. A total of 210 DEGs, including 142 upregulated and 68 downregulated genes, were determined in H/R-induced AC-16 cells treated with or without T1AM. A Venn diagram showed 135 common DEGs. The FoxO signaling pathway was identified via KEGG enrichment analysis of these 135 DEGs. Moreover, T1AM mediated hypometabolism and reduced the apoptosis of H/R-induced AC-16 cells via the Akt/FoxO1 pathway.

Conclusions: Exogenous T1AM protects against cell injury induced by H/R in AC-16 cells via regulation of the FoxO signaling pathway. Our results suggest that T1AM can play a preventive role in myocardial H/R injury and also provide new insight for clinical management of AMI patients.
Background

Coronary heart disease is the leading cause of sudden cardiovascular death worldwide [1]. Myocardial ischemia/reperfusion (I/R) injury-induced myocardial cell injury is considered a critical cause of acute myocardial infarction (AMI), resulting in oxidative stress and cardiac myocyte apoptosis [2]. Drugs such as tenecteplase and streptokinase have been widely used for reducing I/R injury, but several complications still exist. Therefore, discovering new drugs for treatment of AMI patients to alleviate myocardial I/R injury remains a challenge.

3-iodothyronamine (T1AM) is a metabolite product of the thyroid hormones (TH), which exert an opposite physiological function of classical TH such as a decrease of body temperature and metabolic rate in rodents [3]. Several studies have shown that T1AM is presumed to act on various organs and different receptors rapidly [4]. Moreover, it has therapeutic potential for treatment of metabolic diseases [5], myocardial vascular diseases [6], and neurological diseases [7]. Thyroid hormones and their metabolite products play a critical role in the development of cardiovascular diseases [8–10]. Of note, T1AM, a decarboxylated and deiodinated thyroid hormone derivative, can decrease heart rate and lower cardiac output [11]. Studies reported that after administration of exogenous T1AM in the isolated heart, the resistance to ischemic injury was increased [12], but the underlying mechanism between T1AM and myocardial vascular diseases is unclear.

Cardiomyocyte apoptosis is regarded as a pivotal pattern of cell death in I/R injury. Hypoxia/reoxygenation (H/R) is used as an essential model in vitro to establish myocardial I/R injury [13]. This model can induce apoptosis in cardiomyocytes. In our study, we investigated the role of exogenous T1AM in cardiac myocyte apoptosis following H/R injury. Subsequently, in order to predict the potential transcriptional mechanism used by T1AM to protect from H/R-induced myocardial injury, RNA-Seq assay was performed. The data presented here suggest that T1AM ameliorates hypometabolism and reduces H/R-induced AC-16 cell apoptosis via the Akt/FoxO1 pathway.

Material and Methods

Cell cultures

AC-16 cells were purchased from ATCC. AC-16 cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/ml, Gibco), and streptomycin (100 U/ml, Gibco) in a humidified cell incubator providing 5% CO2/95% fresh air at 37°C. Cells were exposed to T1AM for 24 h. AC-16 cells cultured in T1AM-free growth medium were used as controls.

Hypoxia/reoxygenation treatment

AC-16 cells were collected and cultured in 6-well plates at 37°C, 5% CO2, and 95% air. Then, the hypoxia AC-16 cells were cultured in glucose-free Earle’s buffer (Gibco) with 95%N2, 5%CO2, and 1% O2 at 37°C for 24 h. Subsequently, the cells were re-oxygenated in 5% CO2/95% air for 4 h with DMEM containing 10% FBS. Then, the cells were collected and processed for further analysis.

Cell viability assessments

The cell viability was assessed by CCK-8 assay. Briefly, cells were seeded into a 96-well culture plate at density of 6×104/well. Cells were treated with various doses of T1AM for 24 h. AC-16 cells cultured in T1AM-free growth medium were used as controls. Then, cells in each well were incubated with CCK-8 at 37°C for 4 h. The absorbance was determined by a plate reader.

Total RNA extraction

Total RNA in both groups (Control and T1AM treatment) was extracted with RNAiso Plus (Takara) according to the manufacturer’s instructions. The cDNA library was constructed based on the instructions of Illumina. RNA-Seq of each sample was performed by Sangon Biotech Co. (Shanghai). RNA-Seq analysis was performed according to a previously described method [14].

Construction of RNA-seq Library for Illumina sequencing

Sequencing library preparations were constructed using the manufacturer’s protocol (VAHTSTM mRNA-seq V2 Library Prep Kit for Illumina®). The digest RNA samples were used for first- and second-strand cDNA synthesis with random hexamer primers. After the first- and second-strand cDNA synthesis, the double-stranded products were end repaired, a single ‘A’ base was added, and adaptors were ligated onto the cDNA products. CDNA libraries were constructed. Then, the paired-end sequencing was performed on an Illumina HiSeqTM 2500 [15].

Identification of differentially-expressed genes (DEGs)

After sequencing, raw data were obtained in the fastq format. FastQC was used for validating the quality of the data. Trimming of sequences was performed using Trimmomatic. Then, Quality check using FastQC was performed again on the trimmed sequences. Reads per kilobase of exon mode (RPKM) was employed to quantify transcript levels [16]. Differentially-expressed genes in 4 samples were found and conditions for differences in genes were filtered. The adjusted P value <0.05 and log2 (fold change) >1 were set as the cutoff criteria.
**Gene ontology and KEGG pathway enrichment analysis**

The Database for Annotation, Visualization and Integrated Discovery (DAVID 6.8) gene annotation tool (http://david.ncifcrf.gov/tools.jsp) was used to perform GO and KEGG analysis [17]. Gene ontology terms analysis of the DEGs, including biological process (BP), cellular component (CC), and molecular function (MF), were identified using the gene ontology (GO) project (http://www.geneontology.org). KEGG pathway enrichment analysis was performed based on the Kyoto Encyclopedia of Genes and Genomes database (http://www.kegg.jp) [18]. In addition, GO terms and pathways with P values less than 0.05 were considered to be statistically significant.

**Apoptosis assay**

After H/R treatment, apoptosis kits were used to measure the proportion of apoptotic cells in each group according to the manufacturer’s instructions (BD). After collecting the cells and washing them with PBS, the cells were incubated with 5 μl Annexin-V-FITC and 5 μl propidium iodide (PI) working solution for 30 min in the dark. Cell fluorescence was measured using a Navios flow cytometer (Beckman Coulter, Inc).

**Oxygen consumption rate (OCR)**

The AC-16 cell lines were seeded at a density of 2.0×10⁵ cells per well in 24-well XF microplates (SeaHorse Bioscience, Billerica, MA, U.S.A.). The time courses in the oxygen consumption rate were carried out for 24 h treated with 0.5 μM, 2 μM, 5 μM, 7.5 μM T1AM, 5 μM T1AM+30 nM AS1842856 (the FoxO1 inhibitor), and vehicle (DMSO), respectively. OCR was measured by a Seahorse XFe24 analyzer (Seahorse Bioscience, Billerica, MA, U.S.A.). A Mito-stress kit (Vazyme, Nanjing) was used as a control for normalization of RT-qPCR results. The sequences of primers designed by NCBI Primer Blast are shown in Supplementary Table 1. Three independent replicates were conducted for this experiment.

**Western blot**

After H/R treatment, AC-16 cells were prepared and lysed using cell lysis buffer for determining the total protein concentration. First, a total of 30 μg protein was subjected to 12% (v/v) SDS-PAGE and then samples were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking, the membranes were probed with anti-FoxO1, anti-phosphorylated FoxO1, anti-Akt, anti-gck, anti-PPARα, and anti-β-actin antibodies at 4°C overnight. Then, the secondary antibodies were HRP-conjugated antibodies against mouse or rabbit. Finally, band intensities were quantified using Image J software (Rawak Software).

**Results**

**Effects of T1AM on H/R-induced cell viability in AC-16 cells**

AC-16 cells were exposed to 60, 20, 6.67, 2.22, 0.74, 0.25, 0.08 μM T1AM, and vehicle (DMSO). The cell viability of AC-16 cell lines with or without T1AM exposed to H/R were analyzed by CCK-8. Our data indicated that doses below 6.67 μM T1AM increased the cell viability of AC-16 cells exposed to H/R after 24-h treatment (Figure 1, *P*<0.05). Hence, 2.5 and 5 μM T1AM, which increased AC-16 cell viability, were used for the following experiments.
Figure 2. Functional analysis of the DEGs. (A) Volcano plots of significant differential expression of genes between H/R and AC-16 group. (B) Volcano plots of significant differential expression of genes between H/R and T1AM+H/R group. (C) Volcano plots of significant differential expression of genes between AC-16 and T1AM group. (D) Volcano plots of significant differential expression of genes between T1AM and T1AM+H/R group. The X-axis represents Log2 (fold change) and the y-axis represents -Log10 (P value). Red dots are Log2 >1 and P value <0.05. Blue dots are Log2<-1 and P value <0.05. (E) The 135 common DEGs were identified by Venn diagram. (F) Analysis of GO enrichment for the 135 common DEGs. The light blue colors represent significant biological processes, the deep blue colors represent significant cellular components, and the black colors represent significant molecular functions. (G) KEGG enrichment analysis for the 135 common DEGs. The pathways of significant differentially-expressed genes are enriched.
Identification of differentially-expressed genes associated with T1AM treated

RNA sequencing was performed on poly(A)-enriched RNA extracted from AC-16 cells, AC-16 cells treated with T1AM, and AC-16 cells treated with or without T1AM exposed to H/R. The raw data of sequencing were evaluated and are shown in Supplementary Table 2. The Q30 data, the probability of an error base call 1 in 1000 times, were over 98%. The proportion of Q20, the probability of an error base call 1 in 100 times, were 100%, which suggests the quality of sequencing data was appropriate for the following data analysis.

The transcriptome profiles of 4 groups, including AC-16 cells, AC-16 cells treated with T1AM, and H/R-induced AC-16 cells with or without T1AM, were compared. Differently-expressed genes (DEGs) were identified according to Log2-fold changes and P value. Volcano plots were used to analyze DEGs, in which red spots and blue spots represent upregulated and downregulated genes, respectively. In the T1AM group, 98 DEGs, including 89 upregulated and 9 downregulated genes, were determined in comparison to the control group (Figure 2C). There were 210 DEGs, including 142 upregulated and 68 downregulated genes, determined in AC-16 cells treated with or without T1AM exposed to H/R (Figure 2B). Compared with the control group, 1667 DEGs were identified in the H/R group, including 1215 upregulated and 452 downregulated genes (Figure 2A). Compared with the T1AM group, 2645 DEGs were identified in H/R-induced AC-16 cells treated with T1AM, including 1669 upregulated and 976 downregulated genes (Figure 2D). In analysis of DEGs, 135 common genes were identified as being differentially expressed in T1AM-treated AC-16 cells exposed to H/R, as shown by a Venn diagram (Figure 2E).

Analysis of gene ontology (GO)

GO enrichment analyses were carried out to define the biological function of the 135 DEGs, divided into 3 sub-ontologies: biological process (BP), cellular component (CC), and molecular function (MF). In biological process, terms such as cellular process, metabolic function, and single-organism process (GO: 0009987, GO: 0008152, GO: 0044699, GO: 0065007, GO: 0050789, GO: 0071840, GO: 0050896, GO: 0048518, GO: 0051179, GO: 0032501, GO: 0032502, GO: 0048519, GO: 0051234, GO: 0023052, GO: 0051704, GO: 0022241, GO: 0000303, GO: 0023376, GO: 0040011, GO: 0040007, GO: 0022610, GO: 0007610, GO: 0048511, GO: 0098754, GO: 001906, GO: 0044848, GO: 0098743) were significantly enriched. In the cellular component, sub-ontology, terms related to cell, cell part and organelle (GO: 0005623, GO: 0044646, GO: 0043226, GO: 0044422, GO: 0032991, GO: 0016020, GO: 0031974, GO: 0044425, GO: 0055576, GO: 0044421, GO: 0030054, GO: 0099512, GO: 0045202, GO: 0044456, GO: 0031012) were significantly enriched. In the molecular function sub-ontology, terms including binding, catalytic activity, and structural molecular activity (GO: 0005488, GO: 003824, GO: 0005198, GO: 0098777, GO: 0005215, GO: 0030234, GO: 0001071, GO: 0009988, GO: 0044871, GO: 0004872, GO: 0060089, GO: 0009055, GO: 0016209, GO: 0016247, GO: 0045182, GO: 0045499, GO: 0030545, GO: 0016015, GO: 0016530, GO: 0031386, GO: 0042056, GO: 0045735) were significantly enriched (Figure 2F).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment reveals that T1AM attenuates H/R injury via FoxO signaling pathway

KEGG enrichment analysis was performed to identify relative pathway in which the 71 DEGs were involved. A scatter plot was
used to depict significantly enriched pathway terms. Pathways such as FoxO signaling pathway, HIF-1 signaling pathway, and P53 signaling pathway were the most significantly enriched terms in T1AM-treated AC-16 cells exposed to H/R (Figure 2G).

Hypometabolism in T1AM treated AC-16 cells

Assessment of the hypometabolism effects of T1AM in AC-16 cells showed that the OCR was decreased after T1AM treatment compared with the vehicle (DMSO) group. Interestingly, 5 μM T1AM and 7.5 μM T1AM significantly lowered the OCR of AC-16 cells (*p<0.05, Figure 3). Moreover, 30 nM AS1842856, the FoxO1 inhibitor, combined with 5 μM T1AM, increased OCR (**p<0.05, Figure 3).

T1AM attenuates AC-16 cell apoptosis exposed by H/R

Cellular apoptosis rate was detected by Annexin V/PI staining. Our results showed that the cellular apoptosis rate was significantly higher in the H/R group (**p<0.01, Figure 4). Different concentrations of T1AM can markedly increase cellular apoptosis in H/R-exposed AC16 cells (**p<0.05, Figure 4). On the contrary, 30 nM AS1842856 and 2 μM T1AM obviously decreased the cellular apoptosis compared with H/R-exposed AC-16 cells only treated with 2 μM T1AM (**p<0.05, Figure 4).

T1AM mediates hypometabolism of AC-16 cells via Akt/FoxO1 pathway

To investigate whether T1AM regulates OCR in AC-16 cells via the Akt/FoxO1 pathway, the protein levels of phosphorylated FoxO1, Akt, FoxO1, PPARα, and GCK were examined (Figure 5A). As shown in Figure 5D, 2 μM T1AM significantly decreased the expression of Akt compared with AC-16 cells exposed to H/R. Notably, increasing concentration of T1AM markedly decreased FoxO1 phosphorylation (Figure 5B) and significantly increased the expression of PPARα and FoxO1 (**p<0.05, Figure 5F, Figure 5C). Interestingly, our results also found that AS1842856, the FoxO1 inhibitor, can reverse the expression of PPARα, but does not affect the expression of Akt. Moreover,
the expression of GCK was not affected by T1AM or AS1842856 (P>0.05, Figure 5E). To verify the mRNA expression, RT-qPCR was performed to quantify mRNA expression levels of these 4 genes. The results showed that T1AM can mediate hypometabolism of AC-16 cells via the Akt/FoxO1 pathway, which was similar to the results of Western blot (Figure 6). Furthermore, these results show that variation tendencies between the RNA-Seq and RT-qPCR data were identical, demonstrating the reliability of the RNA-Seq data.

Discussion

Previous studies have indicated that T1AM is an effective metabolite products of the TH that can decrease heart rate, lower cardiac output, and lower body temperature [19–21]. The effects of T1AM via TAAR1/TAAR2 at nanomolar concentrations were reported. Of note, in TAAR1 knock-out mice, the observed body temperature-reducing effect of T1AM still persists, which indicated that other targets of T1AM still exist. Furthermore,
Functional effects have been observed after administration of exogenous T1AM: in the isolated heart, the resistance to ischemic injury was increased. Therefore, we speculated that cardiomyocytes would be an essential target site for T1AM. In our investigation, we found that low concentrations of T1AM could protect cardiac myocytes from H/R injuries. Then, 135 DEGs and the FoxO signaling pathway were identified using RNA-Seq and appropriate transcriptome analysis, which indicates that T1AM protects the heart from H/R injuries via this pathway. Finally, oxygen consumption rates and cellular apoptosis rates were significantly reduced by T1AM in AC-16 cells exposed to H/R injuries via the Akt/FoxO1 pathway. The present study is the first to report that these effects can be significantly reversed by AS1842856, the FoxO1 inhibitor.

Using a CCK8 assay, our research substantiated that T1AM can increase the cell viability of AC-16 cells exposed to H/R. Studies showed that doses of T1AM used in in vitro studies were different in various cell lines [5,22–24]. In our study, concentrations of T1AM below 6.67 μM increased the cell viability of AC-16 cells exposed to H/R after 24-h treatment, which suggests that relatively low concentrations of T1AM protect cardiac myocytes from H/R injury. Hence, 0.5, 2, and 5 μM T1AM were used to identify the potential mechanism of decreasing cell injuries exposed to H/R.

Furthermore, in order to specify the detailed targets on AC-16 cells of T1AM, RNA-Seq profiles analysis was performed. RNA-Seq profiles analysis is an efficacious assay for uncovering the potential molecular mechanisms of T1AM, and has been extensively accepted as a method to find the therapeutic targets of drugs [25]. A total of 135 genes were identified as being differentially expressed in T1AM-treated AC-16 cells exposed to H/R injuries via the Akt/FoxO1 pathway. The present study is the first to report that these effects can be significantly reversed by AS1842856, the FoxO1 inhibitor.

It is widely known that T1AM exerts a physiological function of lowering body temperature in rodents. Studies suggest that

Figure 6. (A–D) RT-qPCR analysis of Akt, FoxO1, GCK and PPARα. Data are presented as the mean±standard deviation of 3 experiments. * p<0.05; ** p<0.01; ***p<0.001; ns – not significant.

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T1AM acts through sirtuin-mediated pathways to metabolically reprogram fatty acid and glucose metabolism, possibly through small-molecules signaling [26]. To understand the biological functions of the T1AM-induced DEGs in AC-16 cells, several GO terms related to metabolism were explored, including the biological process category “metabolic function”, and the molecular function category “catalytic activity”. In addition, the FoxO signaling pathway was found by KEGG analysis. These finding suggest that T1AM affects cardiomyocytes by metabolic function.

Evidence was reported suggesting that T1AM treatment decreased the OCR of muscle cells [27]. Notably, our study is the first to demonstrate that T1AM treatment decreases the OCR of AC-16 cells in vitro, suggesting a hypometabolic effect of T1AM in AC-16 cells, which indicates that T1AM affects cardiomyocytes by metabolic function. Moreover, our results also revealed that the cellular apoptosis rates were remarkably increased by T1AM in H/R-exposed AC16 cells. On the contrary, inhibition of FoxO1 significantly aggravated the apoptosis rate and increased the OCR of AC-16 cells.

Regarding the metabolic process, transcription factor forkhead box O proteins and peroxisome proliferator-activated receptor (PPARα) play a key role in cardiomyocytes. Akt activation leads to the phosphorylation and nuclear exclusion of the FoxO1. Based on these results, we hypothesized that H/R-induced AC-16 cells treated with T1AM significantly regulate FoxO signaling pathways through target genes such as Akt, FoxO1, pFoxO1, and PPARα. Our study showed reduced levels of Akt, p-FoxO1, and elevated levels of FoxO1 and PPARα, as detected by Western blot analysis. Our results suggest that T1AM can reduce the expression of Akt. Downregulation of Akt can lead to reduced phosphorylation and nuclear accumulation of FoxO1, resulting in overexpression of PPARα (Figure 7). Moreover, our study indicated that AS1842856, the FoxO1 inhibitor, can reverse the expression of PPARα, and even increase OCR and H/R-induced apoptosis.

Taken together, our study suggests that T1AM mediates hypometabolism and reduces the apoptosis of H/R-induced AC-16 cells via the Akt/FoxO1 pathway, which indicates that the target of T1AM in H/R-induced AC-16 cells might be the Akt/FoxO1 signaling pathway. In addition, AS1842856, the FoxO1 inhibitor, can reverse this phenomenon. The present findings indicate that T1AM plays a preventive role in H/R injury. A limitation of our results is that our study was performed on isolated cells. More sophisticated experimental models such as animal model should be performed to confirm the interesting and promising results obtained here.

Conclusions

In conclusion, exogenous T1AM protects against cell injury induced by H/R in AC-16 cells via regulation of the FoxO signaling pathway. Our results suggest that T1AM plays a preventive role in myocardial H/R injury and also provide new insight for clinical management of AMI patients.

Competing interests

None.
Supplementary Data

Supplementary Table 1. Primers used for RT-qPCR.

| Gene  | Forward primer (5'-3') | Reverse primer (5'-3') |
|-------|------------------------|------------------------|
| Akt   | GCACAAACGAGGGGAGTACA    | AAGTGGCCTGCTGATGACAGT  |
| FoxO1 | ACAGTGAGATCTCAAGGAC     | AAGTATGAATCTCAGCCCGG   |
| PPARα | CGCGTCTTCGCAGGTT        | GCGGCACCTCAAGACTTCTTT  |
| GCK   | GCCCTCCAAGGATCTACCTC    | GATCGCTCACTGGGGAGTT    |

Supplementary Table 2. Quality control of RNA-Seq.

| Sample       | Raw_bases (G) | Raw_reads | Clean_reads | UID_reads | Q20 (%) | Q30 (%) | GC content (%) |
|--------------|---------------|-----------|-------------|-----------|---------|---------|----------------|
| AC-16_1      | 10.89         | 72582764  | 54938490    | 45822902  | 100     | 98.6    | 53.27          |
| AC-16_2      | 12.6          | 83976656  | 63891836    | 52669992  | 100     | 98.55   | 53.48          |
| AC-16_3      | 11.07         | 73782720  | 54715958    | 46471218  | 100     | 98.55   | 53.51          |
| T1AM_1       | 12.27         | 81782532  | 63072746    | 53331582  | 100     | 98.55   | 53.51          |
| T1AM_2       | 12.96         | 86400346  | 64988416    | 55036614  | 100     | 98.5    | 52.38          |
| T1AM_3       | 10.87         | 72460776  | 55274770    | 46842470  | 100     | 98.6    | 53.41          |
| H/R_1        | 13.47         | 89788652  | 6785342     | 56164928  | 100     | 98.65   | 53.53          |
| H/R_2        | 12.83         | 85510102  | 64040788    | 53620442  | 100     | 98.45   | 53.24          |
| H/R_3        | 13.88         | 92549964  | 70968566    | 57977662  | 100     | 98.5    | 53.94          |
| H/R+T1AM_1   | 11.85         | 79008198  | 58364134    | 47963310  | 100     | 98.6    | 52.9           |
| H/R+T1AM_2   | 12.07         | 80449148  | 59323994    | 48870858  | 100     | 98.55   | 53.4           |
| H/R+T1AM_3   | 10.66         | 71073952  | 52212294    | 43004914  | 100     | 98.6    | 53.52          |

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