Class II transactivator (CIITA) mediates transcriptional repression of pdk4 gene by interacting with hypermethylated in cancer 1 (HIC1)  

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Abstract

Increased accumulation and/or impaired utilization of fatty acid in extra-adipose tissues are implicated in the pathogenesis of insulin resistance and type 2 diabetes. Pyruvate dehydrogenase kinase 4 (Pdk4) is a key enzyme involved in fatty oxidation and energy expenditure, and its expression can be repressed by pro-inflammatory stimuli. Previously, we have shown that class II transactivator (CIITA) mediates the adverse effect of interferon gamma (IFN-γ) in skeletal muscle cells by cooperating with hypermethylated in cancer 1 (HIC1) to repress silent information regulator 1 (SIRT1) transcription. Building upon this finding, we report here that CIITA interacted with HIC1 via the GTP-binding domain (GBD) while HIC1 interacted with CIITA via the BTB/POZ domain. The GBD domain was required for CIITA to repress SIRT1 transcription probably acting as a bridge for CIITA to bind to HIC1 and consequently to bind to the SIRT1 promoter. IFN-γ stimulation, CIITA over-expression, or HIC1 over-expression repressed Pdk4 promoter activity while silencing either CIITA or HIC1 normalized Pdk4 expression in the presence of IFN-γ. An increase in SIRT1 expression or activity partially rescued Pdk4 expression in the presence of CIITA, but SIRT1 inhibition abrogated Pdk4 normalization even in the absence of CIITA. Taken together, our data have identified a HIC1-CIITA-SIRT1 axis that regulates Pdk4 transcription in response to IFN-γ stimulation.

Keywords: CIITA, HIC, SIRT1, IFN gamma, PDK4, transcriptional regulation

Introduction

Changes in lifestyle have contributed to the development of metabolic syndrome in humans over the past several decades. Type 2 diabetes, which has become a global pandemic, is a major form of metabolic syndrome. Type 2 diabetes is characterized by insulin resistance in peripheral organs including the liver, adipose tissues, and skeletal muscles. Chronic, low-magnitude inflammation (dubbed as metabolic inflammation) has been extensively investigated as a primary force to promote insulin resistance. Pro-inflammatory cytokines, including interleukins (ILs), macrophage chemoattractant protein 1

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doi: 10.7555/JBR.29.20150055
(MCP1), tumor necrosis factor (TNF-γ), and interferon gamma (IFN-γ), are considered culprits in metabolic inflammation and insulin resistance. How these pro-inflammatory mediators disrupt normal cellular metabolic programs and consequently cause metabolic syndrome is not entirely clear.

Silent information regulator 1 (SIRT1) is the founding member of the mammalian family of class III lysine deacetylases. Numerous studies have demonstrated the beneficial role of SIRT1 in maintaining the homeostasis of cellular metabolism. SIRT1 gain-of-function transgene or activation of SIRT1 with small-molecule agonists has been shown to antagonize both diet-induced and hereditary obesity and the development of type 2 diabetes in mice. In contrast, SIRT1 ablation promotes metabolic inflammation and exacerbates insulin resistance in mice. One of the mechanisms by which SIRT1 prevents insulin resistance is to stimulate cellular energy expenditure by re-programming cellular metabolic circuits. For instance, SIRT1 activation in cells increases fatty acid oxidation by deacetylation of PGC1-α, FOXO1, and p53.

Previously, we have reported that class II transactivator (CIITA) is activated by IFN-γ in skeletal muscle cells. CIITA interacts with hypermethylated in cancer 1 (HIC1), a sequence-specific transcription factor, to repress SIRT1 transcription. Consequently, genes involved in energy expenditure such as Mcad, Ppargc-1a, Cpt1, and Cox4 are down-regulated in response to IFN-γ stimulation, leading to a skewed metabolic phenotype mimicking insulin resistance. In the present study, we have addressed several lingering issues: 1) which domains within CIITA and HIC1 mediate their interaction? 2) Do CIITA and HIC1 mediate the repression of Pdk4, a key enzyme involved in fatty acid oxidation, by IFN-γ? 3) Is SIRT1 required for CIITA to mediate the disruptive effect of IFN-γ? Our data re-affirm the HIC1-CIITA-SIRT1 axis in regulating cellular metabolism in skeletal muscle cells.

Materials and methods

Cell culture and treatment

Mouse myotube/myoblast cells (C2C12) and human embryonic kidney cells (HEK293) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). Cells were serum starved overnight and then performed with murine recombinant interferon-gamma (IFN-γ, R&D Systems, Abingdon, UK), resveratrol (Sigma, St Louis, MO, USA), or nicotinamide (Sigma) for 24-48 hours before the treatment.

Plasmids, transient transfection, and reporter assay

FLAG-tagged CIITA constructs, GFP-tagged HIC1 constructs, SIRT1 promoter-luciferase construct, Pdk4 promoter-luciferase construct. SIRT1 expression constructs, siRNA sequences for mouse C2ta and Hic1 have been previously described. Transient transfections were performed with Lipofectamine 2000 (Invitrogen). Luciferase activities were assayed 24-48 hours after transfection using a luciferase reporter assay system (Promega, Madison, WI, USA). Experiments were routinely performed in triplicate wells and repeated three times.

Protein extraction, immunoprecipitation and Western blotting assay

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, and 1% Triton X-100) with freshly added protease inhibitor (Roche, Indianapolis, IN, USA). FLAG-conjugated beads (M2, Sigma) were added to and incubated with lysates overnight. Precipitated immune complex was eluted with 3X FLAG peptide (Sigma). Western blotting assays were performed with anti-FLAG or anti-GFP (Sigma) antibodies.

RNA isolation and real-time PCR

RNA was extracted with the RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA). Reverse transcriptase reactions were performed using a SuperScript First-strand Synthesis System (Invitrogen). Real-time PCR was performed on an ABI Prism 7500 system. Primers and Taqman probes used for real-time reactions were purchased from Applied Biosystems.

Chromatin immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) assays were performed essentially as described before. In brief, chromatin in control and treated cells were cross-linked with 1% formaldehyde. Cells were incubated in lysis buffer (150 mmol/L NaCl, 25 mmol/L Tris pH 7.5, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) supplemented with protease inhibitor tablet and phenylmethanesulfonyl fluoride (PMSF). DNA was fragmented into -500 bp fragments using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-FLAG (Sigma). Precipitated genomic DNA was amplified by real-time PCR using previously described primers.
Statistical analysis

One-way ANOVA with post-hoc Scheffe analyses were performed using an SPSS package. $P<0.05$ was considered statistically significant.

Results

**CIITA is a de novo co-repressor for HIC1**

Previously, we have found that CIITA mediated SIRT1 repression as a result of IFN-γ stimulation by interacting with HIC1. We made an attempt to delineate the domains within HIC1 that might mediate the interaction with CIITA. To this end, full-length (FL) HIC1 expression construct or deletion mutant HIC1 constructs, all with a GFP tag, were transfected into HEK293 cells with FLAG-tagged CIITA expression construct. Whole lysates extracted from transfected cells were immunoprecipitated with an anti-FLAG antibody. As shown in **Fig. 1A**, FL HIC1 interacted with CIITA as expected. A deletion mutant lacking the CtBP domain, which was reported to bridge the HIC1-CtBP interaction, retained the ability to interact with CIITA. On the contrary, a mutant missing the N-terminal BTB/POZ domain failed to interact with CIITA. Similarly, we determined the domain within CIITA necessary for interaction with HIC1. As shown in **Fig. 1B**, while FL CIITA, an N-terminal deletion CIITA mutant without the acidic domain (AD), acetyltransferase domain (AT), and proline-serine-threonine domain (PST), and a C-terminal deletion CIITA mutant without the leucine-rich...
repeat domain (LRR) all interacted with HIC1, a more extensive C-terminal deletion CIITA mutant lacking the GTP-binding domain (GBD) exhibited diminished HIC1 interaction. Thus, we concluded that HIC1 may interact with CIITA through its N-terminal BTB/POZ domain while CIITA may interact with HIC1 through its GBD domain in the middle.

Next, we tackled the question whether CIITA could serve as a genuine co-factor for HIC1-dependent transcriptional repression. A reporter, constructed by fusing five copies of HIC1 consensus sequences with the luciferase gene, was transfected into HEK293 cells. CIITA repressed the HIC1 reporter in a dose-dependent manner suggesting that CIITA might indeed facilitate HIC1-dependent transcriptional repression in cells (Fig. 1C).

The GBD domain of CIITA is required for SIRT1 repression

Having determined that the GBD domain of CIITA mediates its interaction with HIC1 (Fig. 1B), we

![Graph A: SIRT1 promoter](image)

A SIRT1 promoter

|            | CIITA WT | CIITA (1–612) | CIITA (1–852) | CIITA (519–1130) |
|------------|----------|---------------|---------------|-------------------|
| Relative luciferase activity | 0.6 ± 0.1 | 1.4 ± 0.2 | 1.2 ± 0.1 | 1.0 ± 0.1 |

![Graph B: SIRT1 mRNA](image)

B SIRT1 mRNA

|            | CIITA WT | CIITA (1–612) | CIITA (1–852) | CIITA (519–1130) |
|------------|----------|---------------|---------------|-------------------|
| Relative mRNA levels | 0.4 ± 0.1 | 1.2 ± 0.2 | 1.0 ± 0.1 | 0.8 ± 0.1 |

![Graph C: SIRT1 ChIP](image)

C SIRT1 ChIP

|            | CIITA WT | CIITA (1–612) | CIITA (1–852) | CIITA (519–1130) |
|------------|----------|---------------|---------------|-------------------|
| Relative enrichment | 0.2 ± 0.1 | 1.2 ± 0.2 | 1.0 ± 0.1 | 0.8 ± 0.1 |

Fig. 2 The GBD domain of CIITA is required for SIRT1 repression. A: A SIRT1 promoter construct was transfected into HEK293 cells with or without indicated CIITA expression contracts. Luciferase activities were normalized to the control group and expressed as relative luciferase unit (RLU). B: HEK293 cells were transfected with or without indicated CIITA expression constructs. Endogenous SIRT1 message levels were measured by qPCR. C: HEK293 cells were transfected with or without indicated FLAG-tagged CIITA expression constructs. ChIP assay was performed with anti-FLAG.
proceeded to examine whether this domain might also be required for SIRT1 repression. Over-expression of either FL CIITA or deletion CIITA mutants that retained the GBD domain (1-852 and 519-1130) were able to, while the CIITA mutant that lacked the GBD domain (1-612) failed to, repress SIRT1 promoter activities in HEK293 cells (Fig. 2A). Consistent with this result, endogenous SIRT1 messages were downregulated by ectopic CIITA constructs with the GBD domain but not by the GBD domain deletion CIITA construct (Fig. 2B). Furthermore, ChIP assays showed that FL CIITA and GBD-containing CIITA were recruited to the SIRT1 promoter, but GBD-null CIITA lost the binding to the SIRT1 promoter apparently due to its inability to interact with HIC1 (Fig. 2C). In conclusion, these data confirm our previous observations that CIITA relies on HIC1 to bind to the SIRT1 promoter to repress transcription.

CIITA and HIC1 mediate repression of Pdk4 gene by IFN-γ

Pyruvate dehydrogenase kinase 4 (pdk4) is a key enzyme involved in fatty acid oxidation and can be activated by SIRT1 during starvation and/or fasting to maintain metabolic homeostasis[13]. Previously, we have demonstrated that CIITA and HIC1 cooperate to mediate SIRT1 repression by IFN-γ[12]. Therefore, we

Fig. 3 CIITA and HIC1 mediate the repression of Pdk4 gene by IFN-γ. A: A Pdk4 promoter construct was transfected into C2C12 cells followed by treatment with IFN-γ (50-200U/mL) for 48 hours. Luciferase activities were normalized to the control group and expressed as relative luciferase unit (RLU). B and C: A Pdk4 promoter construct was transfected into C2C12 cells with increasing amounts of CIITA (B) or HIC1 (C) constructs. Luciferase activities were normalized to the control group and expressed as relative luciferase unit (RLU). D and E: C2C12 cells were transfected with siRNA targeting either CIITA (siC2ta, D) or HIC1 (siHic1) followed by treatment with IFN-γ (200U/mL) for 48 hours. Pdk4 mRNA levels were measured by qPCR.
examined the possibility that IFN-γ might disrupt Pdk4 transcription via CIITA/HIC1. IFN-γ treatment repressed Pdk4 promoter activity in a dose-dependent manner (Fig. 3A). Alternatively, over-expression of CIITA (Fig. 3B) or HIC1 (Fig. 3C) directly repressed Pdk4 promoter activity in a dose-dependent manner. Finally, knockdown of CIITA or HIC1 using small interfering RNA (siRNA) abrogated the repression of Pdk4 messages by IFN-γ (Fig. 3D and 3E). In summary, we propose that CIITA and HIC1 might mediate IFN-γ-induced repression of Pdk4 gene.

CIITA represses Pdk4 transcription through SIRT1

Finally, we assessed the hypothesis that CIITA might repress Pdk4 transcription indirectly through SIRT1. As shown in Fig. 4A, over-expression of CIITA repressed the Pdk4 promoter activity; co-expression of a wild type (WT) form, but not an enzyme deficient mutant (HY), of SIRT1 partially abrogated the effect of CIITA. In addition, pre-treatment of cells with a SIRT1 agonist resveratrol also alleviated the
repression of Pdk4 promoter activity by CIITA (Fig. 4B). In contrast, siRNA-mediated knockdown of CIITA normalized Pdk4 expression in the presence of IFN-γ, which was reversed by the addition of a SIRT1 inhibitor nicotinamide (Fig. 4C). Together, these data suggest that CIITA targets SIRT1 to regulate Pdk4 transcription.

Discussion

Metabolic inflammation contributes to insulin resistance in part by disrupting normal cellular metabolism[22]. Our data as summarized in this report echo our previous finding that IFN-γ could modify cellular metabolism by decreasing energy expenditure in skeletal muscle cells[12]. Of note, the detrimental effects of IFN-γ have been observed in other insulin-sensitive organs as well. For instance, McGillicuddy et al. have reported that IFN-γ attenuates insulin signaling in human adipocytes by activating the JAK/STAT pathway[10]. In addition, Wensveen et al. demonstrated that natural killer cell-derived IFN-γ promotes insulin resistance in visceral fat tissue by promoting M1 macrophage differentiation[19]. Accordingly, mice with deficiency in either IFN-γ gene itself or IFN-γ receptor gene (Ifngr1) display improved metabolic profile and resistance to obesity upon high-fat diet challenge[20-21].

Therefore, though relying on various mechanisms, these data together argue for a unified role for IFN-γ in the development of insulin resistance and type 2 diabetes, which is consistent with epidemiology studies that show increased serum levels of IFN-γ in obese population[22-25].

CIITA was initially identified as a co-activator of major histocompatibility complex (MHC II) genes. Follow-up studies have revealed that CIITA is capable of both activating and repressing gene expression[26,27]. Previously, we showed that CIITA relies on HIC1 to be brought down to the SIRT1 promoter to repress transcription. Here, we show that the GTP-binding domain (GBD) is required for CIITA to interact with HIC1. Ting et al. have reported that GBD is necessary for the nuclear import of CIITA[27]. This observation, according to our new finding, alludes to the possibility that the CIITA-HIC1 complex is assembled in the nucleus and probably on the chromatin. CIITA has the ability to bridge sequence-specific transcription factors to the epigenetic machinery[26,31]. CIITA might help recruit certain epigenetic factors (e.g., HDAC2) to facilitate SIRT1 repression by HIC1. Alternatively, the GBD domain is shown to mediate CIITA oligomerization and stability of CIITA[32]; binding to HIC1 may promote CIITA self-assembly and/or stability increasing its transcriptional activity. Clearly, the interaction between HIC1 and CIITA needs to be further examined in the context of different pathophysiological processes.

We show here that HIC1 and CIITA repress Pdk4 transcription by directly targeting SIRT1. A reasonable extrapolation would be that targeting HIC1 and/or CIITA may be beneficial in metabolic disorders. Indeed, a recent discovery suggesting that mice with MHC II deficiency develop resistance to diet-induced obesity and type 2 diabetes seems to support this hypothesis[33]. However, systemic inhibition of HIC1 or CIITA may lead to either carcinogenesis or immune insufficiency, rendering the strategy undesirable[34-36]. Since CIITA activation is a pre-requisite for HIC1 to repress SIRT1 transcription in metabolically challenged situations, an alternative strategy would be to modify/temper CIITA activity rather than abolish it. This could be achieved by either post-translational modifications or, as suggested by several recent reports, by modulating the IFN-γ-STAT1 pathway[26,37].

In summary, we re-affirm our previous finding that HIC1-CIITA-SIRT1 axis modulates cellular metabolism in response to pro-inflammatory stress. Future studies should focus on unveiling a genome-wide role for the CIITA-HIC1 complex in this process to provide a more solid basis for the development of interventional strategies against type 2 diabetes.

Acknowledgements

This work was supported, in part, by the National Natural Science Foundation of China (31200645), the Natural Science Foundation of Jiangsu Province (BK20141498), and a grant from Jiangsu Jiankang Vocational University (JK201405). Y.X. is a Fellow at the Collaborative Innovative Center for Cardiovascular Translational Medicine.

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